Title of the Thesis: Bioremediation of Phenol and Phenolic Compounds using mixed microbial culture in a bioreactor

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by

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Dedicated to

Mr. Tarun Kanti

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&

Mrs. Lekha Maity

& my supervisors

"Statement of Originality"

I, Debapriya Maity, registered on 03.01.02019 do hereby declare that this thesis entitled **"Bioremediation of Phenol and Phenolic Compounds using mixed microbial culture in a bioreactor"** contains literature survey and original research work done by the undersigned candidate as part of Doctoral studies. All information in this thesis have been obtained and presented in accordance with existing academic rules and ethical conduct. I declare that, as required by these rules and conduct, I have fully cited and referred all materials and results that are not original to this work. I also declare that I have checked this thesis as per the "Policy and Anti Plagiarism, Jadavpur University, 2019", and the level of similarity as checked by iThenticate software is 6 %.

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Patents

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Abstract

Phenol and Phenolic substances are very hazardous in nature. The point source of these compounds is mainly industrial effluents. The toxicity of the Phenolic derivatives lasts even in very low concentration (< 10 – 20 mg/L). According to WHO, up to 1 mg/L conc. of Phenol in drinking water is permissible limit. But unfortunately, up to 1000 mg/L conc. of Phenol and its derivatives can be found in the effluents of Pharmaceutical waste water. To reduce the high conc. of Phenol and its compounds, biological treatment method has been adopted. To achieve the goal, soil sample was gathered from the hospital area and six bacterial strains were isolated from that soil via enrichment method to treat Phenol, 4- Chloro Phenol & catechol respectively. After isolation, those strains were morphologically, biochemically characterized and finally phylogenetically identified by involving 16 s rDNA assay. *Brevibacillus formosus* & *Pseudomonas otitidis* were isolated to deal with Phenol. *Bacillus timonensis* & *Bacillus cereus* were isolated to treat 4- Chloro Phenol and *Bacillus pseudomycoides* & *Bacillus paramycoides* were isolated to deal with Catechol. Simultaneously, those strains were continuously acclimated in the Mineral Salt Medium (MSM) to prepare large volume of inoculums as well as to deal with higher conc. of Phenol, 4- Chloro Phenol and Catechol. After that, parameter optimization study was conducted where six parameters were selected to be studied viz. Temperature, pH of the media, Incubation time (residence time), olume of the media, Inoculums size of the strains & Initial conc. of the Phenol or its derivatives. Six parameters were optimized in case of each of the six bacterial strains respectively. Later statistical analysis of those optimized parameters was conducted via Response Surface Methodology (RSM) for all the six microbial strains respectively. Haldane kinetics parameters were estimated in case of the six microbial strains respectively to deduce the inhibition effects of Phenol, 4- Chloro Phenol & Catechol on those strains respectively. Now, as the main focus of the study was to degrade these three Phenolic compounds together (Phenol, 4- Chloro Phenol, Catechol), bi- solute (Phenol & 4- Chloro Phenol, 4- Chloro Phenol & Catechol, Phenol & Catechol) and tri- solute (Phenol & 4- Chloro Phenol & Catechol) mixtures were considered in stead of degrading those compounds singly. To degrade these mixtures, microbial consortiums were prepared by mixing those isolated strains. Same six parameters were taken into account again to optimize their values in case of the four different consortiums while treating with the three bi- solute and a tri- solute mixture respectively. Statistical analysis of those optimized parameters was performed

in case of the four respective consortiums by involving Response Surface Methodology (RSM). Haldane kinetics was studied in case of the four respective consortiums to evaluate the inhibition effects of the bi- or tri- solute mixtures on them. In the next step, only the tri-solute mixture was considered. Up to this step, 1:1:1 ratio was maintained while mixing Phenol, 4- Chloro Phenol & Catechol. Now, the individual concentrations of the three Phenolic compounds were varied in seeral ways while preparing the tri- solute mixture and those mixtures were treated by the particular consortium. Finally, some selected experiments of biodegradation of the tri- solute mixture were performed in very large media volume i.e. 2L to evaluate whether this bioreactor will be effective in the pilot scale. The results were satisfactory indicating the effectiveness of the bioreactor in the field scale.

Key Words: point source, biological treatment, enrichment method, 16srDNA assay**,** *Brevibacillus formosus*, *Pseudomonas otitidis*, *Bacillus timonensis*, *Bacillus cereus*, *Bacillus pseudomycoides*, *Bacillus paramycoides*, Mineral Salt Medium (MSM), parameter optimization, Response Surface Methodology (RSM), Haldane kinetics, inhibition effect, microbial consortium, bioreactor.

Chapter I

Introduction & Review of Literature

In our planet Earth, life was originated in water at first. Also, water is very much essential to support lives on Earth. Around ~71percent of the Earth surface is covered with water. But, out of this total coverage, only ~3percent water is usable to human beings. Rest ~ 97percent water is totally marine and hence, can't be used. Out of this ~3percent of usable water, maximum portion is present in glacial form into the Arctic and Antarctic poles and in mountain tops. Rest portion is present in the form of ground water, river water and others which is used to serve human beings. So it is a clear scenario that the amount of usable or drinking water is very much low in this planet though the total amount of water is infinite. But now-a-days, water pollution has been a big issue on the planet. Water is being polluted throughout the world in different ways e.g. by industrial effluents, by domestic wastes and activities, by municipal wastes, by nuclear wastes and many other ways. The predominant water pollution is caused by the industrial effluents. With exclusive urbanization as well as industrialization, the environmental pollution with anthropogenic organic compounds, became a big issue (Ghisalba O., 1983).

Surface water and ground water has been adversely polluted by the industrial and domestic activities (Armour M.A., 1991). Waste water, released after being used in domestic and industrial purposes, contains several pollutants which are chemically or biologically hazardous and also may be radioactive in nature (Mohanty et al., 2012). These pollutants cause harmful effects to the nature including the living creatures inhabit it. They enter into the water cycle and there by spread in broad scale. The pollutants may enter into the food chain and cause serious injury. As the water is very much valuable to human beings as well as the whole ecosystem, pollutants released from the domestic or industrial sources into the water, can bring severe damages to the ecosystem.

I.1Xenobiotics:

Xenobiotics are referred to the anthropogenic chemicals which are occurred in nature at very high rate with high concentration. These are mainly insecticides, herbicides, various kinds of drugs, carcinogenic compounds etc. These compounds cannot be decomposed in the nature easily and possess long time persistence in the nature (Mohanty et al., 2012).

Among those, Phenol and Phenolic substances are one of the major Xenobiotics found into the nature. Phenol and its derivatives have adverse impacts on the nature. Phenol as well as

the derivatives of Phenol possesses the basic structural unit of a large variety of synthetic organic compounds (Annaduari et al., 2000 and Agarry et al., 2008). Phenolic substances enter into the ecosystem via the wastewater, discharged from the various industries like coking plants, coal processing, petroleum and oil refineries, resins, dyes, paints, production of petrochemicals, varnish, plastic manufacturing, manufacturing of steel, textiles, paper pulps, pharmaceuticals, production of insecticides and pesticides, cosmetics, disinfectants, metallurgy etc (Ghadi et al., 1995, Young et al., 1995, Mahadevaswamy et al., 1997, Bandyopadhyay et al., 1998, Mahesh et al., 1999, Bandyopadhyay et al., 2001, Kim et al., 2002, ATSDR 2003, Kumar et al., 2004, Kumar et al., 2005, Agarry et al., 2008, Edalatmanesh et al., 2008, Mollaei et al., 2010, Zhang et al., 2013, Al- Khalid and El- Naas; 2014, Hasan et al.,2015 and Szczyrba et al., 2016). Now a day, Phenol and its derivatives have become a big issue during the protection of our environment. According to US EPA 1979, Phenol and its compounds have been classified with respect to their adverse impact into the aquatic environment.

I.2 Phenol and its derivatives:

There are many pollutants present in the water e.g.: particulate, soluble, insoluble, organic, inorganic etc. Some of the most hazardous organic pollutants present in the water are Phenol, Chlorophenols, Nitrophenol, Catechol, Cresol etc. The Phenol (C_6H_5OH) and its derivatives stand for the basic structural unit of a variety of synthetic organic compounds (Annaduari et al., 2000, Agarry et al., 2008). These are organic as well as aromatic compounds mainly produced in several industries (Prpich et al., 2005, Agarry et al., 2008).

The chemical name of Phenol is Hydroxybenzene, possessing a hydroxyl group attached to the Benzene ring. Phenol can also be termed as Phenic acid, Carbolic acid or Phenylic acid (Nair et al., 2008). IUPAC name of Phenol is 1- Hydroxyl Benzene. Origin of Phenol can be both natural as well as anthropogenic (Cheela et al., Annaduari et al., 2007). Naturally Phenol can be produced by distillation of coal tar; on the other hand, oxidation of cumene produces more than 95percent of Phenol throughout the world artificially (Mohanty S.S, 2012). Also, Phenol may be formed in the environment by the natural degradation of organic wastes such as Benzene (Agency for Toxic Substances and Disease Registry, 2006). It is whitish in colour, crystalline and soluble in a large variety of organic solvents (ATSDR 2008, EPA 1979). It is volatile in nature to some extent mainly in the normal room temperature (Calabrase et al., 1991). Phenol also possesses a strong odor with a threshold level of 0.04

PPM (Amoore et al., 1983). Phenol may explode above 78^{o}C temperature (Central pollution Control Board, 2016). Physicochemical properties of the Phenol have been displayed in the Table no 1.1.

Table I.1: Physicochemical properties of Phenol (Central pollution Control Board, 2016)

Property	Phenol
Chemical formula	C_6H_5OH
Molecular weight	94.11 gm/mol
Solubility in water	87 gm/L at 25^{o}C
Boiling point	181.7^{o}C
Melting point	40.5 ^{o}C
Auto ignition point	715^{o}C
Flash point	79^{o}C
pK_a	9.95 in water
	29.1 in acetonitrile

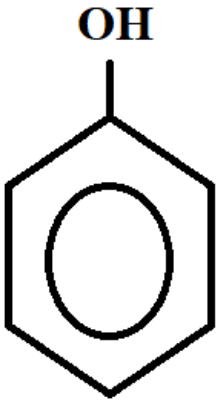


Figure I.1: Molecular structure of Phenol

The chlorinated phenols involve a gathering of 19 congeners, comprising of mono-, di-, tri-, tetra-and penta- Chlorophenol. Chlorinated phenols have moderate instability, empowering them to circulate between air, land, and water. Chlorophenols are the most boundless and the

biggest gathering of phenols (Central pollution Control Board, 2016). Chlorophenols are a group of synthetics where chlorine molecules (somewhere in the range of one and five) have been added to phenol. Chlorophenols are framed in the environment by chlorination of mono and poly aromatic compounds present in the soil and water. The most well-known Chlorophenols are 2-Chlorophenol and 2, 4-dichlorophenol, tri-Chlorophenols, tetra-Chlorophenols and pentachlorophenol (Toxicology, 1999).

4- Chlorophenol is a one of the well known Chlorinated Phenolic compounds. It may also be termed as p- Chlorophenol as Chlorine molecule is present in the para position. The origin of 4- Chlorophenol is mainly anthropogenic. The presence of this compound in the environment is mainly due to the degradation of pesticides, herbicides etc. It is light sensitive as well as heat sensitive in nature. 4- Chlorophenol degrades while heating and thereby produces very hazardous fumes of Hydrochloric acid (HCl) and Chlorine (Cl) (Central pollution Control Board, 2016). Vapour pressure of this compound is 13 pa (Central pollution Control Board, 2016). Physicochemical properties of the 4- Chlorophenol have been displayed in the Table no 1.2.

Table I.2: Physicochemical properties of 4-Chlorophenol (Central pollution Control Board, 2016)

Property	**Phenol**
Chemical formula	C_6H_5ClO
Molecular weight	128.6 gm/mol
Appearance	Solid, white & crystal form
Boiling point	220^oC
Melting point	43^oC
Solubility in water	27.1 gm/L at 25^oC
Flash point	121^oC
pK_a	9.41

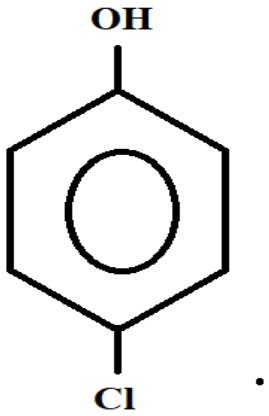


Figure I.2: Molecular structure of 4-Chlorophenol

Catechol is also called Pyrocatechol, 1, 2- Benzene diol, o-Benzenediol, o-Dihydroxybenzene, 2-Hydroxyphenol etc. IUPAC name of the Catechol is 1, 2 – Dihydroxy Benzene. Catechol is white in appearance and crystalline (USCG, 1999). It can be dissolved both in water and many other organic solvents (Central pollution Control Board, 2016). It is mostly soluble in pyridine, chloroform, benzene etc (O' neil, 2013). Naturally, Catechol is occurred in different kinds of food items like apple, onion, and also in some trees like Oak, Pine etc (Marshall et al., 2000, Brenes et al., 2004, Sternitzke et al., 1992, Singh et al., 1994 and McDonald et al., 2001). It is mainly produced by a process of catalytic hydrolysis of 2-Chlorophenol in a very high temperature. Catechol and Chlorocatechols are the main outcomes of the environmental transformation of the Phenols and Chlorophenols (Michalowicz and Duda, 2007). Catechols are delegate items from the degradation of aromatic mixtures and lignin by microorganisms (Crawford; 1981, Van der Meer et al., 1992 and Schweigert et al., 2001). Unhealthy odour and fumes are formed during the combustion of the Catechol (Central pollution Control Board, 2016). Catechol is mainly intermediate product, forms during the degradation of other aromatic compounds and lignin, when microorganisms are involved (Schweigert et al., 2001). Physicochemical properties of the Catechol have been displayed in the Table no 1.3.

Table I.3: Physicochemical properties of Catechol (Schweigert et al., 2001 and Central pollution Control Board, 2016)

Property	Phenol
Chemical formula	$C_6H_5O_2$
Molecular weight	110.112 gm/mol
Appearance	Brownish white crystal or powder
Boiling point	245.5 ^oC
Melting point	105^oC
Solubility in water	430 gm/L
Density	1.34 gm/Cm^3
pK_a	9.45

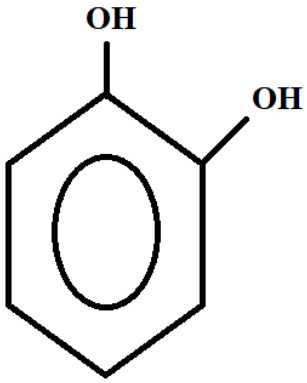


Figure I.3: Molecular structure of Catechol

I.3 Uses of Phenolic compounds:

Pure Phenol is used in various aspects viz. manufacturing of disinfectants, antiseptics, production of ear drops, nose drops and mouthwash, lozenges for throat etc (Hodgson and Wooley; 1991, ATSDR; 2008, Mohanty et al., 2012 and Central pollution Control Board, 2016). Moreover, Phenol is utilized in different sectors such as production of bisphenol A, phenolic resins, Caprolactam, alkylphenols, adipic acid etc. Out of these products, bisphenol A is an important chemical that is used to produce polycarbonates. Cyclohexanol is catalytically reduced form of Phenol, used in the production of polyamides. Thermosetting polymers can also be produced from Phenol. Aniline, also called phenylamine, is produced from Phenol which in turn is used in the manufacturing of dyes (Central pollution Control Board, 2016).

Phenol is used in pharmaceutical industry in a wide scale. Phenol itself used as a safety chemical namely Carbolic acid to drive away snakes. Phenol is also used in the production of antiseptics and slimicide (refers to the chemical which is utilized to kill bacteria & fungi in slimes). Phenol has also usage in the manufacturing of lotions, ointments, salves, creams, shaving soaps (Central pollution Control Board, 2016 and Mohanty ., 2012). In veterinary sector, Phenol is used as the gastric anaesthetic and internal antiseptic. Phenol is very important in case of producing aspirin (Busca et al., 2008). Some oral anaesthetic sprays, used to treat sores of throat, possess Phenol as an important material.

As general disinfectants, Phenol is widely used in different sectors like food, pharmaceuticals, medical sectors etc. Several disinfectants such as Matar, O-Syl, Environ, Septicol, Hexachlorophene, One- stroke, Discan, Pantek, Lysovet etc belong to Phenolic substances. Phenol is extensively effective in case of gram positive bacteria and some enveloped viruses like Corona virus, Pox, Rabies, PI3, Leukemia, BVD, BRS and stomatitis virus (Mohanty., 2012).

Moreover, Phenol is used in glue as peptizing agent, used in oil refineries as extracting solvent, used as a reagent in many chemical analysis, used as a primary petrochemical intermediate (Wang et al., 2012 and ATSDR, 2005).

Role of the mixture of Phenol and Chlorophenol is widely used in cellular biology as the mixture is used to purify DNA and RNA from proteins and also for cell lysis and disruption of cells (Sambrook et al., 2001).

Chlorophenols are widely used in paper pulp industries; 2, 4- dichlorophenols are used significantly as preservatives of wood, important raw materials to produce pesticides, insecticides and herbicides (Stoilova et al., 2006 and Rape C., 1980). Different isomers of Chlorinated Phenols are mainly used in pesticides and insecticides. Some usages of Chloro phenols have been listed below:

Table I.4: Uses of some Chlorophenols (Central pollution Control Board, 2016)

Isomers of Chlorophenols	Uses
2,4- Di Chlorophenol	Herbicide
2,4,5-Tri Chlorophenol	Herbicide
2,4,6- Tri Chlorophenol	Herbicide
2,3,4,5 Tetra Chlorophenol	Fungicide
2,3,4,6- Tri Chlorophenol	Pesticide ,Wood preservatives,
2,4,5,6- Tri Chlorophenol	Fungicide
2,5- Di Chlorophenol	Herbicide , used as chemical intermediate
2,6- Di Chlorophenol	Used as the starting material for the manufacture of Tri Chlorophenols and Tetra Chlorophenols
3,5- Di Chlorophenol	Veterinary medicine
3,4- Di Chlorophenol	Chemical intermediate for 2,3,4- TCP and 2-Chloro 1,4- dihydroxyanthraquinone
O-Chlorophenol	Component of disinfectant , soil sterilant , Organic synthesis of dyes
m- Chlorophenol	Intermediate in organic Synthesis ,catalyst for polymers
p- Chlorophenol	Synthesis of dyes , Pharmaceuticals , solvent in refining mineral oils , bacterial agent

Catechol is utilized in various applications viz. It is utilized as a reagent for photography, colouring material, elastic, plastic creation and medication industries (Merck 1989, Milligan and Hagg- Blom; 1998, Schweigert et al., 2001). It is likewise utilized in cosmetics and preparation of insecticides (Central Pollution Control Board, 2016). Catechol is involved in the manufacturing of 4- tert- buthycatechol which in turn has the ability to restrict the polymerization of synthetics. Also, Catechol is utilized to make different synthetics utilized in drugs and horticultural item (Krumenacker; 2001).

Chlorinated Catechols are used in the manufacturing of dichloro aniline and chlorinated bisphenyl (Central Pollution Control Board, 2016).

Nitrophenol is used as building block of some polymers, medicines and itself used in preservatives and photographies. Also, it is used in some dyes, solvents, production of some explosive things, plastics etc.

Now- a- day, the fundamental use for Bisphenol -A is the development of polycarbonates and epoxide tars (Weber and Weber; 2010).

All the isomers of aminophenols and 2, 4-diaminophenol are utilized in dyes used in colouration of hair (Michalowicz and Duda; 2007). Xylenol is used in antiseptics and disinfectants. Phenlophthalein is used as a pH indicator. Orthophenyl phenol is used as a fungicide. BHT (Butylated Hydroxy toluene) is utilized as a fat soluble anti oxidant and food additive. Picric acid is used to produce explosive materials. 4- Nony phenol is a breakdown product of detergent (Central pollution Control Board, 2016).

Moreover, many Phenolic compounds are used to disinfect many non critical medical devices.

I.4 Hazardous effects of Phenol and Phenolic compounds:

As the aromatic compounds, especially Phenolic compounds, released from different industries are not degraded in nature so easily, they can persist in environment for long term and thereby, can easily stay in the long range food chain and also exhibit bioaccumulation property in case of human as well as animal tissues (Al- Khalid and El- Naas; 2014). Organic pollutants address a likely gathering of synthetic substances that can severely be dangerous to the human health (Chung et al., 2003, Nair et al., 2008, Liu et al., 2009, and Al- Khalid and El- Naas; 2014). So many aromatic compounds possess teratogenic, carcinogenic and mutagenic effects (Zhao et al., 2009 and Al- Khalid and El- Naas; 2014).

Now a day, soil, surface water as well as ground water is being polluted by Phenol and Phenolic substances which has become a burning question to the human civilization (Al-Khalid and El- Naas; 2014).

Phenols and Phenolic compounds show very much toxic effects to the human beings, plants and aquatic organisms (Kumar et al., 2005, Stoilova et al., 2006, Agarwal et al., 2008, Shourian et al., 2009, Jiang et al., 2010, Bakhshi et al.,2011, Tabib et al., 2012 and Al-Khalid and El- Naas; 2014). Phenol is very much toxic to the living organisms in a wide range of concentrations (5 – 2000 mg/L) and has been listed as a priority pollutant (Kasikara- Pazarlioglu and Telefoncu; 2005, Han et al., 2006 and Hasan and Jabeen; 2015). Phenolic compounds are supposed to be extremely hazardous pollutants (El- Naas et al., 2009) and their presence in inland water can be heavily stressful to the aquatic environment (Cheela et al., Agarry et al., 2005 and Kumar et al., 2005). Also these compounds are very difficult to be removed from the nature (El- Naas et al., 2009 and Al- Khalid and El- Naas; 2014). Phenol can enter into the living animals as well as human beings by inhalation, contact and ingestion and exhibit its toxic effects to them. Very much low concentration of Phenol (even 5 mg/L) can be lethal to the living organisms (Kumaran et al., 1996, Alon et al., 2012 and Tabib et al., 2012). 0.005 mg/L concentration of Phenolic derivatives can cause severe damage of the aquatic ecosystem and 0.8 PPM concentration is enough to damage the quality of soil (Djokic et al., 2013 and Szczyrba et al., 2016). 5 – 20 mg/L concentration of Phenol can be toxic as well as lethal to the fish population and sometimes the concentration may be 0.1 mg/L also (Nuhoglu and Yalcin; 2005, Mollaei et al., 2010, Tabib et al., 2012, Hasan and Jabeen; 2015, Szczyrba et al., 2016 and Cheela et al.). The adverse effects of Phenol on human being have been studied by Sax; 1984 and Calabrese and Canyon; 1991. Phenolic compounds show adverse effects to the ecosystem and human health, particularly to the human nervous system (mainly Central Nervous system), kidneys, heart, liver and can immediately be absorbed via skin and mucosa (Olujimi et al., 2010, Khare; 2011 and Szczyrba et al., 2016). In turn, if heart is affected by Phenol, reduction in blood pressure, weak pulse may be resulted (Mohanty; 2012). Also, hypothermia, myocardial misery, blistering on skin, bothering of the eyes, gastrointestinal damages, diarrhea and dark urine excretion are a portion of the impacts detailed by the scientists (Tziotzios et al., 2005, Chakraborty et al., 2010, Olujimi et al., 2010 and Khare; 2011). 1 gm of Phenol may be cause of death of a human being (Kumaran and Paruchuri; 1996 and Prpich and Daugulis; 2005). Phenol contaminated water may be resulted into cancer and changes of blood (Bunce; 1994, Kim et al., 2002, Nuhoglu and Yalcin; 2005 and Bakhshi et

al., 2011). At the concentration of 2 µg/L, which is extremely low, Phenols can impart medicinal smell and taste (Kumar et al., 2005 and Szczyrba et al., 2016).

In the list of EPA (1979), Phenol has been mentioned as a major pollutant. Agarry et al., 2008 has also reported the same. According to US EPA 1979, Phenol and its compounds have been classified with respect to their adverse impact into the aquatic environment. Phenol is harmful even at very low fixations and the toxicity of phenols for microbial cells has been explored by Keweloh et al., 1990, 1998 and Kahru et al., 2002.

Chlorophenols create a major group of the pollutants and mainly found in the effluents of paper pulp industries (Stoilova et al., 2006). Chlorophenols are more toxic than that of the unsubstituted Phenolic compounds (Chacon et al., 2004). Toxicity of the Chlorinated Phenols increases with the increase of the degree of Chlorination as well as lipophilicity of Chlorophenols (Krug et al., 1985, Apajalahti and Salkinoja- Salonen; 1986 and Lee et al., 1994). Due to lack of consciousness, Chlorophenols have widely polluted the groundwater and soil and toxicity of these Chlorinated Phenols severely affect the living organisms (Stoilova et al., 2006). During the accidental ingestion of the Chlorophenols, the whole compound can be entered into the body very quickly. Usually the isomers of mono Chlorophenols don't persist inside the body for a long and excreted within a day after being converted into the less harmful products. But di-, tri- and tetra- Chlorophenols exhibit toxic effects and can persist within the body for some days (Central Pollution Control Board, 2016).

Chlorophenols exhibit severe health issues to the human being such as white necrotic sores in mouth, oesophagus and stomach, headache, vomiting, pain in mouth and throat, unpredictable pulse, hypothermia, shortcoming of muscles, hypotension, weakness, pain in abdomen, permanent damage in lungs and gastro intestinal tract etc (Central Pollution Control Board, 2016). Moreover, mixture of the Chlorophenols or sodium salts of the chlorinated Phenols may show carcinogenicity to animals as well as human beings. Tumours, Sarcoma and lung cancer may be resulted due to these compounds. Based on log octanol coeffiecients and bioconcentration values, it has been revealed that all the isomers of Chlorinated Phenols are very much potential to be accumulated in the aquatic living beings (Loehr and Krishnamurthy; 1988 and Kushalatha et al., 2017).

Catechol is a harmful water pollutant in the environment (Lofrano et al., 2009). Catechol is treated to be more harmful than that of the Phenol (Kumar et al., 2005 and Rigo et al., 2010). Catechol is bothering to eyes, nose and throat. It's ingestion causes trouble in breathing (USCG; 1999). The International Agency for Research on cancer (IARC) has placed Catechol as class 2B Cancer-causing agent (Moussavi et al., 2010). On inward breath of Catechol prompts a burning sensation in the throat and lungs and thusly expansion in the pace of breathing (Bingham; 2001). Catechol might deliver harmful toxic gases when heated (USCG; 1999). Catechol is harmful to fish at 5-25mg/L (Kumar et al., 2005 and Rigo et al., 2010). Catechol prompted DNA strand breaks, quality change, chromosomal variation and transformation of cell in non human mammalian cells regardless of metabolic actuation (Brandt; 1986 and doCeu silva et al., 2003).

Table I.5: List of some Phenolic Compounds according to their hazardous property (*CPCB, Ministry of Environment, Forests and Climate Change, August 2016*)

Phenols and its compounds	Nature
Phenol	Corrosive
2- Chlorophenol	Toxic
3- Chlorophenol	Non toxic
4- chlorophenol	Toxic
o- cresol	Toxic and corrosive
p- cresol	Toxic and corrosive
m- cresol	Toxic and corrosive
2,4- Di Chlorophenol	Toxic
2,5- Di Chlorophenol	Toxic
3,5- Di Chlorophenol	Toxic
Hydroquinone	Toxic
Penta Chlorophenol	Toxic
2,3,5,6- tetra chlorophenol	Toxic

For the vast hazardous effects of the Phenol and its derivatives, several norms have been implemented throughout the world. As per environmental protection rules of the Central pollution Control Board and IS: 2490 – 1974, the maximum permissible limit of Phenol in inland water should not exceed more than 1 mg/L and in case of public sewers (IS:3306 –

1974), it should be 5 mg/L (Cheela et al., Lathasree et al., 2004 and Saravanan et al., 2009). The World health Organization (WHO) has permitted maximum 1 mg/L Concentration of Phenol in drinking water due to the unfavourable effects of the Phenol and its substances (Saravanan et al., 2008, Wang et al., 2010 and Bakhshi et al., 2011). Many countries around the world have specified the maximum permissible concentration of the Phenolic substances which is to be < 1mg/L in the effluent streams (Bapat et al., 2008, Kusic et al., 2011 and Al-Khalid and El- Naas; 2014). The legislation of United Arab Emirates has limited the concentration of total Phenolic compounds to 0.1 mg/L in the industrial effluents, which is discharged in the ocean (Al- Zarooni and Elshorbagy; 2006 and Al- Khalid and El- Naas; 2014).

But unfortunately, concentrations of Phenolic compounds can be found as excessively high in the effluents, discharged from different industries (as shown in the Table no. 1.6).

Table I.6: Concentrations of some Phenolic substances, discharged from various industries (Busca et al., 2008)

Industry	Concentration of the Phenolic substances (mg/L)
Coak	Up to 3900
Coal	Up to 6800
Petrochemicals	Up to 1200
Oil refineries	Up to 500
Pharmaceuticals	Up to 1000

I.5 Treatment methodology to remove the Phenolic compounds:

The above scenario reveals that very low concentration of Phenolic compounds have the ability to exhibit toxicity to the environment, living creatures (both terrestrial and aquatic) and human beings also. Moreover, Phenolic substances are discharged and mixed along with the effluents to the nearest water bodies, rivers or oceans from different industries in very high concentrations as shown in the above (Table no. 1.6). Therefore, it is very much important to treat the wastewater, contaminated by Phenol and Phenolic compounds to deal with this burning environmental issue. Several treatment pathways have been

suggested by different researchers throughout the world (klein and Lee; 1978, Koyama et al., 1994, Danis et al., 1998, Reardon et al., 2000, Backhaus et al., 2001 and Ajay et al., 2004). The treatment method may be, physical or chemical or biological or the combination of the above mentioned types but the maximum degradation of the Phenolic substances should be achieved so that the concentration of those harmful compounds in the water will be reduced to the permissible levels as guided by the different health and environmental organizations throughout the world including WHO (World Health Organization).

I.5.1 Physico- Chemical treatment methods:

Different kinds of Physico- Chemical treatment methods are there such as ion exchange, wet oxidation, adsorption, chemical oxidation, flotation and coagulation, reverse osmosis and so on. By applying those methods, Phenolic compounds may be removed from the liquid solutions including water.

I) Chemical oxidation:

Chemical treatment includes the utilization of synthetic reagents to totally obliterate or convert the pollutants to innocuous or less poisonous mixtures, or intermediates that can further be degraded by microorganisms (Hamby D. M., 1996). Usually strong oxidants are used in this procedure as reagents. The most common oxidant, used in this process is Hydrogen peroxide (H_2O_2) (Dias- Machado et al., 2006 and ksibi; 2006).

II) Ion exchange:

The fundamental tenet of the ion exchange method of xenobiote degradation is the degradation of one ion from an aqueous solution by substituting another ionic species. For the purpose of purifying and decontaminating the industrial effluent, ion exchange is performed using natural and synthetic materials that are particularly created to permit ion exchange activities at high levels. According to Zorpus et al., 2010, porosity, density and capacity of adsorption are the main features of the ionic resins.

I.5.2 Limitations of the Physico- Chemical techniques:

Some secondary toxic intermediates are formed in many times while performing the physico- chemical techniques and also these methods are costly (klein and Lee; 1978). Complete degradation cannot be achieved and so, further treatment methods are required even after performing these methods.

In case of ion exchange method, the ion exchange resins are too costly to be utilized easily. Also, resins are pollutant specific in nature i.e. one particular resin removes usually one particular pollutant. According to Caetano et al. (2009), ion exchange resins can only remove phenol in an alkaline media while non-functionalized resins can remove the most amount of phenol in an acidic medium.

Instead of being completely removed from the wastewater, phenol is selectively transported into the solid phase (adsorbent) while performing adsorption and so, it generates a lot of solid waste once more, which needs to be disposed of safely. Furthermore, in case of the chemical oxidation process, chemicals are highly cost effectives. Moreover, some toxic by products can be produced during this technique. Emission of harmful gases is also a drawback of this technique (Jena et al., 2005).

Due to these limitations of the physico- chemical processes, biological treatment method can be considered to be an alternative to deal with the treatment of wastewater, containing Phenol and Phenolic derivatives as no harmful end products are formed (Banerjee et al., 2001 and abuhamed et al., 2004).

I.5.3 Bioremediation:

Bioremediation refers to the biological procedure by which toxins or hazardous wastes are captured from nature by some microorganisms (I.e. bacteria, algae etc) and used in their physiological and cellular processes without causing any harmful effects to them (Glazer et al., 1995 and Megharaj et al., 2011). In short, it is a remedy to reduce the wastes from the nature involving the biological mechanism. The principle of microbiological infallibility states that all naturally existing chemicals can be broken down by microorganisms (Alexander; 1965).

I.5.3.1 Types of Bio remediation:

There are several types of bio remediation. Based on the biotic factors involved in this process, it can be classified mainly in three (3) types.

I) Microbial remediation: Refers to the bio remediation where microorganisms are involved such as bacteria. Here the pollutants are mainly used as their energy sources.

II) Phytoremediation: Refers to the bio remediation procedure where floral species are incorporated to bind and extract hazardous materials. E.g. Pesticides, herbicides are removed by phytoremediation.

III) Mycoremediation: Refers to the bio remediation process where fungal species are involved. E.g. Hydrocarbons and some heavy metals can be removed by mycoremediation.

I.5.3.2 Advantages of bioremediation:

Bioremediation is widely accepted throughout the globe mainly because of its eco-friendly nature. It is economically helpful, usually no harmful by products are formed rather less- toxic end products are formed (Chang et al., 1998, Aleksieva et al., 2002 and Nweke and Okpokwasili; 2014). Particularly in case of bioremediation of Phenol & Phenolic derivatives, complete degradation can be achieved by the microbes and thereby, the final concentration of the compounds decrease below their permissible limit, suggested by different health and environmental organizations around the world. According to El- Naas et al., 2009, Liu et al., 2009 and Al- Khalid and El-Naas; 2014, biological treatment methods are more acceptable because there are chances for complete mineralization of Phenolic compounds.

I.6 Microorganisms, involved in the bioremediation of Phenol and Phenolic substances:

In the last two or three decades, several microorganisms, including bacterial strain, algae, fungi have been isolated by many researchers throughout the world. These microorganisms mainly use the Phenolic substances as their carbon source. Many microorganisms are vulnerable to the antimicrobial properties of Phenolic compounds and so, the bioremediation of Phenolic compounds is often obstructed (Yap et al., 1999 and

Ahuatzi - Chacon et al., 2004). However, there are some microorganisms that can breakdown Phenolic compounds and are resistant to those compounds.

Several experiments have been carried out regarding the bioremediation of Phenol and Phenolic substances both in aerobic and anaerobic situation (Harwood and parales; 1996, Caldeira et al., 1999 and Ahuatzi - Chacon et al., 2004). There is a large variety of microbes which are able to remove Phenol and Phenolic compounds. Those are *Alcaligenes* sp. (Schwien et al., 1982, Hughes & Bayly; 1983 and Hill et al., 1996), *Acinebacter* sp. (Paller et al., 1995, Abd- El- Halim et al., 2003), *Arthrobacter* sp. (Baradarajan et al., 1995 and Westerberg et al., 2000), *Alcaligenes eutrophus* (Leonard and Lindley; 1998), *Pseudomonas* sp. (Knackmuss et al., 1978, Kiyohara et al., 1992, Armenante & Kafkewitz; 1995 and Bandyopadhyay et al., 1998), *Comamonas* sp. (Hollender et al., 1994), *Bacillus stearothermophilus* (Buswell; 1975 and Gurujeyalakshmi & Oriel; 1989), *Rhodococcus* sp. (Briglia et al., 1996, Moiseeva et al., 1999), *Ralstonia* sp. (Steinle et al., 1998), *Nocardioidess* (Cho et al., 2000), *Psedomonas aeruginosa* (Oboirien et al., 2005 and Ojumu et al., 2005), *Pseudomonas fluorescens* (Oboirien et al., 2005 and Ojumu et al., 2005), *Pseudomonas pictorum* (Annadurai et al., 2000), *Pseudomonas putida* (Hill & Robinson; 1975, Yang & Humphrey; 1975, Bettman & Rehm; 1984, Hinteregger et al., 1992, Gotz & Reuss; 1997 and Reardon et al., 2000), *Pseudomonas resinovorans* (Dikshitulu et al., 1993 and yang & Lee; 2007), *Ralstonia eutropha* (Leonard et al., 1999), *Streptomyces setonii* (Antai & Crawford et al., 1983), *Azotobacter* sp. (Wieser et al., 1994), *Arthrobacter ureafaciens* (Bae et al., 1996) etc. The most of the above mentioned bacterial strains are mesophilic in nature. All of these strains are aerobic bacteria and remove Phenol and Phenolic compounds aerobically. *Desulfobacterium phenolicum* sp. is an anaerobic strain which was used by Bak & Widdel in 1986 to degrade Phenol.

Furthermore, the degradation of Phenolic substances mainly Phenol and Chlorophenols, has been reported for algae (Semple & Cain; 1997, semple et al., 1999 and Lovell et al., 2002), filamentous fungi (Yadav & Reddy; 1993 and Zouari et al., 2002), Yeasts (Ivoilov & Karasevich; 1983, krug et al., 1985, Polnisch et al., 1991, Katayama- Hirayama et al., 1994, Kurtz & Crow; 1997 and Shivarova et al., 1999). Mostly studied Yeast strains are *Candida tropicalis* (Ivoilov & Karasevich; 1983, Ehrhardt & Rehm; 1985, Krug et al., 1985, Ruiz- Ordaz et al., 1998, 2000, 2001, Shivarova et al., 1999, Juarez- Ramirez et al., 2001 and Ahuatzi- Chacon et al., 2004), Candida *maltosa*, *Fusarium flocciferium* (Anselmo et al., 1985 and Mendoca et al., 2004), *Trichosporon oivide* (Polnisch et al.,

1991 and Kurtz & Crow; 1997), *Trichosporon cutaneum* (Neujahr & Varga; 1970, Neujahr & Gal; 1973, Yang & Humphrey; 1975, Neujahr & Kjellen; 1978 and Gaal & Neujahr; 1980),*Rhodotorula glutinis* (Katayama- Hirayama et al., 1994), *Fusarium* sp.(Anselmo & Novais; 1992, Cai et al., 2007 and Li et al., 2011), *Aspergillus* sp. (Jones et al., 1995 and Stoilova et al., 2006), *Graphium* sp. (Santos et al., 2003) etc.
Among all these microorganisms, Pseudomonas is an important genus which comprises a large number of bacteria, able to remove pollution load from the nature (Agarry et al., 2008). The presence of hazardous chemical substances can be adapted by microorganisms utilising a variety of adaptation processes. The most significant responses of bacteria among the adaptive mechanisms are changes in the fatty acid content of membrane lipids (Neumann et al., 2004). The isomerisation of cis-unsaturated fatty acids to trans-unsaturated fatty acids is one adaptation mechanism that allows some *Pseudomonas* bacteria to flourish in the presence of membrane-disrupting substances.

Pseudomonas pictorum NICM-2077, a strain that is efficient in the biodegradation of phenol, was employed by Balan et al. (1999). The microorganism was cultivated on several nutritional compounds which protect it when confronting shock loads of concentrated harmful contaminants during waste water treatment. An artificial neural network (ANN) model was created to predict phenol degradation after research on the impact of glucose, yeast extract, (NH4)2SO4, and NaCl on phenol degradation. After that, the network model was contrasted with an MRA model created using the same training data.

Kim et al., (2002) performed a study with mixed culture in shake- flasks and a packed bed reactor (PBR). *Pseudomonas testosterone* CPW301 was able to degrade Phenol and 4-Chlorophenol where degradation rate was stimulated by Phenol as it helped to increase the biomass of the strain which indeed, stimulated the degradation of 4- Chlorophenol. *Pseudomonas solanacearum* TCP114 degraded 2, 4, 6- trichlorophenol. Finally, a mix culture of *Pseudomonas testosterone* CPW301 and *Pseudomonas solanacearum* TCP114 degraded Phenol, 4- Chlorophenol and 2, 4, 6- trichlorophenol together, completely and here no inhibition effects of the substrate to the strains were observed. The degradation in a PBR was much better than a continuously shook reactor. However, PBR was not suitable for aerobic microorganisms.

Ahuatzi – Chacon et al., (2004) deployed *Candida tropicalis* to perform the kinetic study of biosynthesis of some enzymes such as Phenol hydroxylase and Catechol 1, 2 – dioxygenase. During the growth of the *Candida tropicalis* on Phenol or Catechol or resorcinol, the maximum level of specific activity of the Phenol hydroxylase (EC. 1.14.13.7) and Catechol 1, 2 – dioxygenase (EC. 1.13.11.1) were achieved with Phenol. The yeast cells displayed clear peaks of distinct activity of both the enzymes with respect to the three aromatic compounds examined at certain incubation periods. The cells, which were induced with Phenol as well as possess high levels of both the enzymes, were able to degrade 4- Chlorophenol and 2, 6- dichlorophenol very fast. Comparatively, less efficiency was recorded while degrading pentachlorophenol.

Kumar et al., (2005) involved *Pseudomonas putida* MTCC 1194 to degrade Phenol and Catechol in basal salt medium. Here, the strain was acclimatized for three months and after that, the strain was able to degrade Phenol and Catechol when their initial conc. was 1000 mg/L and 500 mg/L respectively in shake- flasks method. Residence time was 162 hours and 94 hours respectively. Here Phenol and Catechol both showed inhibitory effects. Haldane's growth kinetic model was fitted to the growth kinetics data of this study.

Arthrobacter citreus was isolated from a hydrocarbon-contaminated environment, and Karigar et al. (2006) investigated its capacity to use phenol as its only carbon source. According to the phenol degradation studies they conducted, the chemical was degraded completely in about 24 hours. Phenol was only capable of being metabolised by the organism up to a maximum starting concentration of 22 mM; higher concentrations were inhibiting.

Stoilova et al., (2006) conducted a study where mycelium of *Aspergillus awamori* NRRL 3112 was involved to degrade very high concentrations of Phenol, Catechol, 2, 4-dichlorophenol and 2,6- dimethoxyphenol. *Aspergillus awamori* was able to completely degrade 1 gm/L Phenol within 7- 8 days, 3 gm/L Catechol within 124 hours and 1 gm/L 2, 6- dimethoxyphenol for 7 days. Also, it was able to degrade 85percent of, 2, 4-

dichlorophenol within 6 days. Haldane model was fitted to the growth kinetics data of this study.

Cheela et al., performed a similar experiment where biodegradation of Phenol was done by using pure and mixed culture in ambient room temperature and almost neutral pH. 100 mg/L concentration of Phenol was degraded in 96 hours and 60 hours by pure culture and mixed culture respectively while, 156 hours and 102 hours of incubation was obtained by the pure and mixed culture respectively when the initial concentration of Phenol was increased up to 200 mg/L.

Agarry and Solomon; (2008) conducted microbial degradation of Phenol in batch, by involving *Pseudomonas fluorescence*. Range of the initial conc. of synthetic Phenol in water was maintained between 100 – 500 mg/L and its effect was studied on the biodegradation procedure. Increase in the initial conc. of Phenol from 100 to 500 mg/L resulted into decrease in the rate of degradation. The achievement of the complete degradation was increased from 84 hour to 354 hour with increase in initial Phenol conc. The inhibitory effect of phenol was demonstrated by data fitting into the Monod kinetic model. Up to an initial phenol concentration of 500 mg/L, the kinetic parameters have been determined. As phenol content grew, the rs_{max} fell and K_s rose. The Pseudomonas fluorescence exhibited good potential for using in the bioremediation of phenol waste effluents; according to the bio kinetic constants calculated using the Haldane model.

Cordova-Rosa et al. (2009) revealed about the time-course execution of a phenol degrading native bacterial consortium, and of *Acinetobacter calcoaceticus* var. *anitratus*, isolated from a modern coal wastewater treatment plant. The bacterial mixture had the option to get by within the sight of phenol fixations as high as 1200 mg/L and the consortium was quicker in disposing of the phenol than a pure culture of the *A. calcoaceticus* strain. Very high biodegradation of phenol (above 95percent) by the blended culture in a bioreactor was acquired in both nonstop and batch frameworks. When the same test was done in coke gasification wastewater, no biodegradation was seen following 10 days at pH 9-11 for both the pure strain and the secluded consortium.

Hank et al., (2010) performed a factorial experimental design study where *Pseudomonas aeruginosa* ATTC27853 was involved to degrade Phenol in aerobic batch culture. At first, three parameters were taken into account viz. adaptation of the strain to the Phenol, temperature and nature of the bacteria. It was revealed that 100 mg/L Phenol can be degraded in 30^{o}C temperature. Regeneration of the strain pushed the reactivation of its activity of enzyme which revealed that degradation of 100 mg/L conc. of Phenol at 30^{o}C needed at least 50 hours time span with revivified strains. Adaptation in the increasing conc. of Phenol permits the strain to remove 400 mg/L of Phenol within 350 hours. Furthermore, a substrate degradation model was deduced by deploying factorial design where temperature (30^{o}C to 40^{o}C) and size of the inoculums (260.88 to 521.76 mg/L) were considered. Results were processed via t test, variance analysis and F test. Both the values of R^2 and adjusted R^2 were close to 1 (0.99872 and 0.9962 respectively) indicating a good relationship between expected and observed values. The statistical model was found to be significant as the F- value was greater than 200.

Rigo et al., (2010) used a yeast strain of *Candida parapsilopsis* while degrading Catechol. The strain was acclimatized and it degraded 910 mg/L Catechol completely within 48 hours, at the temperature of 30^{o}C. Haldane's model validated the experimental data of growth kinetics here.

Tabib et al., (2012) performed a biodegradation study of Phenol by involving *Ralstonia* sp. strain PH-S1, which was isolated from oil- contaminated soil. The isolation process was successfully done by screening and enrichment methods in mineral medium which contained 100 mg/L of Phenol as the source of carbon. The growth of the strain was outstanding when the range of the pH was in between 4 to 9 and the range of the temperature was in between 30^{o}c to 40^{o}C. It was found that, the strain had the ability to grow well when the concentration of the NaCl was 10percent while studying the effect of conc. of NaCl on the growth ranging from 10percent to 20percent. 1100 mg/L of Phenol was degraded by *Ralstonia* sp. strain PH-S1 in the above mentioned optimum environmental parameters.

Bakhshi et al., (2011) investigated growth kinetic models for biodegradation of Phenol by using Pseudomonas putida strain PTCC 1694 in a batch culture. It was grown in facultative anaerobic situation at 27^{o}C temperature and neutral pH. Initial conc. of Phenol was varied in the range between 300 to 1000 mg/L. Study was conducted for 7 days with

where samples were examined every day. Rate of the Phenol degradation increased up to 500 mg/L initial conc. and beyond that, rate of the biodegradation slightly decreased because of the inhibition effect of Phenol. Logistic and Haldane model validated the experimental data. The yield coefficient of biomass at conc. of 300, 500, 700 and 1000 mg/L were respectively 0.177, 0.062, 0.035 and 0.012.

Wang et al., (2012) isolated *Candida tropicalis* W1 by enrichment with Phenol and used to degrade Phenol and 4- Chlorophenol in liquid medium where those two Phenolic compounds were utilized by the strain as carbon sources. The strain was capable of degrading 900 mg/L Phenol within 30 hours but no degradation was achieved in case of 4- Chlorophenol. However, the strain degraded Phenol and 4- Chlorophenol together within 20 hours when the concentration of each of the compounds was 150 mg/L indicating that 4-Chlorophenol can be degraded in the presence of Phenol.

Aravindhan et al., (2014) conducted a biodegradation study of Phenolic compounds by using a mixed microbial culture of *Pseudomonas aeruginosa* and *Bacillus subtilis*. Optimum pH and temperature for the growth of the culture was found to be 7 ± 0.2 and $37 \pm 2^{o}C$ respectively. 250 mg/L of Phenol and Wattle was completely degraded by the culture within 36 and 48 hours respectively. A vast range of initial conc. of Phenol and Wattle was deployed to determine the growth kinetics of the mixture by using Haldane's model. Haldane model sufficiently described the degradation of the Phenolic compounds by the mixed bacterial culture where both the Phenol and wattle was found to be inhibitory compounds. Decay coefficient for the growth of the culture for Phenol and wattle was deducted to be 0.0069 h^{-1} and 0.0082 h^{-1} respectively.

Al- Khalid and El- Naas; (2014) studied an aerobic biodegradation where Phenol and 2, 4- dichlorophenol was removed by *Pseudomonas putida* which was immobilized in Polyvinyl Alcohol gel pellets in a bioreactor of bubble column. Here, the strain was acclimatized with phenol and 2, 4- dichlorophenol up to 200 mg/L concentrations with and without glucose. When the initial conc. of the Phenol was 25 – 200 mg/L, a very good result was achieved in the entire conc. by the bacteria which was not acclimatized by glucose than the strain. On the other hand, in case of 25 – 200 mg/L conc. of 2, 4- dichlorophenol, slightly better performance was obtained by the strain, acclimatized by

glucose, was noticed. It indicates that when the glucose was absent in the media, it left either significant effect or no effect in the degradation capacity of *Pseudomonas putida*.

Hasan and Jabeen; (2015) performed a similar experiment by using *Pseudomonas aeruginosa* IES- Ps-1, *Pseudomonas* sp. IES-S and *Bacillus subtilis* IES-B which were able to tolerate 400, 700 and 500 mg/L of Phenol respectively. *Pseudomonas aeruginosa* IES- Ps-1 removed 700, 900 and1050 mg/L Phenol within mineral salt medium (MSM) with a rate of 0.034, 0.075 and0.021 h^{-1} respectively. All the three strains developed by making clusters when presented to Phenol to prevent the harms because of the high substrate conc. These strains changed Phenol into Catechol which in turn, was debased by means of ortho- cleavage pathway. Phenol was degraded according to Monode kinetics during the growth of cell mass in nutrient medium and MSM medium.

Szczyrba et al., (2016) conducted a study to degrade Phenol aerobically in a batch reactor by using *Stenotrophomonas maltophilia* KB2 strain. The experiment mainly focused on the optimization of various process parameters for the cell growth and to deduct an equation which will describe the growth rate of cell as well as the rate of biodegradation of Phenol. Phenol was utilized here as sole carbon and energy source in different initial concentrations in the range between 25 – 500 gm/m^3. 30^oC temperature and 7 pH were found to be optimum in this study. Kinetic experiments were performed in this optimized conditions and Haldane inhibitory model validated the experimental data accurately. The obtained coefficient of biomass yield $(Y_{xs})_{abs}$ was found to be 0.614 and coefficient of endogenous decay (k_d) was 0.05 h^{-1}.

Kushalatha et al., (2017) isolated *Providencia* sp. CJ-3 from contaminated sediment of soil via enrichment method, to degrade 4- Chlorophenol. The metabolic pathway was studied here by involving 4- Chlorophenol as a carbon source. The growth response of the bacterium was observed as increase in the OD value at 660 nm. Accumulated metabolites in the medium during the growth of the strain were separated and characterized. *Providencia* sp. CJ-3 removed 4- Chlorophenol by the activity of Chlorophenol- NADPH- Oxidoreductase. The activity of Chlorophenol- NADPH- Oxidoreductase was assayed and the specific activity was 0.84 µmoles/min/mg of protein.
Parvathy and Prabhakumari; (2017) isolated eleven bacterial strains from cashew industrial soil. Out of them, *Pseudomonas aeruginosa* was found to remove Catechol

when the initial conc. was 100 mg/L within 96 hours. Optimization of parameters was considered by them. pH and temperature was taken into account for this study. Five different pH (5, 6, 7, 8 & 9) and five different temperatures (10, 20, 30, 40 & 50)^{o}C were applied in the experiment where most suitable pH and temperature were found to be 7 and 30^{o}C respectively.

Das and Dey; (2020) separated *Pseudomonas* sp JB163 to perform the biodegradation study in case of Para- Nitrophenol. 16 srRNA assays was carried out to identify the strain and two parameters were considered to reveal the best performance of the strain. pH and temperature were those parameters. 90percent degradation of the Para- Nitrophenol was obtained when the initial conc. of the Para- Nitrophenol was 85 mg/L. Favourable temperature and pH for the survival of the strain were found to be 30^{o}C and 8.5 respectively.

I.7 Mechanism of Biodegradation:

I.7.1 Aerobic degradation:

When phenol is broken down by microorganisms in aerobic conditions, the process is started by oxygenation, and the aromatic ring is first monohydroxylated by a phenol hydroxylase at an ortho location to the already-existing hydroxyl group to produce Catechol. This is the primary intermediate produced when various microbial strains break down phenol. The Catechol then goes through a ring cleavage, which, depending on the strain, can happen either at the ortho position, launching the ortho pathway that produces succinyl Co-A and Acetyl Co-A, or at the meta position, launching the meta pathway that produces Pyruvate and Acetaldehyde. Ghadi and sangodkar; (1995) revealed the meta cleavage pathway of the Phenol biodegradation while Hasan and Jabeen; (2015) have described the ortho cleavage pathway.

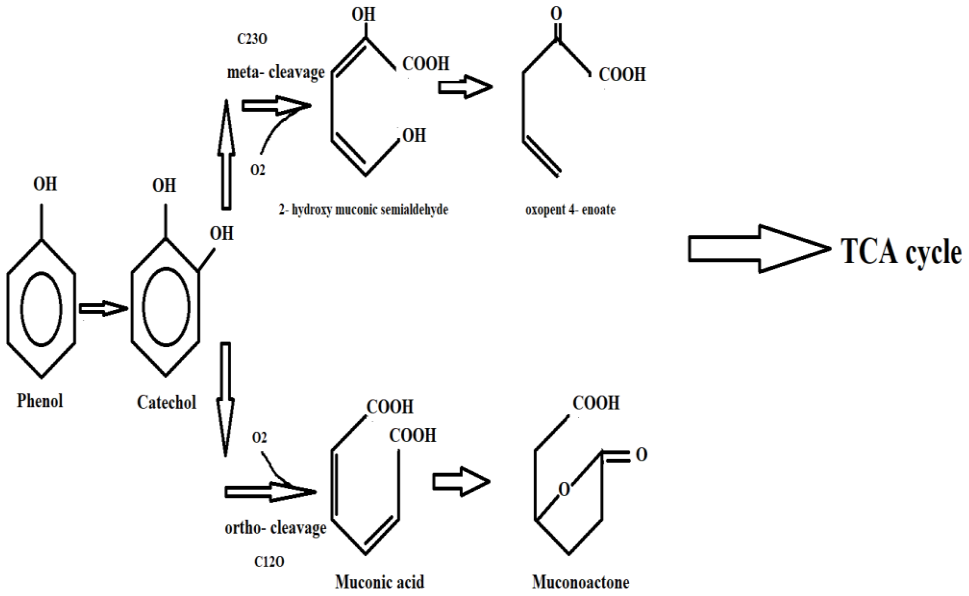


Fig I.4: Metabolic pathways of degradation of Phenol (Hasan and Jabeen; 2015)

I.7.2 Anaerobic degradation:

The carboxylation of phenol starts the anaerobic breakdown process. It is occurred through two steps. The first step is the phosphorylation of the Phenol from an unidentified phosphoryl donor which is catalysed by phenyl phosphate synthase. In the second step, carboxylation of the phenyl phosphate took place via phenyl phosphate carboxylase and thereby, 4- hydroxybenzoate formed.

Thauera aromatica, a denitrifying bacterium, has been used to study the microbial breakdown of phenol in anaerobic environments (Aresta et al., 1998, Lack and Fuchs, 1992 & 1994).

I.8 Scope of the study:

A review of the literature found that significant research has been done on the biodegradation of phenol & Phenolic compounds and the identification of the genes encoding the enzymes involved in the degradation pathways. Several parameters have also been used to optimize the favourable conditions of the strains. However, degradation of more than one Phenolic compound at a time was hardly found. In some literature it has been found that, 7 to 8 days are required to degrade the Phenol (or Phenolic compounds) completely. Moreover, only two or three physico- chemical parameters were considered in previous studies and also, the interaction effects of those parameters were hardly

investigated. Furthermore, maximum degradation studies were performed in small scale, rather than pilot scale. Based on these gaps, the scope of the present study is:

1. Isolation and identification of suitable bacterial strains from enriched soil capable of removing Phenol and Phenolic Compounds.

2. Biodegradation of microbial culture for treatment of Phenolic Compounds in batch reactor.

3. Optimization of process parameters (i.e. pH, temp, percent of inoculums, age of inoculums, time, initial conc. of Phenolic compounds etc.) using isolated strains in batch reactor.

4. Kinetic study & determination of kinetic constants including inhibition constant during batch study for Phenolic compounds as a single substance & bi-substrate conditions.

5. Verification of different substrate inhibition models (Haldane, Han- Levenspiel, Luong, Edward, Yano- Koga, Sum kinetic model) for biodegradation of Phenolic Compounds.

6. Factorial design studies for examining the effects of interaction of different Phenolic Substances.

7. Design and fabrication of bioreactor on the basis of the process parameters obtained in the batch study

8. Performance and kinetic Study for biological degradation of Phenolic compounds in a bio reactor.

9. Development and validation of kinetic model and Response Surface Methodology (RSM) , artificial neural network (ANN) etc.

I.9 Objectives of the study:

In this study, Phenolic substances, found in the effluents of the pharmaceuticals industries, is to be removed. A number of Phenolic substances may be occurred in pharmaceutical effluents. That is why, three major compounds was selected for the study. Those are Phenol, 4- Chloro phenol and Catechol.

Based on the review of available literature, the objective of the present research includes:

1. Isolation & identification of predominant bacterial species from soil or media enriched with Phenol or 4- Chloro phenol or Catechol and to explore the kinetic data for design

and performance of a bioreactor for Phenolic compounds bioremediation either as single substrate (i.e. Phenol, 4- Chloro phenol & Catechol) or bi-substrate mixture (i.e. Phenol+ 4- Chloro phenol, Phenol+ Catechol, 4- Chloro phenol+ Catechol etc) or tri- substrate mixture (Phenol+ 4- Chloro phenol+ Catechol).

2. In the subsequent phase of the research, the experimental results to be obtained have been used to validate various mathematical and statistical model such as Artificial Neural Network (ANN) & Response Surface Method (RSM) by using process parameters.

Chapter II

Isolation & Identification of microorganisms

Introduction:

Aromatic hydrocarbons have become a burning question to the human civilization because of their severe hazardous effects to the aquatic and terrestrial organisms as well as human beings. Phenol, 4- Chlorophenol and Catechol, these three compounds belong to this class. Individually, these three compounds are discharged from different industries like petrochemicals, oil refineries, paper pulp, cosmetics, herbicides and pesticides, textile industries, plastic industries, paint manufacturing industries etc (Nweke and Okpokwasili ,; 2014). But from the pharmaceuticals, these three compounds are discharged together along with many other hazardous components. According to WHO, the maximum permissible level of the Phenolic mixture in drinking water should not exceed 1 mg/L (Yan et al., 2006). So, now-a day, to save the living aquatic creatures and thereby to protect and balance the natural ecosystem of this planet, industries should treat their effluents before the disposal in the nature (Al- Khalid and El- Nass; 2014). There are many physicochemical processes to remove Phenol and its derivatives from the contaminated water but bioremediation is the best treatment method because of its cheapness, eco- friendly nature and moreover, complete degradation can be achieved. Many researchers such as Santos et al., 2003, Collins et al., 2005, Dursun and Tepe; 2005, Marrot et al., 2006, Celik et al., 2008, Laowansiri et al., 2008, Shen et al., 2009, Chakraborty et al., 2010, Mohanty and Jena; 2017 isolated and identified several strains to remove Phenol and Phenolic compounds from the contaminated water.

In the current study, three Phenolic components have been selected to be removed. Those were Phenol, 4- Chlorophenol and Catechol. For each of the compounds, two different bacterial strains were isolated and selected and thereby, six different strains were isolated for the three contaminants to treat them. In case of the each compound, two strains were isolated by two different techniques i.e. one strain was isolated by media enrichment technique and the other one was isolated via soil enrichment technique. Details of the methods & materials, and research findings have been illustrated in three different sub chapters.

Sub chapter IIA

Isolation & Identification of two bacterial strains to remove Phenol

IIA.1 Materials and Method:

Isolation of the bacterial strains was made via media enrichment and soil enrichment technique followed by plate culture. The individual colonies, appeared in the petriplates, were transferred to the individual slants. For the screening of the isolated strains and thereby, to select the two most potent bacterial strain, a fermentation medium was prepared (2.2 Medium preparations). To prepare the inoculums and to keep the isolated strain in continuous culture for the acclimatization and to perform the further experiments, Mineral Salt Medium (MSM) was utilized (2.2 Medium preparations).

IIA.1.1 Reagents and Chemicals:

In this study, Phenol and other chemicals which were utilized belonged to analytical grade. Peptone, beef extract, agar, inorganic salt etc belonged to reagent grade.

IIA.1.2 Collection of the Soil sample:

For the isolation of the organisms, the soil sample was gathered from South Howrah State General Hospital as different kind of bacterial species may be found in the soil sample of a hospital area.

IIA.1.3 sampling procedure:

Soil sample was collected from two different points in the collection site. Removing the upper layer of the soil up to 2 cm, soil profile up to 10 cm was sampled. Sampling was performed by using a sterilized knife and the sampled soil was poured into a clean and disinfected plastic bag. The two sub samples were then mixed with each other and stored in ambient room temperature.

IIA.1.4 Preparation of the media:

IIA.1.4.1 Media for plate and slants:

For the plate and slant culture, media composition used was (g/L): Peptone: 5.0, Beef extract powder: 3.0, agar: 30, pH: 6.8- 7.2. All the ingredients other than agar were solubilised into the distilled water and pH was balanced in the range of 6.8–7.2 using 1 N solution of hydrochloric acid (HCl) and sodium hydroxide (NaOH) drop by drop. At that point, the agar was included in the mixture and in turn, the whole mixture was melted in water bath. 5 ml of the melted medium was transferred to each test tubes, cotton plugged, wrapped with brown paper and sterilized at 121ºC and 15 psig for 15 min. 15 ml of liquid medium was taken in the test tubes for the use in the Petri plate, cotton plugged, wrapped with brown paper and sterilized.

IIA.1.4.2 Media for screening:

Similar medium composition was used (excluding the agar only) for screening – Peptone: 0.5percent; Beef extract: 0.3percent; pH: 6.8–7.2. pH was adjusted in the range of 6.8–7.2. 50 ml of the medium was taken in each of the 250 ml conical flasks, cotton plugged and wrapped with brown paper and sterilized at 121ºC and 15 psig for 15 min.

IIA.1.4.3 Preparation of the acclimatization media:

Readymade powder of Inorganic Salt Media (MSM) was used in this purpose. 4.14 gm of media powder weighed properly and dissolved into 1 L of distilled water and required amount of glucose was added to it and stirred properly with a disinfected glass rod. pH was adjusted by using a digital ph meter. Then, as per requirement, the media was distributed in different volumes viz. 100 ml, 250 ml, 1 L and 2 L etc, plugged with cotton properly and wrapped with brown paper and sterilized in an autoclave at 121ºC and 15 psig for 15 min. As a lot of experiments were to be performed, large size of inoculums was required and so, 2 L volume of the MSM media was used to prepare inoculums. The composition of this media was as follows: Calcium carbonate 3.0 g/L, Calcium chloride hexahydrate 0.446 g/L, Potassium chloride 0.165 g/L, Potassium dihydrogen phosphate 0.2 g/L, Magnesium sulphate heptahydrate 0.7 g/L, Sodium sulphate 0.2 g/L, Potassium iodate 0.75 mg/L, Ferric chloride hexahydrate 2.5 mg/L, Boric acid 1.5 mg/L, Sodium molybdate dehydrate 0.25 mg/L, Manganese sulphate tetrahydrate 6.6 mg/L, Zinc sulphate heptahydrate

2.67 mg/L, Copper sulphate pentahydrate 0.07 mg/L. 10 g/L of glucose was added to this media first time and then the amount of the glucose was reduced gradually and finally after three to four weeks of acclimatization process, addition of glucose to the media, was completely stopped. Hasan and Jabeen; 2015, Ahuatzi-Chacon et al., 2004 and Hank et al., 2010 utilized this kind of Mineral Salt Media (MSM) for their experiments.

IIA.1.5 Enrichment:

Both the media enrichment and soil enrichment process was deployed here.

IIA.1.5.1 Media enrichment:

In this method, 1 gm of soil weighed properly and dissolved into 100 ml sterilized distilled water in a conical flask aseptically and the solution was kept in a shaker for 30 min to the mix the soil properly. Now 1 ml of this solution was taken and serially diluted with sterilized distilled water, up to 10^{-6}. 1 ml from each of the six serially diluted solutions was taken and transferred to six different sterile petriplates respectively. Simultaneously 15 ml of agar mediums, prepared previously (A.1.4.1), was transferred to each plates and in turn, 500 mg/L (500 PPM) of Phenol was added to each of the petriplates respectively and the whole thing was mixed properly in the plates. The whole procedure was performed aseptically. Now, all the petriplates were kept in a BOD incubator at 37^{o}C temperature for 24 hours. After 24 hours, several bacterial colonies were appeared in each of the petriplates. Those colonies were differentiated by their physical appearances very carefully, according to the Bergey's manual and transferred to slants aseptically. Twenty nine (29) colonies, able to tolerate the toxicity of Phenol, were isolated in this way and transferred to twenty nine different slants respectively and those slants were kept into the BOD incubator a 37^{o}C for 24 hours for growth of those strains.

IIA.1.5.2 Soil enrichment:

5 gm of soil weighed properly and kept in a sterile pertriplate. Now, 500 mg/Kg (or 500 PPM) Phenol was added to this soil and mixed properly with a sterilized glass rod. The whole thing was covered and kept in ambient temperature for 2 days. In the mean time, the sample was stirred with the same glass rod with an interval of 2 hours. After 2 days, 1 gm of enriched soil sample from this mixture was taken and thereby, the same process was repeated as mentioned in A.1.5.1 only excluding the enrichment of the media while performing the pour plate method as the soil itself, was enriched previously. After 24 hours of incubation, again several colonies, able to tolerate the Phenol, were obtained which were taken into the slants through the same process as mentioned in A.1.5.1. Total eighty three (83) colonies were isolated by involving soil enrichment technique.

IIA.1.6 Selection and testing of the most effective bacterial strain:

Two most tolerant bacterial strains (D25 & D108) were chosen after the screening process. One strain was selected among those strains which had been segregated via media enrichment technique and the other one was selected among the strains, which had been isolated via soil enrichment technique.

Liquid medium was used for the screening procedure. Each of the colonies, either isolated by media enrichment or soil enrichment, was taken to liquid media and then it was cultured in a BOD incubator in shaking condition at 37^{o}C for 24 hours with 500 mg/L of Phenol. Following the incubation, the broth was centrifuged at 6000 rpm for 15 minutes, and the clear supernatant was utilized to estimate the residual Phenol content spectrophotometrically.

IIA.1.7 Calculation of the Residual content of Phenol:

The Residual concentration of Phenol was measured in the spectrophotometer at 510 nm wavelength followed by 4- Amino Antipyrene Method. This method was suggested by Yang et al., 1975. For that purpose a standard curve as well as a standard equation was made with the known concentrations of Phenol and the residual Phenol content was calculated from that equation. To carry out this process, Ammonium buffer, 4- Amino Antipyrene solution and Potassium ferricyanide solution are required. The preparation of the reagents was as follows:

IIA.1.7.1 Preparation of Buffer Solution:

16.9 gm Ammonium chloride (NH_4Cl) was dissolved in 143ml of Concentrated Ammonium hydroxide (NH_4OH) and diluted to 250 ml with distilled water. The concentration of the buffer was such that 2ml of this buffer was able to adjust 100ml of distillate to pH 10.

IIA.1.7.2 Preparation of Antipyrene solution:

2 gm of properly weighed 4- Amino antipyrene powder was dissolved in to 100 ml distilled water.

IIA.1.7.3 Preparation of Potassium ferricyanide solution:

8 gm of Potassium ferricyanide was weighed properly and dissolved into 100 ml distilled water.

IIA.1.7.4 Procedure:

i) 100 ml of the sample diluted to 100 ml with distilled water and 2 ml buffer solution was added to it.

ii) 2 ml of 4- Amino antipyrene solution added to the mixture.

iii) 2 ml of Potassium ferricyanide was added to the solution and mix properly.

iv) A pinkish red colour appeared which indicates the presence of Phenolic compounds.

v) After 10- 15 minutes, the absorbance was measured in a spectrophotometer at 510 nm wavelength.

vi) However for practical convenience, twenty times dilutions were made for each of the reagents.

IIA.1.7.5 Analytical method:

Here, a standard curve possessing the R^2 value of 0.99, along with a standard equation was prepared with different known concentrations of Phenol starting from 20mg/L to 1000 mg/L. The obtained equation followed the format given below:

$$Y = mX + C \quad (1)$$

Where, Y stands for the optical density (OD) of an unknown sample, m stands for slope of the standard curve, X stands for the residual conc. of Phenol of the sample and C represents a constant.

IIA.1.8 Acclimatization of the strains:

The segregated bacterial strains (D25 & D108) were constantly acclimated in the Mineral Salt Medium to maintain its potentiality regarding the degradation of Phenol as well as to prepare inoculums for the further experiments. In the initial stage, the strain was cultured with 50 mg/L conc. of Phenol. Then 100mg/L, 200 mg/L, 300 mg/L, 400 mg/L, 500 mg/L, 600 mg/L, 700 mg/L and 800 mg/L conc. of Phenol was used to acclimatize the strain properly.

IIA.1.9 Characterization of the segregated strains from morphological, biochemical & phylogenetic perspective:

The selected bacterial strains (D25 & D108) were subjected to several morphological and biochemical characterizations. Simple staining, Gram staining, Spore staining, Carbohydrate tests, nitrate reduction test, catalase reduction test, Voges- Proskauer test, starch hydrolysis, urease and many other tests were successfully done as per Bergey's manual.

IIA.1.10 Phylogenetic assay of the two isolated strains:

As per the standard method, 16S rDNA Sequence and Phylogenetic Analysis of the two isolated strains were performed.

DNA was isolated from the culture **D25**. Quality was evaluated on 1.2percent Agarose Gel, a single band of high-molecular weight DNA has been observed. After that, Isolated DNA was amplified with *16S rRNA* Specific Primer (**8F**and **1492R**) using Veriti® 99 well Thermal Cycler (Model No. 9902). A single discrete PCR amplicon band of **1500 bp** was observed (**Figure 1**). The PCR amplicon was enzymatically purified and further subjected to Sanger Sequencing. Bi-directional DNA sequencing reaction of PCR amplicon was carried out with **M13F** and **M13R** primers using BDT v3.1 Cycle sequencing kit on ABI 3730xl Genetic Analyzer. Consensus sequence of **1407 bp** *16S rDNA* was generated from forward and reverse sequence data using aligner software. Finally, the ***16S rDNA*** sequence was used to carry out BLAST alignment search tool of NCBI Genbank database. Based on maximum identity score first fifteen sequences were selected and aligned using multiple alignment software program ClustalW. Distance matrix was generated using RDP database and the Phylogenetic tree was constructed using MEGAX.

The evolutionary history was inferred using the Neighbour-Joining method (Saitou & Nei; 1987). The bootstrap consensus tree inferred from 1000 replicates (Felsenstein; 1985) is taken to represent the evolutionary history of the taxa analyzed (Felsenstein; 1985). Branches corresponding to partitions reproduced in less than 50percent bootstrap replicates are collapsed. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches (Felsenstein; 1985). The evolutionary distances were computed using the Maximum Composite Likelihood method (Kimura; 1980) and are in the units of the number of base substitutions per site. This analysis involved 16 nucleotide sequences. Codon positions included were 1st+2nd+3rd+Noncoding. All ambiguous positions were removed for each sequence pair (pair wise deletion option). There were a total of 1409 positions in the final dataset. Evolutionary analyses were conducted in MEGA X (Kumar et al., 2018).

IIA.2 Results & Discussions:

Total twenty nine (29) colonies were segregated via media enrichment method. After screening, D25 was found to be the most potential strain to remove Phenol as it was able to remove almost 99.44percent of Phenol (highlighted in the table) from the media within 24 hours at 37^{o}C, when the initial conc. of Phenol was 500 mg/L. In Table No. IIA.1, distinguishing characteristics of all the isolated colonies have been displayed along with the screening results.

Table IIA.1: Screening and colony characterization of the bacterial strains isolated from soil via media enrichment method

Sample strain number	Colony Size	Colony Pigmentation	Colony Form	Colony Margin	Colony Elevation	percent of Phenol degradation
D1	Moderate	White	Circular	Serrate	Flat	83.16
D2	Moderate	White	Irregular	Serrate	Flat	82.86
D3	Small	White	Irregular	Serrate	Flat	80.75
D4	Large	White	Circular	Entire	Flat	80.15
D5	Moderate	White	Circular	Entire	Flat	84.36
D6	Small	Light Yellow	Circular	Entire	Raised	81.35
D7	Small	White	Circular	Serrate	Umbonate	80.15
D8	Pin Point	White	Circular	Serrate	Flat	80.45
D9	Small	Light Yellow	Circular	Serrate	Flat	79.25
D10	Moderate	White	Circular	Serrate	Raised	77.44
D11	Moderate	White	Circular	Entire	Flat	82.86
D12	Moderate	Light Yellow	Circular	Entire	Flat	82.56
D13	Large	Yellowish	Irregular	Entire	Flat	84.06
D14	Small	Reddish Yellow	Circular	Undulate	Flat	84.06
D15	Small	White	Circular	Serrate	Flat	83.76
D16	Moderate	White	Irregular	Serrate	Raised	84.36
D17	Large	White	Circular	Serrate	Raised	84.06
D18	Moderate	Yellowish	Circular	Entire	Umbonate	84.06
D19	Small	Light Yellow	Circular	Entire	Raised	83.46
D20	Pin Point	White	Irregular	Entire	Flat	64.51
D21	Small	Light Yellow	Irregular	Serrate	Raised	80.15
D22	Small	White	Circular	Serrate	Flat	83.46
D23	Moderate	White	Circular	Entire	Flat	83.16
D24	Large	Light Yellow	Circular	Serrate	Flat	94.84
D25	**Large**	**White**	**Circular**	**Serrate**	**Flat**	**99.44**
D26	Large	Light Yellow	Irregular	Serrate	Raised	93.83
D27	Large	Light Yellow	Circular	Serrate	Flat	91.68
D28	Large	White	Circular	Serrate	Flat	90.83
D29	Large	White	Circular	Entire	Flat	88.48

Similarly, total eighty three (83) colonies were segregated via soil enrichment method. After screening, it was found that D108 was the most potential strain to remove Phenol as it was able to remove almost 98.08percent of Phenol from the media within 24 hours at 37^{o}C, when the initial conc. of Phenol was 500 mg/L. In Table No. IIA.2, distinguishing characteristics of all the segregated colonies have been displayed along with the screening results.

Table IIA.2: Screening and colony characterization of the bacterial strains isolated from soil via soil enrichment method

Sample strain number	Colony Size	Colony Pigmentation	Colony Form	Colony Margin	Colony Elevation	percent of Phenol degradation
D30	Moderate	White	Circular	Serrate	Flat	90.53
D31	Moderate	White	Circular	Entire	Umbonate	84.69
D32	Large	White	Irregular	Serrate	Flat	91.22
D33	Small	White	Circular	Entire	Flat	79.76
D34	Small	White	Circular	Serrate	Flat	82.89
D35	Small	White	Irregular	Serrate	Flat	89.35
D36	Large	White	Irregular	Serrate	Flat	87.64
D37	Large	Reddish Yellow	Circular	Serrate	Flat	96.36
D38	Small	White	Circular	Serrate	Flat	82.05
D39	Moderate	White	Circular	Serrate	Flat	88.87
D40	Moderate	White	Irregular	Serrate	Flat	89.41
D41	Moderate	White	Circular	Undulate	Flat	87.34
D42	Large	White	Irregular	Serrate	Flat	87.82
D43	Small	White	Circular	Serrate	Raised	78.47
D44	Moderate	White	Circular	Serrate	Flat	86.47
D45	Large	White	Circular	Entire	Flat	94.71
D46	Moderate	White	Circular	Serrate	Raised	88.54
D47	Large	Reddish Yellow	Circular	Serrate	Flat	97.71
D48	Large	White	Circular	Entire	Flat	91.19
D49	Large	White	Circular	Serrate	Raised	98.41
D50	Moderate	White	Irregular	Serrate	Flat	88.69
D51	Moderate	White	Irregular	Entire	Flat	86.74
D52	Large	White	Irregular	Serrate	Flat	98.47
D53	Large	Light Yellow	Irregular	Serrate	Raised	93.95
D54	Large	White	Circular	Serrate	Flat	95.01
D55	Large	Light Yellow	Circular	Serrate	Raised	95.25
D56	Large	White	Circular	Serrate	Raised	93.5
D57	Moderate	White	Circular	Serrate	Flat	92.84
D58	Moderate	Light Yellow	Circular	Serrate	Flat	93.11
D59	Moderate	White	Irregular	Serrate	Flat	91.32
D60	Moderate	White	Circular	Undulate	Raised	92.17
D61	Moderate	Light Yellow	Circular	Serrate	Flat	86.84
D62	Large	White	Circular	Serrate	Flat	92.23
D63	Moderate	White	Circular	Serrate	Raised	97.29
D64	Moderate	White	Irregular	Entire	Flat	97.74
D65	Moderate	Reddish Yellow	Circular	Serrate	Flat	90.23
D66	Moderate	White	Circular	Entire	Flat	95.85
D67	Moderate	Light Yellow	Circular	Serrate	Raised	91.38
D68	Large	White	Irregular	Serrate	Flat	93.29
D69	Large	White	Circular	Serrate	Flat	86.42
D70	Large	White	Circular	Serrate	Raised	86.31

D71	Large	White	Circular	Entire	Flat	85.91
D72	Moderate	White	Irregular	Serrate	Flat	89.96
D73	Large	White	Circular	Serrate	Flat	8.23
D74	Moderate	White	Circular	Entire	Flat	97.92
D75	Moderate	White	Circular	Serrate	Raised	91.35
D76	Moderate	White	Circular	Serrate	Flat	92.71
D77	Large	White	Circular	Entire	Flat	90.24
D78	Moderate	Light Yellow	Circular	Serrate	Raised	95.55
D79	Moderate	Light Yellow	Irregular	Serrate	Flat	96.93
D80	Large	White	Circular	Serrate	Flat	91.82
D81	Moderate	White	Circular	Serrate	Flat	87.35
D82	Moderate	White	Circular	Entire	Raised	87.35
D83	Moderate	White	Circular	Serrate	Raised	98.22
D84	Large	Light Yellow	Circular	Serrate	Flat	87.41
D85	Large	White	Irregular	Serrate	Flat	84.44
D86	Large	White	Circular	Serrate	Flat	86.87
D87	Large	Light Yellow	Circular	Serrate	Flat	91.81
D88	Large	White	Circular	Serrate	Raised	92.76
D89	Large	White	Irregular	Serrate	Raised	93.71
D90	Large	White	Circular	Entire	Flat	90.36
D91	Large	White	Circular	Serrate	Flat	90.15
D92	Large	Light Yellow	Circular	Entire	Flat	91.03
D93	Large	White	Irregular	Serrate	Umbonate	91.65
D94	Large	White	Circular	Serrate	Flat	87.51
D95	Large	Reddish Yellow	Circular	Serrate	Flat	95.88
D96	Large	White	Irregular	Entire	Flat	94.52
D97	Large	White	Circular	Serrate	Flat	91.79
D98	Large	White	Circular	Entire	Flat	92.47
D99	Large	White	Circular	Serrate	Flat	92.83
D100	Moderate	White	Circular	Serrate	Flat	91.84
D101	Large	White	Circular	Undulate	Raised	86.87
D102	Moderate	White	Circular	Serrate	Flat	84.66
D103	Large	White	Circular	Serrate	Flat	87.38
D104	Large	Light Yellow	Circular	Entire	Flat	76.84
D105	Large	White	Irregular	Undulate	Flat	95.85
D106	Large	White	Irregular	Serrate	Flat	94.92
D107	Large	White	Circular	Serrate	Raised	95.13
D108	**Large**	**White**	**Circular**	**Serrate**	**Flat**	**98.08**
D109	Moderate	Light Yellow	Irregular	Serrate	Flat	97.56
D110	Moderate	White	Circular	Undulate	Flat	97.11
D111	Moderate	White	Circular	Serrate	Flat	97.14
D112	Large	Light Yellow	Circular	Serrate	Raised	97.17

From table IIA.1 and IIA.2, it can be displayed clearly that D25 and D108 are the most potential strains (as highlighted in the tables respectively). These two strains were selected for further studies i.e. morphological, biochemical and phylogenetic identification.

Morphological & Biochemical Characterizations are displayed in the Table No. IIA.3, IIA.4, IIA.5 and IIA.6.

Table IIA.3: Colony characterization of D25 & D108 studied on Petri plate

Colony characteristics	D25	D108
Size	~ 1 mm	> 1mm
Opacity	Opaque	Translucent
Surface growth	Smooth	Smooth
Edge	Sharp	Diffuse
Consistency	Good	Good
Pigmentation	Nil	Nil

Table IIA.4: Colony characterization of D25 & D108 studied on Slant

Colony characteristics	D25	D108
Opacity	Opaque	Translucent
Surface growth	Smooth	Smooth
Consistency	Good	Good
Colour	Good	Good
Pigmentation	Nil	Nil

Table IIA.5: Physicochemical Characteristics of D25 & D108

Characteristics	D25	D108
Arrangement	Cell shape: rod/bacillus	Cell shape: rod/bacillus
	Cell size: > 6 micron	Cell size: 5- 6 micron
Gram staining	Positive	Positive
Spore staining	Positive	Positive
Motility	Non motile	Non motile

Table IIA.6: Growth Characteristics of D25 & D108 in stationary and shaking condition

Experiment	Observation	
	D25	**D108**
Growth in 50 ml medium taken in 250 ml Erlenmeyer flask	A. Stationary condition (a) After 24 hr : - Poor growth, no ring formation, no pellicle formation, sedimentation at the bottom, upper portion of the broth was clear.	Same as D25
	(b) After 48 hr : - Same as after 24 hr but the growth was fair.	
	B. Shaking condition. (a) Fair growth, turbid, no sedimentation, no pellicle formation, no pigmentation, and no ring formation.	Same as D25
	(b) After 48 hr : - Same as after 24 hr	

Results of the Carbohydrate fermentation as well as other physicochemical characteristics of the two particular strains D25 and D108 have been shown in Table No. IIA.7.

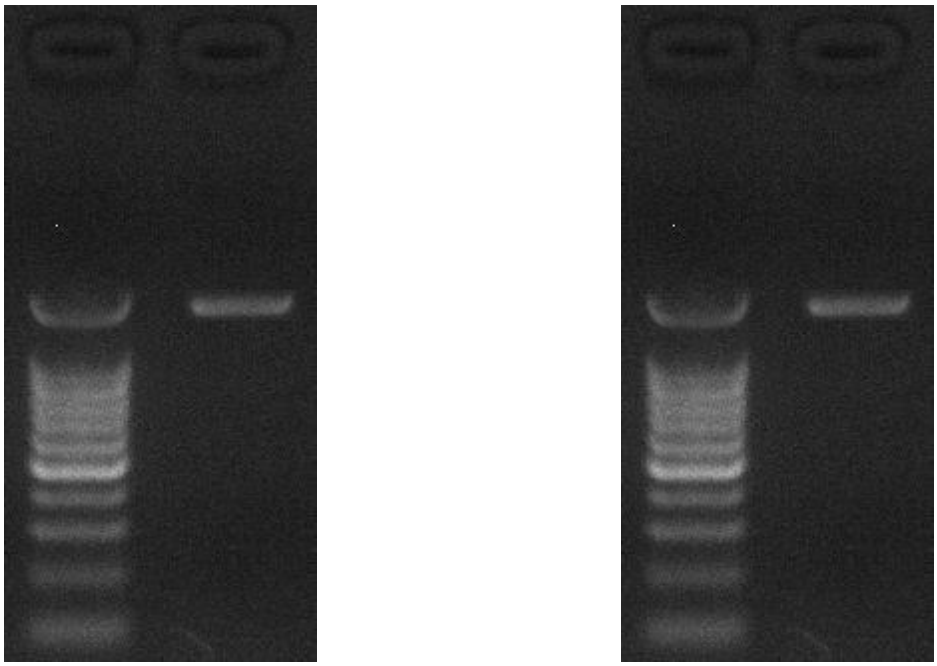
Table IIA.7: Results of Biochemical tests of D25 & D108

Parameters	**Characteristics of D25**		**Characteristics of D108**	
Carbohydrate fermentation at 37˚C	Acidity	Gas formation	Acidity	Gas formation
Fructose	Positive	Positive	Negative	Negative
Arabinose	Negative	Positive	Positive	Negative
Galactose	Positive	Positive	Negative	Negative
Xylose	Positive	Negative	Negative	Negative
Glucose	Positive	Positive	Positive	Negative
Lactose	Positive	Positive	Negative	Negative
Raffinose	Positive	Positive	Negative	Negative
Sucrose	Negative	Positive	Negative	Positive
Maltose	Positive	Positive	Negative	Negative
Dextrin	Positive	Positive	Negative	Negative
Salicin	Positive	Positive	Negative	Negative
Mannitol	Positive	Positive	Negative	Negative
Glycerol	Positive	Positive	Negative	Negative
Inositol	Negative	Positive	Negative	Positive
Sorbitol	Negative	Positive	Positive	Negative
Catalase reduction	Positive		Negative	
Litmus milk test	Positive		Positive	
Starch hydrolysis test	Negative		Negative	
Urease test	Positive		Negative	
Ammonia from Arginine	Negative		Positive	
Arginine as sole source of energy	Negative		Positive	
Nitrate reduction	Positive		Positive	
Indole formation	Positive		Positive	
Voges-Proskaurer test at pH < 6	Negative		Negative	
Voges-Proskaurer test at pH > 7	Positive		Negative	
Growth under anaerobic	Negative		Negative	

condition		
Growth at 5˚ C	Poor	Poor
Growth at 10˚C	Poor	Poor
Growth at 20˚C	Poor	Poor
Growth at 30˚C	Vigorous	Vigorous
Growth at 35˚C	Vigorous	Vigorous
Growth at 40˚ C	Vigorous	Vigorous
Growth at 50˚ C	Poor	Poor
Growth at 2percent NaCl concentration	Vigorous	Vigorous
Growth at 5percent NaCl concentration	poor	Vigorous
Growth at 7percent NaCl concentration	Poor	Poor
Growth at 10percent NaCl concentration	Poor	Poor
Growth at extreme pH 9.6 & 6.5 percent NaCl conc.	Positive	Positive
Growth at extreme pH 6.8 & 6.5 percent NaCl conc.	Positive	Positive
Growth at extreme pH 5.6 & 6.5 percent NaCl conc.	Positive	Negative

From the biochemical tests, it can be summarised that, the two isolated bacterial strains are **gram positive, spore forming, non motile, rod shaped (bacillus) and obligatory aerobic** in nature.

In case of identification of the two isolated strains i.e. D25 and D108, 16s rDNA assay was involved (A.1.10). After obtaining the gel eletrogram images (Fig: IIA.1), the remaining results were explored.



(a) (b)

Figure IIA.1: 1.2percent Agarose gel showing single 1500 bp of *16S rDNA* amplicons of isolated D25 and D108 (a & b respectively). Lane 1: 100bp DNA ladder; Lane 2: *16S rDNA* amplicon)

Sequence alignment views of the two strains i.e. D25 and D108 have been showed in Table IIA.8 and IIA.9 respectively. The distance matrixes of the two strains have been provided in the Table IIA.10 and IIA.11 respectively.

Table IIA.8: Sequence alignment view of D25 strain using combination of NCBI Gene Bank

Accession	Description	Max score	Total score	Query coverage	E value	Max ident
NR_041524.1	*Brevibacillus brevis* strain NBRC 15304	2501	2501	99percent	0.0	99.14percent
NR_040979.1	*Brevibacillus formosus* strain DSM 9885	2501	2501	99percent	0.0	99.07percent
NR_112204.1	*Brevibacillus brevis* strain DSM 30	2501	2501	99percent	0.0	99.07percent
NR_113801.1	*Brevibacillus formosus* strain NBRC 15716	2497	2497	99percent	0.0	99.07percent
NR_040980.1	*Brevibacillus choshinensis* strain DSM 8552	2471	2471	99percent	0.0	98.71percent
NR_113763.1	*Brevibacillus choshinensis* strain NBRC 15518	2468	2468	99percent	0.0	98.71percent
NR_112926.1	*Brevibacillus nitrificans* strain DA2	2455	2455	99percent	0.0	98.50percent
NR_040983.1	*Brevibacillus agri* strain DSM 6348	2446	2446	99percent	0.0	98.35percent
NR_113767.1	*Brevibacillus agri* strain NBRC 15538	2438	2438	99percent	0.0	98.28percent
NR_040981.1	*Brevibacillus parabrevis* strain IFO 12334	2438	2438	99percent	0.0	98.28percent
NR_113589.1	*Brevibacillus parabrevis* strain NBRC 12334	2431	2431	99percent	0.0	98.21percent
NR_113802.1	*Brevibacillus reuszeri* strain NBRC 15719	2429	2429	99percent	0.0	98.21percent
NR_040982.1	*Brevibacillus reuszeri* strain DSM 9887	2429	2429	99percent	0.0	98.14percent
NR_024822.1	*Brevibacillus limnophilus* strain DSM 6472	2416	2416	99percent	0.0	98.00percent
NR_115591.1	***Brevibacillus formosus* strain NRRL NRS-863**	**2359**	**2359**	**99percent**	**0.0**	**98.65percent**

Table IIA.9: Sequence alignment view of D108 strain using combination of NCBI Gene Bank

Accession	Description	Max score	Total score	Query coverage	E value	Max ident
NR_043289.1	***Pseudomonas otitidis* strain MCC10330**	**2516**	**2516**	**96percent**	**0.0**	**99.71percent**
NR_117678.1	*Pseudomonas aeruginosa* strain DSM 50071	2427	2427	96percent	0.0	98.55percent
NR_114471.1	*Pseudomonas aeruginosa* strain ATCC 10145	2409	2409	95percent	0.0	98.53percent
NR_112062.1	*Pseudomonas resinovorans* strain ATCC 14235	2405	2405	95percent	0.0	98.32percent
NR_113599.1	*Pseudomonas aeruginosa* strain NBRC 12689	2394	2394	94percent	0.0	98.53percent
NR_114957.1	*Pseudomonas guezennei* strain RA26	2383	2383	91percent	0.0	99.47percent
NR_103934.2	*Pseudomonas stutzeri* ATCC 17588 = LMG 11199	2350	2350	96percent	0.0	97.53percent
NR_117827.1	*Pseudomonas alcaligenes*	2342	2342	95percent	0.0	97.66percent
NR_116489.1	*Pseudomonas stutzeri* strain VKM B-975	2338	2338	96percent	0.0	97.39percent
NR_026078.1	*Pseudomonas aeruginosa* strain DSM 50071	2331	2331	96percent	0.0	97.31percent
NR_113646.1	*Pseudomonas alcaligenes* strain NBRC 14159	2329	2329	94percent	0.0	97.64percent
NR_113652.1	*Pseudomonas stutzeri* strain NBRC 14165	2318	2318	94percent	0.0	97.50percent
NR_043419.1	*Pseudomonas alcaligenes* strain IAM 12411	2316	2316	96percent	0.0	97.10percent
NR_037000.1	*Pseudomonas pseudoalcaligenes* strain Stanier 63	2316	2316	96percent	0.0	97.09percent
NR_118798.1	*Pseudomonas stutzeri* strain CCUG 11256	2316	2316	94percent	0.0	97.56percent

Finally, the phylogenetic trees of the two isolated strains have been displayed in figure IIA.2 and figure IIA.3 respectively. The trees revealed that D25 strain exhibits its similarities mostly with *Brevibacillus formosus* strain NRRL NRS- 863 (Fig: IIA.2). On the other hand, D108 possess its similarities mostly with *Pseudomonas otitidis* strain MCC10330 (Fig: IIA.3).

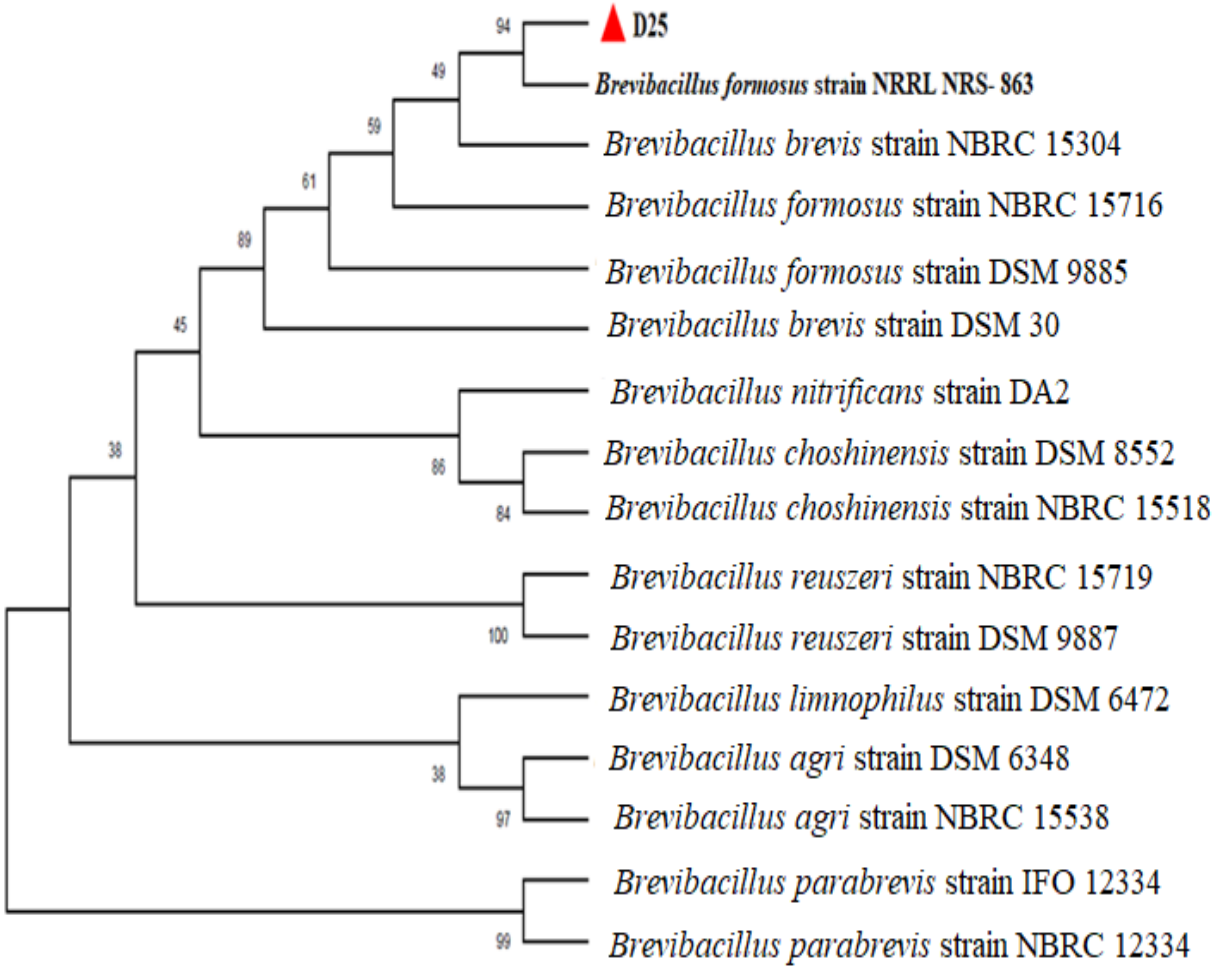


Fig IIA.2: Unrooted phylogenetic tree based on 16S rDNA sequences obtained by the Neighbour-Joining (NJ) method showing the position of D25 strain among its Phylogenetic Neighbours. NCBI Accession Numbers are provided in Parentheses

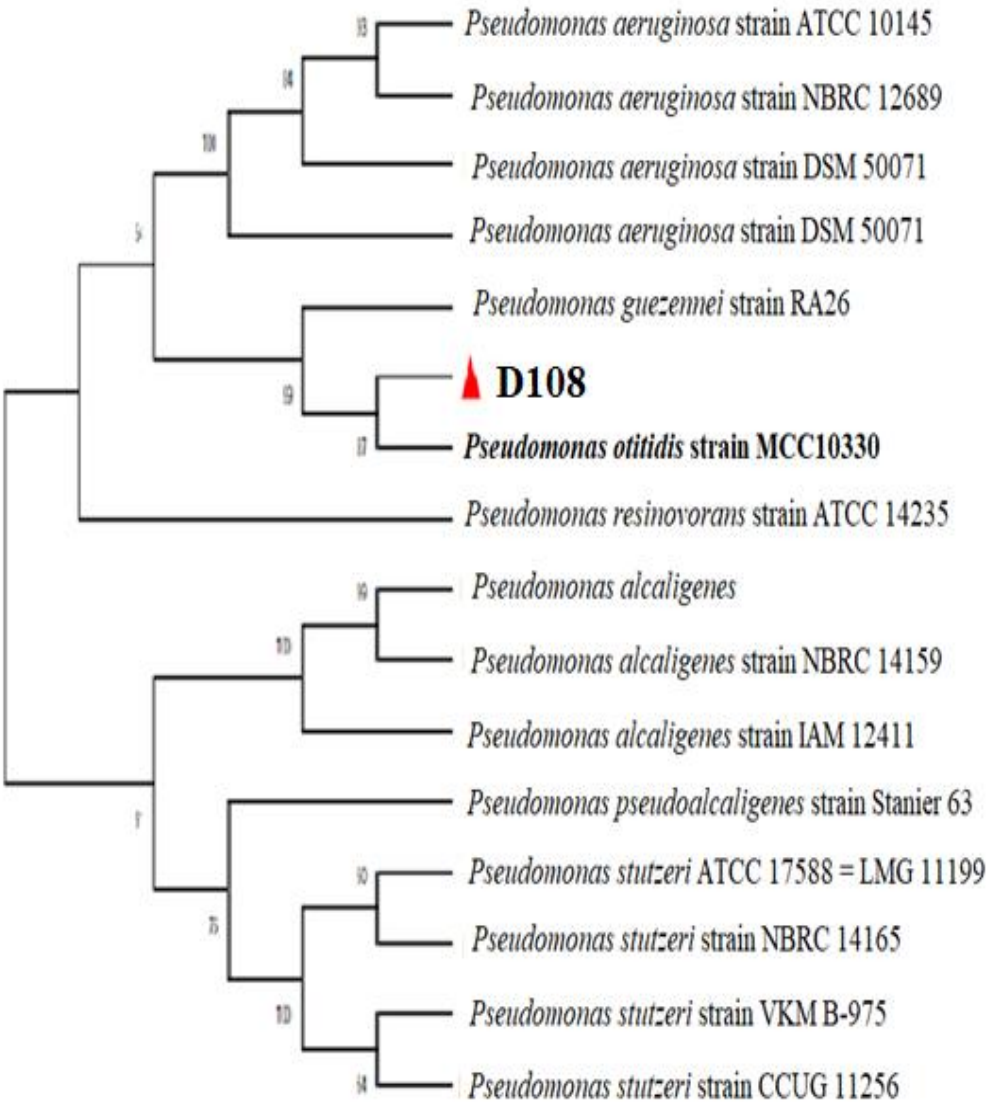


Fig IIA.3: Unrooted phylogenetic tree based on 16S rDNA sequences obtained by the Neighbour-Joining (NJ) method showing the position of D108 strain among its Phylogenetic Neighbours. NCBI Accession Numbers are provided in Parentheses

Sub chapter IIB

Isolation & Identification of two bacterial strains to remove 4- Chloro phenol

IIB.1 Materials and Method:

All the materials used here, are same as the materials, used while isolating and identifying the two strains *Brevibacillus formosus* strain NRRL NRS- 863 and *Psudomonas otitidis* strain MCC10330 regarding the degradation of the Phenol (Sub chapter A). All the methodologies are also same as mentioned in the **A.1**. Only in case of the enrichment process of the media and soil, 500 PPM of 4- Chloro Phenol was used here instead of the Phenol.

Also in case of the standard curve preparation along with the standard equation to deduct the residual 4- Chloro Phenol, known concentrations of 4- Chloro Phenol were utilized starting from 20 mg/L to 1000 mg/L instead of Phenol. Rest of the procedure was same as that of the IIA.1.7.

IIB.2 Results & Discussions:

Total eighteen (18) colonies were segregated via media enrichment method. After screening, C17 was found to be the most potential strain (highlighted in the table) to remove 4- Chloro Phenol as it was able to remove almost 99.93percent of 4- Chloro Phenol from the media within 24 hours at 37^{o}C, when the initial conc. was 500 mg/L. In Table No. IIB.1, distinguishing characteristics of all the isolated colonies have been displayed along with the screening results.

Table IIB.1: Screening and colony characterization of the bacterial strains isolated from soil to remove 4- Chloro Phenol via media enrichment method

Sample Strain No.	Colony Size	Colony Pigmentation	Colony Form	Colony Margin	Colony Elevation	percent of 4-Chlorophenol degradation
C1	Large	White	Circular	Serrate	Flat	92.52
C2	Large	Light Yellow	Irregular	Serrate	Flat	92.25
C3	Moderate	White	Irregular	Serrate	Raised	93.92
C4	Large	Reddish Yellow	Irregular	Undulate	Flat	93.47
C5	Moderate	White	Circular	Serrate	Flat	81.25
C6	Large	Light Yellow	Circular	Undulate	Raised	92.43
C7	Small	White	Circular	Entire	Raised	89.75
C8	Moderate	White	Circular	Serrate	Flat	97.77
C9	Large	Light Yellow	Irregular	Entire	Raised	96.74
C10	Large	White	Circular	Undulate	Flat	95.91
C11	Small	White	Irregular	Serrate	Raised	94.74
C12	Small	Reddish Yellow	Circular	Serrate	Flat	94.71
C13	Moderate	White	Irregular	Serrate	Raised	79.43
C14	Large	Light Yellow	Irregular	Serrate	Flat	84.63
C15	Moderate	White	Irregular	Entire	Umbonate	78.94
C16	Pin point	White	Irregular	Serrate	Flat	90.78
C17	**Large**	**Light Yellow**	**Circular**	**Undulate**	**Flat**	**99.93**
C18	Large	Reddish Yellow	Circular	Entire	Raised	99.98

Similarly, total twenty seven (27) colonies were segregated via soil enrichment method. After screening, it was found that C19 was the most potential strain to remove 4- Chloro Phenol as it was able to remove almost 99.97percent of 4- Chloro Phenol from the media within 24 hours at 37^{o}C, when the initial conc. was 500 mg/L. In Table No. IIB.2, distinguishing characteristics of all the separated colonies have been displayed along with the screening results.

Table IIB.2: Screening and colony characterization of the bacterial strains isolated from soil to remove 4- Chloro Phenol via soil enrichment method

Sample Strain No.	Colony Size	Colony Pigmentation	Colony Form	Colony Margin	Colony Elevation	percent of 4-Chlorophenol degradation
C19	**Small**	**White**	**Circular**	**Entire**	**Flat**	**99.97**
C20	Large	White	Irregular	Serrate	Raised	96.87
C21	Moderate	Light Yellow	Circular	Serrate	Flat	96.07
C22	Large	Reddish Yellow	Irregular	Undulate	Raised	95.28
C23	Large	White	Irregular	Entire	Flat	96.08
C24	Small	White	Irregular	Serrate	Flat	95.43
C25	Small	Light Yellow	Circular	Serrate	Raised	95.42
C26	Small	White	Circular	Entire	Flat	95.05
C27	Small	White	Circular	Undulate	Flat	95.74
C28	Large	White	Circular	Entire	Flat	96.22
C29	Small	Reddish Yellow	Circular	Serrate	Flat	96.04
C30	Large	White	Irregular	Undulate	Flat	96.35
C31	Large	Light Yellow	Circular	Entire	Raised	98.02
C32	Large	White	Irregular	Serrate	Umbonate	97.2
C33	Moderate	White	Circular	Entire	Flat	96.55
C34	Small	White	Circular	Entire	Raised	96.91
C35	Small	White	Irregular	Serrate	Flat	97.98
C36	Moderate	Light Yellow	Circular	Serrate	Raised	97.04
C37	Large	White	Irregular	Entire	Raised	96.85
C38	Small	Light Yellow	Circular	Serrate	Raised	97
C39	Small	White	Circular	Undulate	Flat	96.84
C40	Moderate	Light Yellow	Circular	Serrate	Flat	96.89
C41	Pin point	White	Irregular	Entire	Raised	97.17
C42	Pin point	White	Irregular	Entire	Flat	97.58
C43	Small	Reddish Yellow	Circular	Serrate	Flat	97.37
C44	Small	Reddish Yellow	Irregular	Serrate	Flat	96.29
C45	Moderate	Reddish Yellow	Circular	Serrate	Flat	96.23

From table IIB.1 and IIB.2, it can be displayed clearly that C17 and C19 are the most potential strains (as highlighted in the tables respectively). These two strains were selected for further studies i.e. morphological, biochemical and phylogenetic identification.

Morphological & Biochemical Characterizations are showed in the Table No. IIB.3, IIB.4, IIB.5 and IIB.6.

Table IIB.3: Colony characterization of C17 & C19 studied on Petri plate

Colony characteristics	C17	C19
Size	~ 1 mm	> 1mm
Opacity	Opaque	Translucent
Surface growth	Smooth	Smooth
Edge	Sharp	Diffuse
Consistency	Good	Good
Pigmentation	Nil	Nil

Table IIB.4: Colony characterization of C17 & C19 studied on Slant

Colony characteristics	C17	C19
Opacity	Opaque	Translucent
Surface growth	Smooth	Smooth
Consistency	Good	Good
Colour	Good	Good
Pigmentation	Nil	Nil

Table IIB.5: Physicochemical Characteristics of C17 & C19

Characteristics	C17	C19
Arrangement	Cell shape: rod/bacillus	Cell shape: rod/bacillus
	Cell size: > 6 micron	Cell size: 5- 6 micron
Gram staining	Positive	Positive
Spore staining	Positive	Positive
Motility	Non motile	Motile

Table IIB.6: Growth Characteristics of C17 & C19 in stationary and shaking condition

Experiment	Observation	
	C17	**C19**
Growth in 50 ml medium taken in 250 ml Erlenmeyer flask	A. Stationary condition (a) After 24 hr : - Poor growth, no ring formation, no pellicle formation, sedimentation at the bottom, upper portion of the broth was clear.	Same as C17
	(b) After 48 hr : - Same as after 24 hr but the growth was fair.	
	B. Shaking condition. (a) Fair growth, turbid, no sedimentation, no pellicle formation, no pigmentation, no ring formation.	Same as C17
	(b) After 48 hr : - Same as after 24 hr	

Results of the Carbohydrate fermentation as well as the other physicochemical characteristics of the two particular strains C17 and C19 have been shown in Table No. IIB.7.

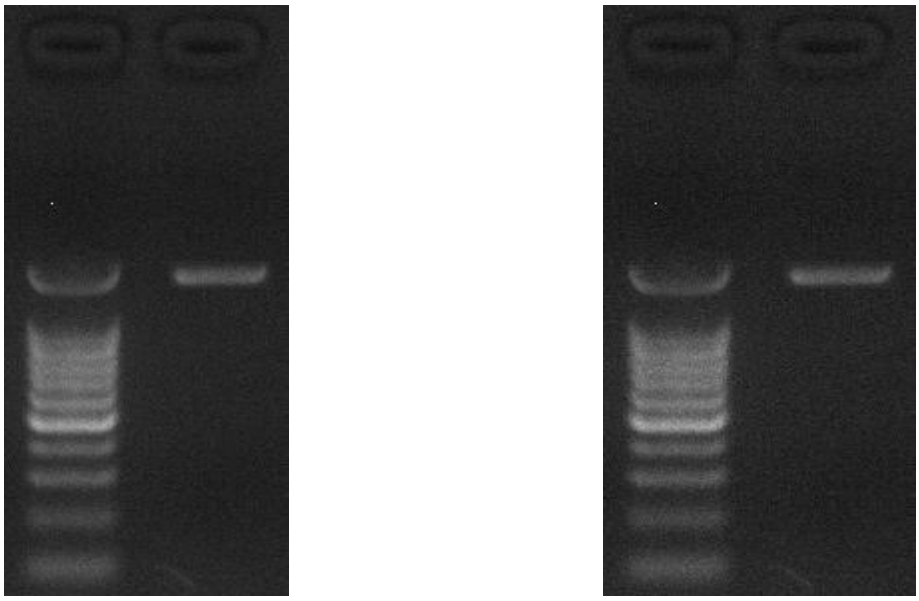
Table IIB.7: Results of Biochemical tests of C17 & C19

Parameters	**Characteristics of C17**		**Characteristics of C19**	
Carbohydrate fermentation at 37˚C	Acidity	Gas formation	Acidity	Gas formation
Fructose	Positive	Negative	Positive	Negative
Arabinose	Negative	Negative	Negative	Negative
Galactose	Negative	Negative	Negative	Negative
Xylose	Negative	Negative	Negative	Negative
Glucose	Positive	Negative	Positive	Negative
Lactose	Negative	Negative	Positive	Negative
Raffinose	Negative	Negative	Positive	Negative
Sucrose	Positive	Negative	Positive	Positive
Maltose	Positive	Negative	Negative	Negative
Dextrin	Negative	Negative	Positive	Negative
Salicin	Negative	Negative	Negative	Negative
Mannitol	Negative	Negative	Positive	Negative
Glycerol	Negative	Negative	Positive	Negative
Inositol	Negative	Negative	Negative	Negative
Sorbitol	Negative	Negative	Negative	Negative
Catalase reduction	Positive		Positive	
Litmus milk test	Positive		Positive	
Starch hydrolysis test	Positive		Positive	
Urease test	Positive		Positive	
Ammonia from Arginine	Negative		Positive	
Arginine as sole source of energy	Negative		Positive	
Nitrate reduction	Negative		Positive	
Indole formation	Negative		Negative	
Voges-Proskaurer test at pH < 6	Positive		Positive	
Voges-Proskaurer test at pH > 7	Positive		Positive	
Growth under anaerobic condition	Negative		Negative	
Growth at 5˚	Poor		Poor	

C		
Growth at 10˚C	Poor	Poor
Growth at 20˚C	Poor	Slightly good
Growth at 30˚C	Vigorous	Vigorous
Growth at 35˚C	Vigorous	Vigorous
Growth at 40˚ C	Vigorous	Vigorous
Growth at 50˚ C	Poor	Poor
Growth at 2percent NaCl concentration	Vigorous	Vigorous
Growth at 5percent NaCl concentration	Vigorous	Vigorous
Growth at 7percent NaCl concentration	Poor	Poor
Growth at 10percent NaCl concentration	Poor	Poor
Growth at extreme pH 9.6 & 6.5 percent NaCl conc.	Positive	Positive
Growth at extreme pH 6.8 & 6.5 percent NaCl conc.	Positive	Positive
Growth at extreme pH 5.6 & 6.5 percent NaCl conc.	Positive	Positive

From the biochemical results, it can be determined that the two isolated strains are **rod shaped (bacillus)**, **gram positive, spore forming and obligatory aerobic** in nature. Only in case of motility, C17 strain is **non motile** whereas, C19 strain is **motile**.

In case of identification of the two isolated strains i.e. C17 and C19, 16s rDNA assay was involved (A.1.10). After obtaining the gel eletrogram images (Fig: IIB.1), the remaining results were explored.



(a) (b)

Figure IIB.1: 1.2percent Agarose gel showing single 1500 bp of *16S rDNA* amplicons of isolated C17 and C19 (a & b respectively). Lane 1: 100bp DNA ladder; Lane 2: *16S rDNA* amplicon)

Sequence alignment views of the two strains i.e. C17 and C19 have been showed in Table IIB.8 and IIB.9 respectively. The distance matrixes of the two strains have been provided in the Table IIB.10 and IIB.11 respectively.

Table IIB.8: Sequence alignment view of C17 strain using combination of NCBI Gene Bank

Accession	Description	Max score	Total score	Query coverage	E value	Max ident
NR_133024.1	***Bacillus timonensis* strain 10403023**	2585	2585	94percent	0.0	98.98percent
NR_025626.1	*Bacillus humi* strain LMG 22167	2545	2545	95percent	0.0	97.93percent
NR_147383.1	*Bacillus sinesaloumensis* strain Marseille-P3516	2473	2473	94percent	0.0	97.35percent
NR_149252.1	*Bacillus onubensis* strain 0911MAR22V3	2454	2454	91percent	0.0	98.25percent
NR_108491.1	*Bacillus gottheilii* strain WCC 4585	2436	2436	97percent	0.0	96.05percent
NR_149175.1	*Bacillus mesophilus* strain SA4	2406	2406	97percent	0.0	95.57percent
NR_041942.1	*Bacillus acidicola* strain 105-2	2406	2406	97percent	0.0	95.52percent
NR_117050.1	*Bacillus kochii* strain WCC 4582	2390	2390	97percent	0.0	95.25percent
NR_109443.1	*Bacillus songklensis* strain CAU 1033	2388	2388	96percent	0.0	95.61percent
NR_036766.1	*Bacillus bataviensis* strain IDA1115	2386	2386	95percent	0.0	95.66percent
NR_042168.1	*Bacillus novalis* strain IDA3307	2377	2377	95percent	0.0	95.53percent
NR_156041.1	*Bacillus maritimus* strain KS16-9	2376	2376	97percent	0.0	95.13percent
NR_145534.1	*Bacillus salitolerans* strain KC1	2374	2374	96percent	0.0	95.17percent
NR_043334.1	*Bacillus niabensis* strain 4T19	2374	2374	94percent	0.0	95.88percent
NR_146005.1	*Bacillus malikii* strain NCCP-662	2373	2373	95percent	0.0	95.58percent

Table IIB.9: Sequence alignment view of C19 strain using combination of NCBI Gene Bank

Accession	Description	Max score	Total score	Query coverage	E value	Max ident
MK789657.1	***Bacillus cereus* strain K1**	**1988**	**1988**	**99percent**	**0.0**	**91.73percent**
MF346121.1	Uncultured *Bacillus* sp. clone 53	1971	1971	99percent	0.0	91.46percent
MN833549.1	*Bacillus anthracis* strain DHL56	1954	1954	99percent	0.0	91.24percent
MF967405.1	*Bacillus cereus* strain DM-5	1954	1954	99percent	0.0	91.24percent
KF623095.1	*Bacillus* sp. strain FP 1	1954	1954	99percent	0.0	91.32percent
MK053896.1	*Bacillus* sp. strain Eb9	1953	1953	99percent	0.0	91.24percent
KX057625.1	*Bacillus cereus* strain b53	1951	1951	99percent	0.0	91.19percent
KX343987.1	*Bacillus cereus* strain G28	1949	1949	99percent	0.0	91.25percent
KY293394.1	*Bacillus cereus* strain EVRAPSUBAC-1	1947	1947	99percent	0.0	91.17percent
MZ311868.1	*Bacillus subtilis* strain X-c	1940	1940	99percent	0.0	91.09percent
MH985192.1	*Bacillus cereus* strain H18SacTM1	1938	1938	99percent	0.0	90.88percent
MF157568.1	*Bacillus cereus* strain TJ-1-5	1936	1936	99percent	0.0	91.02percent
MF346119.1	Uncultured *Bacillus* sp. Clone 48	1934	1934	99percent	0.0	90.96percent
MN448455.1	*Bacillus* sp. Strain HSW-1	1930	1930	99percent	0.0	90.95percent
MK949150.1	*Bacillus cereus* strain MPF-A1	1930	1930	98percent	0.0	91.11percent
MZ004949.1	*Bacillus* sp. Strain BH11	1930	1930	99percent	0.0	91.08percent
FJ611966.1	*Bacillus cereus* strain GCH-1	1930	1930	99percent	0.0	90.92percent
MN213356.1	*Bacillus* sp. Strain GF-1	1927	1927	99percent	0.0	90.86percent

Finally, the phylogenetic trees of the two isolated strains have been displayed in figure IIB.2 and figure IIB.3 respectively. The trees revealed that C17 strain exhibits its similarities mostly with *Bacillus timonensis* strain 10403023 (Fig: IIB.2). On the other hand, C19 possess its similarities mostly with *Bacillus cereus* strain K1 (Fig: IIB.3).

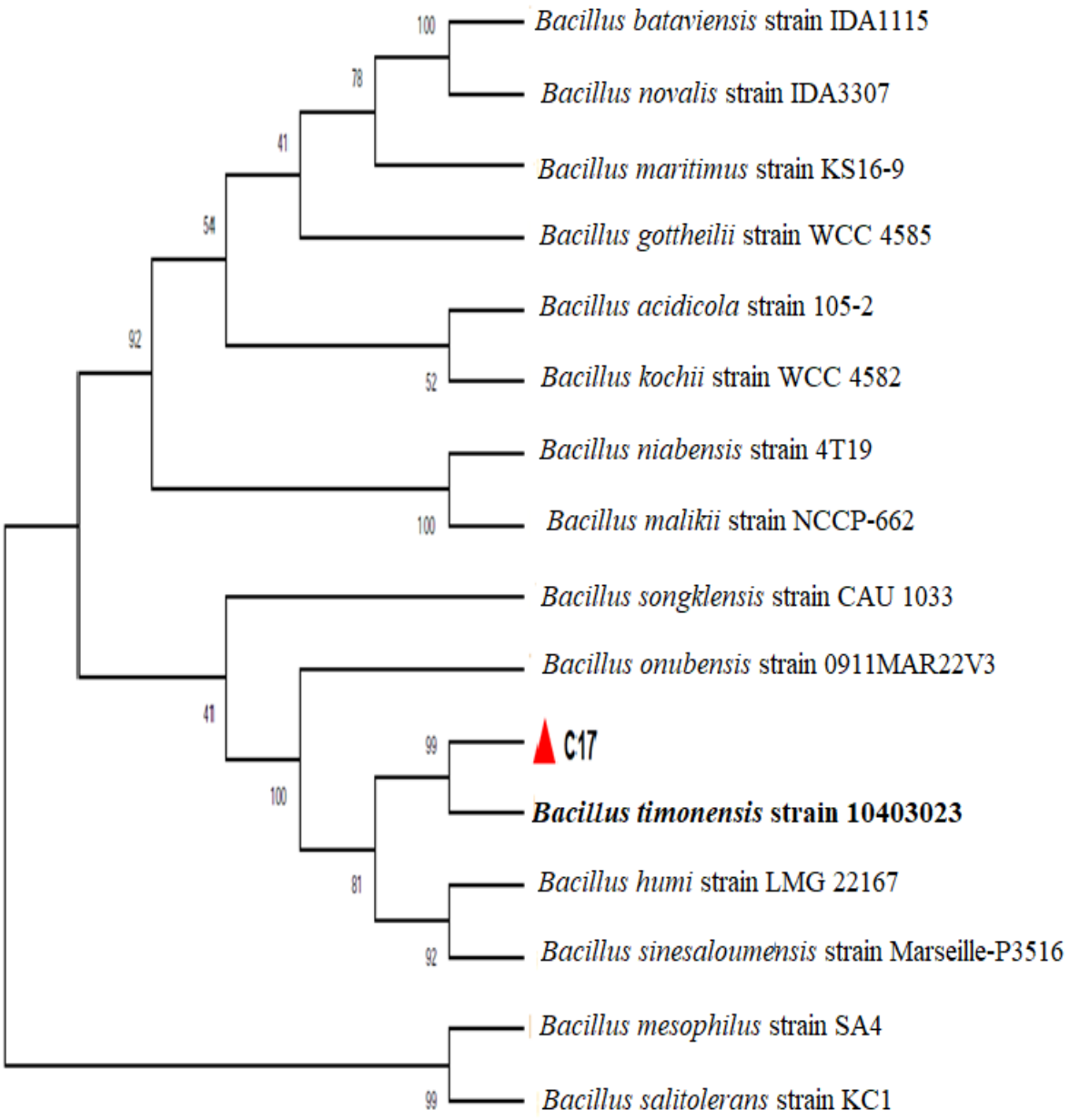


Fig IIB.2: Unrooted phylogenetic tree based on 16S rDNA sequences obtained by the Neighbour-Joining (NJ) method showing the position of C17 strain among its Phylogenetic Neighbours. NCBI Accession Numbers are provided in Parentheses

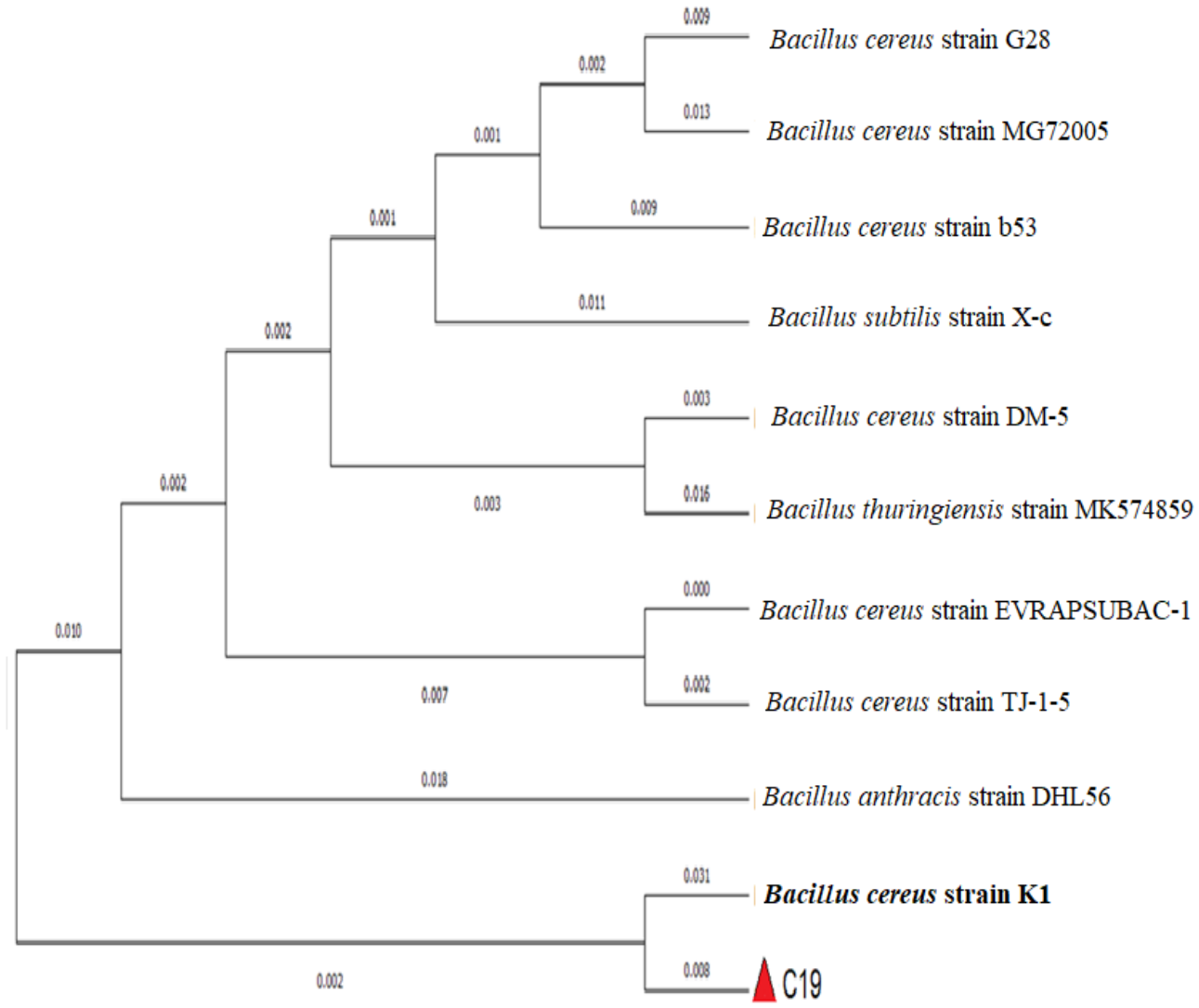


Fig IIB.3: Unrooted phylogenetic tree based on 16S rDNA sequences obtained by the Neighbour-Joining (NJ) method showing the position of C19 strain among its Phylogenetic Neighbours. NCBI Accession Numbers are provided in Parentheses

Sub chapter IIC

Isolation & Identification of two bacterial strains to remove Catechol

IIC.1 Materials and Method:

All the materials used here, are same as mentioned in **A.1** and **B.1**. All the methodologies are also same as mentioned in the **A.1**. Only in case of the enrichment process of the media and soil, 500 PPM of Catechol was used here instead of the Phenol or 4- Chloro Phenol.

In case of reparation of the standard curve along with the standard equation to measure the residual Catechol, known concentrations of Catechol were used instead of Phenol or 4-Chloro Phenol ranging between 20 mg/L to 1000 mg/L. Rest of the procedures were same as mentioned in IIA.1.7.

IIC.2 Results & Discussions:

Total twenty seen (27) colonies were segregated via media enrichment method. After screening, S12 was found to be the most potential strain to remove Catechol as it was able to remove almost 93.61percent of Catechol from the media within 24 hours at 37^{o}C, when the initial conc. was 500 mg/L. In Table No. IIC.1, distinguishing characteristics of all the isolated colonies have been displayed along with the screening results.

Table IIC.1: Screening and colony characterization of the bacterial strains isolated from soil to remove Catechol via media enrichment method

Sample Strain No.	Colony Size	Colony Pigmentation	Colony Form	Colony Margin	Colony Elevation	percent of Catechol degradation
S1	Large	white	Irregular	Serrate	Flat	78.94
S2	Moderate	Light yellow	Circular	Entire	Flat	84.21
S3	Moderate	white	Circular	Entire	Umbonate	89.08
S4	Small	white	Circular	Entire	Raised	82.31
S5	Large	white	Irregular	Undulate	Flat	80.21
S6	Small	white	Irregular	Entire	Raised	87.75
S7	Moderate	Light yellow	Circular	Entire	Flat	86.56
S8	Moderate	Reddish yellow	Circular	Entire	Flat	83.89
S9	Moderate	white	Circular	Entire	Flat	84.66
S10	Pin point	white	Circular	Entire	Raised	85.29
S11	Large	white	Irregular	Serrate	Flat	74.38
S12	**Moderate**	**Light yellow**	**Circular**	**Entire**	**Flat**	**93.61**
S13	Moderate	white	Circular	Entire	Umbonate	76.21
S14	Small	white	Circular	Entire	Raised	84.03
S15	Large	white	Irregular	Undulate	Flat	81.43
S16	Small	white	Irregular	Entire	Raised	74.94
S17	Moderate	Light yellow	Circular	Entire	Flat	85.78
S18	Moderate	Reddish yellow	Circular	Entire	Flat	82.98
S19	Moderate	white	Circular	Entire	Flat	87.12
S20	Pin point	white	Circular	Entire	Raised	38.91
S21	Large	white	Irregular	Serrate	Flat	20.7
S22	Moderate	Light yellow	Circular	Entire	Flat	43.29
S23	Moderate	white	Circular	Entire	Umbonate	32.91
S24	Small	white	Circular	Entire	Raised	34.03
S25	Large	white	Irregular	Undulate	Flat	29.96
S26	Small	white	Irregular	Entire	Raised	23.57
S27	Moderate	Light yellow	Circular	Entire	Flat	26.31

Similarly, total sixty five (65) colonies were segregated via soil enrichment method. After screening, it was found that S37 was the most potential strain to remove Catechol as it was able to remove almost 95.08percent of Catechol from the media within 24 hours at 37^{o}C, when the initial conc. of Catechol was 500 mg/L. In Table No. IIC.2, distinguishing characteristics of all the segregated colonies have been displayed along with the screening results.

Table IIC.2: Screening and colony characterization of the bacterial strains isolated from soil to remove Catechol via soil enrichment method

Sample Strain No.	Colony Size	Colony Pigmentation	Colony Form	Colony Margin	Colony Elevation	percent of Catechol degradation
S28	Moderate	Reddish yellow	Circular	Entire	Flat	7.08
S29	Moderate	white	Circular	Entire	Flat	28.98
S30	Pin point	white	Circular	Entire	Raised	24.56
S31	Large	white	Irregular	Serrate	Flat	24.84
S32	Moderate	Light yellow	Circular	Entire	Flat	2.17
S33	Moderate	white	Circular	Entire	Umbonate	73.75
S34	Small	white	Circular	Entire	Raised	10.59
S35	Large	white	Irregular	Undulate	Flat	37.26
S36	Small	white	Irregular	Entire	Raised	53.26
S37	**Moderate**	**Light yellow**	**Circular**	**Entire**	**Flat**	**95.08**
S38	Moderate	Reddish yellow	Circular	Entire	Flat	27.41
S39	Moderate	white	Circular	Entire	Flat	10.31
S40	Pin point	white	Circular	Entire	Raised	21.96
S41	Large	white	Irregular	Serrate	Flat	53.26
S42	Moderate	Light yellow	Circular	Entire	Flat	24.63
S43	Moderate	white	Circular	Entire	Umbonate	20.42
S44	Small	white	Circular	Entire	Raised	12.7
S45	Large	white	Irregular	Undulate	Flat	23.57
S46	Small	white	Irregular	Entire	Raised	76.59
S47	Moderate	Light yellow	Circular	Entire	Flat	33.19
S48	Moderate	Reddish yellow	Circular	Entire	Flat	36.84
S49	Moderate	white	Circular	Entire	Flat	15.64
S50	Pin point	white	Circular	Entire	Raised	12.7
S51	Large	white	Irregular	Serrate	Flat	32.63
S52	Moderate	Light yellow	Circular	Entire	Flat	24.21
S53	Moderate	white	Circular	Entire	Umbonate	17.08
S54	Small	white	Circular	Entire	Raised	30.1
S55	Large	white	Irregular	Undulate	Flat	15.78
S56	Small	white	Irregular	Entire	Raised	25.68
S57	Moderate	Light yellow	Circular	Entire	Flat	28.42
S58	Moderate	Reddish yellow	Circular	Entire	Flat	36.28
S59	Moderate	white	Circular	Entire	Flat	19.43
S60	Pin point	white	Circular	Entire	Raised	34.03
S31	Large	white	Irregular	Serrate	Flat	35.85
S62	Moderate	Light yellow	Circular	Entire	Flat	50.59
S63	Moderate	white	Circular	Entire	Umbonate	11.29
S64	Small	white	Circular	Entire	Raised	38.66
S65	Large	white	Irregular	Undulate	Flat	35.43
S66	Small	white	Irregular	Entire	Raised	40.07
S67	Moderate	Light yellow	Circular	Entire	Flat	32.45

S68	Moderate	Reddish yellow	Circular	Entire	Flat	12.14
S69	Moderate	white	Circular	Entire	Flat	62.73
S70	Pin point	white	Circular	Entire	Raised	51.47
S71	Large	white	Irregular	Serrate	Flat	13.68
S72	Moderate	Light yellow	Circular	Entire	Flat	32.31
S73	Moderate	white	Circular	Entire	Umbonate	57.78
S74	Small	white	Circular	Entire	Raised	43.15
S75	Large	white	Irregular	Undulate	Flat	29.26
S76	Small	white	Irregular	Entire	Raised	73.57
S77	Moderate	Light yellow	Circular	Entire	Flat	39.36
S78	Moderate	Reddish yellow	Circular	Entire	Flat	69.47
S79	Moderate	white	Circular	Entire	Flat	45.64
S80	Pin point	white	Circular	Entire	Raised	41.05
S81	Large	white	Irregular	Serrate	Flat	10.31
S82	Moderate	Light yellow	Circular	Entire	Flat	52.56
S83	Moderate	white	Circular	Entire	Umbonate	18.17
S84	Small	white	Circular	Entire	Raised	18.59
S85	Large	white	Irregular	Undulate	Flat	42.14
S86	Small	white	Irregular	Entire	Raised	2.59
S87	Moderate	Light yellow	Circular	Entire	Flat	24.07
S88	Moderate	Reddish yellow	Circular	Entire	Flat	31.22
S89	Moderate	white	Circular	Entire	Flat	16.07
S90	Pin point	white	Circular	Entire	Raised	32.63
S91	Large	white	Irregular	Serrate	Flat	38.8
S92	Moderate	Light yellow	Circular	Entire	Flat	28.28

From table IIC.1 and IIC.2, it can be displayed clearly that S12 and S37 are the most potential strains (as highlighted in the tables respectively). These two strains were selected for further studies i.e. morphological, biochemical and phylogenetic identification.

Morphological & Biochemical Characterizations are showed in the Table No. IIC.3, IIC.4, IIC.5 and IIC.6 respectively.

Table IIC.3: Colony characterization of S12 & S37 studied on Petri plate

Colony characteristics	S12	S37
Size	~ 1 mm	~ 1mm
Opacity	Opaque	Opaque
Surface growth	Smooth	Smooth
Edge	Sharp	Sharp
Consistency	Good	Good
Pigmentation	Nil	Nil

Table IIC.4: Colony characterization of S12 & S37 studied on Slant

Colony characteristics	S12	S37
Opacity	Opaque	Opaque
Surface growth	Smooth	Smooth
Consistency	Good	Good
Colour	Good	Good
Pigmentation	Nil	Nil

Table IIC.5: Physicochemical Characteristics of S12 & S37

Characteristics	S12	S37
Arrangement	Cell shape: rod/bacillus Cell size: > 6 micron	Cell shape: rod/bacillus Cell size: 5- 6 micron
Gram staining	Positive	Positive
Spore staining	Negative	Negative
Motility	Non motile	Non motile

Table IIC.6: Growth Characteristics of S12 & S37 in stationary and shaking condition

Experiment	Observation	
	S12	**S37**
Growth in 50 ml medium taken in 250 ml Erlenmeyer flask	A. Stationary condition (a) After 24 hr : - Poor growth, no ring formation, no pellicle formation, sedimentation at the bottom, upper portion of the broth was clear.	Same as S12
	(b) After 48 hr : - Same as after 24 hr but the growth was fair.	
	B. Shaking condition. (a) Fair growth, turbid, no sedimentation, no pellicle formation, no pigmentation, and no ring formation.	Same as S12
	(b) After 48 hr : - Same as after 24 hr	

Results of the Carbohydrate fermentation as well as the other physicochemical characteristics of the two particular strains S12 and S37 have been shown in Table No. IIC.7.

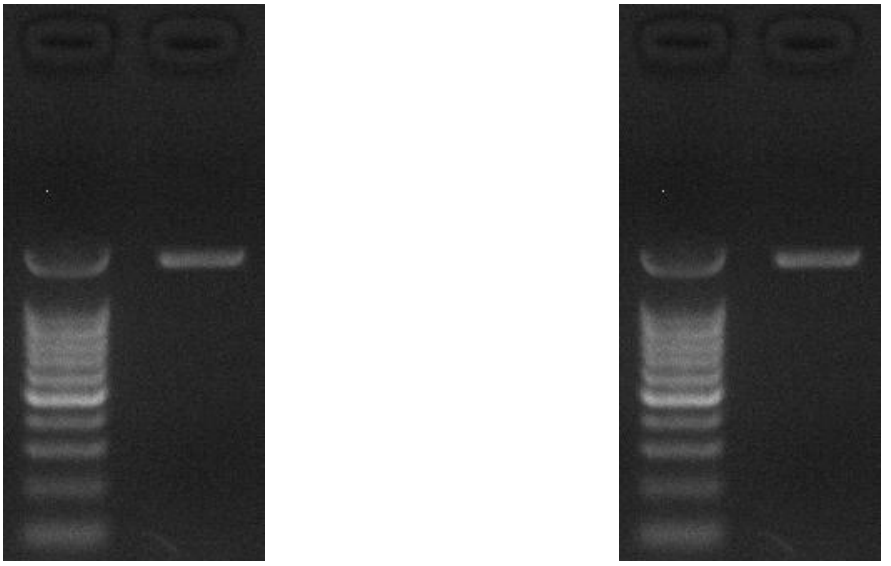
Table IIC.7: Results of Biochemical tests of S12 & S37

Parameters	Characteristics of S12		Characteristics of S37	
Carbohydrate fermentation at 37˚C	Acidity	Gas formation	Acidity	Gas formation
Fructose	Positive	Positive	Positive	Positive
Arabinose	Negative	Negative	Negative	Negative
Galactose	Negative	Negative	Negative	Negative
Xylose	Negative	Negative	Negative	Negative
Glucose	Positive	Positive	Positive	Negative
Lactose	Negative	Negative	Negative	Negative
Raffinose	Negative	Negative	Negative	Negative
Sucrose	Negative	Negative	Negative	Negative
Maltose	Positive	Positive	Positive	Positive
Dextrin	Negative	Negative	Positive	Negative
Salicin	Negative	Positive	Positive	Positive
Mannitol	Negative	Negative	Negative	Negative
Glycerol	Negative	Negative	Negative	Negative
Inositol	Negative	Negative	Negative	Negative
Sorbitol	Negative	Negative	Negative	Negative
Catalase reduction	Positive		Positive	
Litmus milk test	Positive		Positive	
Starch hydrolysis test	Positive		Positive	
Urease test	Positive		Positive	
Ammonia from Arginine	Positive		Positive	
Arginine as sole source of energy	Positive		Positive	
Nitrate reduction	Positive		Positive	
Indole formation	Negative		Negative	
Voges-Proskaurer test at pH < 6	Positive		Positive	
Voges-Proskaurer test at pH > 7	Positive		Positive	
Growth under anaerobic condition	Negative		Negative	
Growth at 5˚ C	Poor		Poor	
Growth at	Poor		Poor	

10˚C		
Growth at 20˚C	Poor	Poor
Growth at 30˚C	Vigorous	Vigorous
Growth at 35˚C	Vigorous	Vigorous
Growth at 40˚ C	Vigorous	Vigorous
Growth at 50˚ C	Poor	Poor
Growth at 2percent NaCl concentration	Vigorous	Vigorous
Growth at 5percent NaCl concentration	Vigorous	Vigorous
Growth at 7percent NaCl concentration	Poor	Poor
Growth at 10percent NaCl concentration	Poor	Poor
Growth at extreme pH 9.6 & 6.5 percent NaCl conc.	Positive	Positive
Growth at extreme pH 6.8 & 6.5 percent NaCl conc.	Positive	Positive
Growth at extreme pH 5.6 & 6.5 percent NaCl conc.	Positive	Positive

From the biochemical studies, it can be deducted that both of the isolated strains are **rod shaped (bacillus), gram positive, non motile** and **obligatory aerobic** in nature. Both of them did not form spores.

In case of identification of the two isolated strains i.e. S12 and S37, 16s rDNA assay was deployed (A.1.10). After obtaining the gel eletrogram images (Fig: IIC.1), the remaining results were explored.



Lane: 1 Lane:2 Lane: 1 Lane:2

(a) (b)

Figure IIC.1: 1.2percent Agarose gel showing single 1500 bp of *16S rDNA* amplicons of isolated S12 and S37 (a & b respectively). Lane 1: 100bp DNA ladder; Lane 2: *16S rDNA* amplicon)

Sequence alignment views of the two strains i.e. S12 and S37 have been showed in Table IIC.8 and IIC.9 respectively. The distance matrixes of the two strains have been provided in the Table IIC.10 and IIC.11 respectively.

Table IIC.8: Sequence alignment view of S12 strain using combination of NCBI Gene Bank

Accession	Description	Max score	Total score	Query Coverage	E value	Max ident
NR_157734.1	*Bacillus paramycoides* strain MCCC 1A04098	2615	2615	98percent	0.0	98.91per cent
NR_157729.1	*Bacillus albus* strain MCCC 1A02146	2610	2610	98percent	0.0	98.84per cent
NR_115714.1	*Bacillus cereus* strain CCM 2010	2610	2610	98percent	0.0	98.71per cent
NR_074540.1	*Bacillus cereus* ATCC 14579	2604	2604	98percent	0.0	98.84per cent
NR_115526.1	*Bacillus cereus* strain IAM 12605	2604	2604	98percent	0.0	98.84per cent
NR_152692.1	*Bacillus wiedmannii* strain FSL W8-0169	2599	2599	98percent	0.0	98.58per cent
NR_157735.1	*Bacillus proteolyticus* strain MCCC 1A00365	2599	2599	98percent	0.0	98.71per cent
NR_112630.1	*Bacillus cereus* strain NBRC 15305	2595	2595	97percent	0.0	98.90per cent
NR_113266.1	*Bacillus cereus* strain JCM 2152	2590	2590	97percent	0.0	98.90per cent
NR_157728.1	*Bacillus paranthracis* strain MCCC 1A00395	2588	2588	98percent	0.0	98.57per cent
NR_114582.1	*Bacillus cereus* ATCC 14579	2586	2586	97percent	0.0	98.90per cent
NR_157733.1	*Bacillus pacificus* strain MCCC 1A06182	2582	2582	98percent	0.0	98.50per cent
NR_121761.1	*Bacillus toyonensis* strain BCT-7112	2582	2582	98percent	0.0	98.37per cent
NR_043403.1	*Bacillus thuringiensis* strain IAM 12077	2582	2582	98percent	0.0	98.57per cent
NR_113991.1	***Bacillus pseudomycoides* strain NBRC 101232**	**2573**	**2573**	**97percent**	**0.0**	**98.63per cent**

Table IIC.9: Sequence alignment view of S37 strain using combination of NCBI Gene Bank

Accession	Description	Max score	Total score	Query Coverage	E value	Max ident
NR_157734.1	***Bacillus paramycoides* strain MCCC 1A04098**	**2776**	**2776**	**96percent**	**0.0**	**99.87percent**
NR_157729.1	*Bacillus albus* strain MCCC 1A02146	2771	2771	96percent	0.0	99.80percent
NR_115714.1	*Bacillus cereus* strain CCM 2010	2771	2771	97percent	0.0	99.67percent
NR_152692.1	*Bacillus wiedmannii* strain FSL W8-0169	2760	2760	97percent	0.0	99.54percent
NR_157735.1	*Bacillus proteolyticus* strain MCCC 1A00365	2760	2760	96percent	0.0	99.67percent
NR_074540.1	*Bacillus cereus* ATCC 14579	2758	2758	96percent	0.0	99.73percent
NR_157728.1	*Bacillus paranthracis* strain MCCC 1A00395	2748	2748	96percent	0.0	99.54percent
NR_157733.1	*Bacillus pacificus* strain MCCC 1A06182	2743	2743	96percent	0.0	99.47percent
NR_121761.1	*Bacillus toyonensis* strain BCT-7112	2737	2737	97percent	0.0	99.34percent
NR_157731.1	*Bacillus mobilis* strain MCCC 1A05942	2732	2732	96percent	0.0	99.34percent
NR_115526.1	*Bacillus cereus* strain IAM 12605	2723	2723	95percent	0.0	99.73percent
NR_114582.1	*Bacillus cereus* ATCC 14579	2715	2715	95percent	0.0	99.73percent
NR_024697.1	*Bacillus mycoides* strain DSM 11821	2715	2715	96percent	0.0	99.14percent
NR_112630.1	*Bacillus cereus* strain NBRC 15305	2704	2704	94percent	0.0	99.73percent
NR_113266.1	*Bacillus cereus* strain JCM 2152	2700	2700	94percent	0.0	99.73percent

Finally, the phylogenetic trees of the two isolated strains have been displayed in figure IIC.2 and figure IIC.3 respectively. The trees revealed that S12 strain exhibits its similarities mostly with *Bacillus Psudomucoides* strain NBRC 101232 (Fig: IIC.2). On the other hand, S37 possess its similarities mostly with *Bacillus paramycoides* strain MCCC 1A04098 (Fig: IIC.3).

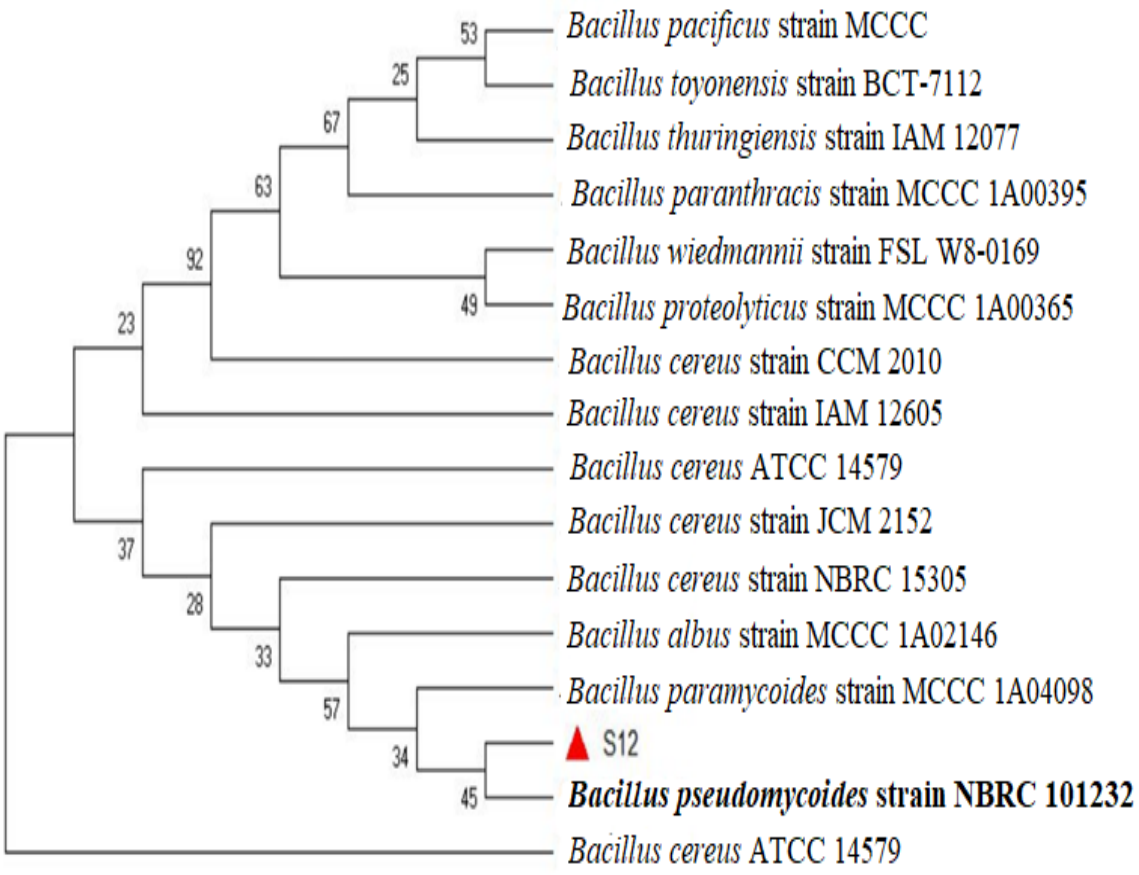


Fig IIC.2: Unrooted phylogenetic tree based on 16S rDNA sequences obtained by the Neighbour-Joining (NJ) method showing the position of S12 strain among its Phylogenetic Neighbours. NCBI Accession Numbers are provided in Parentheses

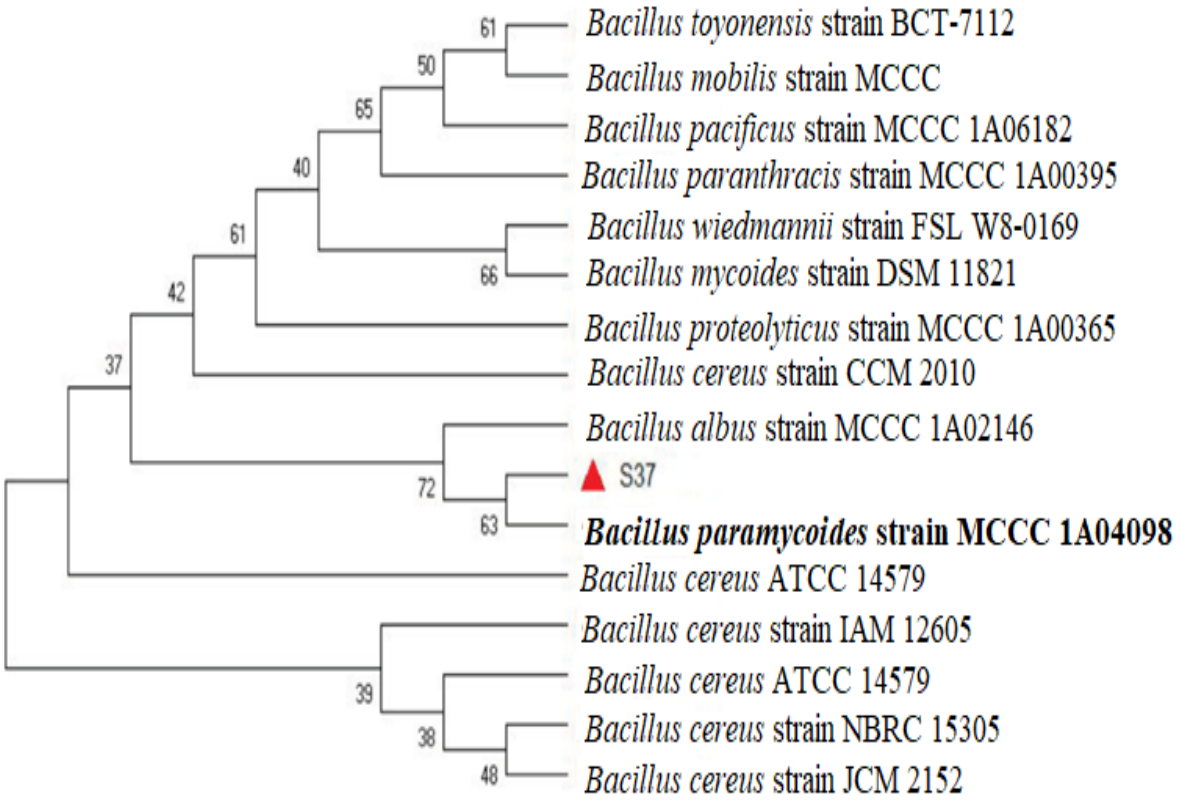


Fig IIC.3: Unrooted phylogenetic tree based on 16S rDNA sequences obtained by the Neighbour-Joining (NJ) method showing the position of S37 strain among its Phylogenetic Neighbours. NCBI Accession Numbers are provided in Parentheses

Conclusions:

Now a day, water pollution caused by aromatic hydrocarbons has become a burning question. As the Phenolic compounds are highly water soluble, these can be mixed with aquatic bodies within very short time and possess the ability to collapse the entire aquatic ecosystem and can also exhibit its harmful effects to the terrestrial creatures, even to the human beings (Mohanty and Jena; 2017). So, Phenolic compounds should immediately be removed from the water bodies, particularly from their point sources. Bioremediation is widely accepted throughout the world because of its eco friendliness nature. To remove three Phenolic compounds (Phenol, 4- Chloro Phenol & Catechol) from the waste water, six bacterial strains were finally selected via isolation and screening process. *Brevibacillus formosus* strain NRRL NRS- 863 and *Pseudomonas otitidis* strain MCC10330 were selected to dispose of Phenol, *Bacillus timonensis* strain 10403023 & *Bacillus cereus* strain K1 were chosen to remove 4- Chloro Phenol, *Bacillus Psudomucoides* strain NBRC 101232 & *Bacillus paramycoides* strain MCCC 1A04098 were chosen to remove Catechol. All of these strains were able to remove Phenol, 4- Chloro Phenol and Catechol respectively when the initial concentrations of the each compound were 500 mg/L. Then morphological and biochemical tests were performed for each of the six strains and finally 16 s rDNA assay was deployed to identify those strains. Mineral Salt Medium was utilized to prepare a large size of inoculums of all these strains for further studies.

Mohanty and Jena; 2017 isolated *Pseudomonas* sp. NBM11 which was able to degrade 1000 mg/L Phenol in some optimized parameters. Aravindhan R et al., 2014, suggested a mix microbial culture of *Pseudomonas aeruginosa* and *Bacillus subtilis*, isolated from the soil and microbiology laboratory of the Central Leather research Institute, Chennai, India. These two strains were reported to degrade 250 mg/L Phenol and a polyphenolic compound Wattle completely (100percent) within 36 hr and 48 hr respectively in favourable conditions.

So in case of future study, consideration of some physico - chemical parameters is strongly recommended to obtain better performance of these strains in batch reactor to remove these toxic phenolic compounds when the initial conc. will be higher. Moreover, treatment of these compounds as bi-solute (i.e. Phenol & 4- Chloro Phenol, Phenol & Catechol and 4- Chlorophenol & Catechol) and tri- solute (Phenol, 4- Chlorophenol & Catechol) mixtures is also recommended for the future study.

Chapter III

Optimization of process parameters in batch culture

Introduction:

Biodegradation of any substance by some bacteria or algae or fungi, depends upon some physico – chemical or biological conditions always. So it is very important to understand how those conditions affect the biodegradation process. To identify the perfect values of the parameters, many experiments are required. Normally, pH, temperature, time, initial conc. of the substance etc are taken into account while performing the biodegradation study. Favourable conditions may enhance the mechanism. Moreover, outstanding performances of the strains can be achieved in suitable parameters and thereby, target can be achieved very easily i.e. satisfactory degradation of the substance may be obtained within a low time span. So, to optimize the parameters is very much useful in case of biodegradation.

Chakraborty et al. (2010) conducted an examination to evaluate the biodegradation of phenol by native bacterial strain segregated from the effluents of a coke handling plant. The rate of phenol evacuation by the oppressed strain ESDSPB2 was researched to optimize the different physiological boundaries like pH, temperature and glucose conc. of the medium. The ideal circumstances for phenol evacuation were viewed as pH 7, 30^{o}C of incubation temperature and 0.25 percent level of glucose.

Nor Suhaila et al., (2010) performed a study to degrade Phenol in shake flask method deploying *Rhodococcus* UKM- P strain. Five parameters were considered there viz. pH, temperature, time, source of nitrogen and salt concentration. 0.5 g/L of Phenol was degraded at 30^{o}C, 7.5 pH, at 21 hour of time period. 0.4 g/L ammonium sulphate and 0.1 g/L conc. of NaCl was favourable for this degradation.

Sreeja Mole et al., (2021) studied the biodegradation of P- Nitro Phenol using *Achromobacter denitrifacians,* isolated from the industrial waste water. Several parameters were justified there (i.e. pH, temperature, conc. of glucose, peptone and metal ions) and the optimal conditions for the degradation of P- Nitro Phenol were found to be 7.5 pH, 35^{o}C temperature, 0.25 g/L conc. of glucose, 0.25 g/L conc. of peptone and 0.01 g/L zn^{+2} ions.

In the current study, parameters were to be optimized for all the six isolated strains (described in the chapter II). Now, the optimal parameters are always species specific. Furthermore, in case of same substance to be degraded, optimal parameters may vary from species to species. So, the optimization study was to be conducted individually for all the six strains. Primarily, seven (7) parameters were considered to be studied viz. temperature, pH of the media,

residence time, volume of the media, inoculums size, initial conc. of the substance and shaking speed of the flaks (in RPM). However after some experiments, it was found that shaking speed had no significant effects on the bacterial strains while removing the Phenolic substances. That's why, this parameter was excluded. Other six (6) parameters were found to be very much significant.

In this study, optimization of various process parameters was required to remove higher concentrations of Phenolic substances. As in the chapter II, it was revealed that isolated strains *Brevibacillus formosus* strain NRRL NRS- 863 & *Pseudomonas otitidis* strain MCC10330 were able to remove 500 mg/L Phenol, *Bacillus timonensis* strain 10403023 & *Bacillus cereus* strain K1were able to remove 4- Chloro Phenol and *Bacillus Psudomucoides* strain NBRC 101232 & *Bacillus paramycoides* strain MCCC 1A04098 were able to dispose of Catechol respectively, within 24 hours at 37^{o}C temperature at 7 ± 0.2 pH. But when the initial conc. of the Phenolic substances increased from 500 mg/L up to 600 or 700 mg/L, the degradation efficacy of the strains decreased rapidly. To overcome this phenomenon, parameter optimization study was conducted for all the six bacterial strains individually.

Sub chapter III A

Optimization of parameters of the Phenol degrading strains

IIIA.1 Materials and Methods:

IIIA.1.1 Materials:

Brevibacillus formosus strain NRRL NRS- 863 and *Pseudomonas otitidis* strain MCC10330 were isolated from the contaminated soil, collected from South Howrah state General Hospital by enrichment method as described in the chapter II, section IIA.1.5. A large volume of inoculums of the two above mentioned strains was already prepared via acclimatization process in Mineral Salt Medium (MSM) as described in chapter II, section IIA.1.8.

Phenol solution was prepared synthetically in the laboratory. Conc. of Phenol stock solution was maintained as 10 g/L (or 10,000mg/L).

IIIA.1.2 Experimental set up:

All the experiments were performed in 250 mL, 500 mL and 1L conical flasks with cotton plugged. Readymade Mineral Salt Medium (MSM) was utilized as the culture media. Composition and preparatory method of this medim has been described in chapter II, section IIA.4.3. pH of the media was adjusted by using 1N HCl and 1N NaOH as per requirement. To maintain the temperature and dynamic condition, BOD incubator shaker was used for the experiments with an average speed of 140- 150 RPM. Different residence time, media volume and inoculums size of the two strains (*Brevibacillus formosus* strain NRRL NRS- 863 & *Pseudomonas otitidis* strain MCC10330) were maintained properly for each experiments. Finally, initial conc. of Phenol also played a pivotal role in all the experiments

IIIA.1.3 Analytical Method:

Percentage of degradation of the Phenol from the waste water was measured in the UV-VIS spectrophotometer at 510 nm wavelength followed by 4- Amino Antipyrene method as suggested by Yang, R.D., et al. 1975. Details of this process has been mentioned in chapter II, section IIA.1.7.

IIIA.2 Results and Discussions:

Six parameters were considered for this study. Moreover, the study was conducted for the two strains simultaneously. In case of the two strains, some of the optimized parameters were found to be different from each other whereas, some parameters found to be same.

IIIA.2.1 Effect of temperature:

Brevibacillus formosus strain NRRL NRS- 863 was cultured in six different temperatures ranging between 20^{o}C to 45^{o}C with an interval of 5^{o}C to find out the most suitable one. 30^{o}C temperature was found to be the most favourable temperature for this particular strain while removing Phenol. 99.96% degradation was achieved in this temperature when the initial conc. was 600 mg/L (higher than the initial conc. of Phenol which was maintained during screening). Below that temperature, the degradation efficacy of the strain decreased. In 25^{o}C, 94.25% degradation was obtained and in 20^{o}C, 82.54% degradation was achieved. Similarly, when temperature increased, gradual decrease in the degradation percentage was observed. 96.24%, 87.25% and 78.2% degradations were achieved in 35^{o}C, 40^{o}C and 45^{o}C temperature respectively (Fig: IIIA.1 (a)).

On the other hand, in case of *Pseudomonas otitidis* strain MCC10330, 40^{o}C temperature was found to be most suitable during degradation of the Phenol. 99.38% degradation was achieved in this temperature when the initial Phenol conc. was 600 mg/L. Here, the strain was cultured in five different temperatures from 25^{o}C to 45^{o}C with an interval of 5^{o}C. Below and above the optimal temperature, the degradation percentage decreased gradually. A slight decrease in degradation percentage was noted at 45^{o}C. Also at 35^{o}C and 30^{o}C, 94.25% and 88.29% degradation was obtained respectively. But when the temperature reduced to 25^{o}C, only 76.26% degradation was achieved by the strain (Fig: IIIA.2 (a)).

IIIA.2.2 Effect of pH:

In case of both the strains, pH 7.0 was found to be the most suitable pH for the degradation of Phenol. Degradation percentage decreased gradually, both in acidic and basic pH in case of both the strains.

In case of *Brevibacillus formosus* strain NRRL NRS- 863; almost 99.8% degradation was achieved at pH 7. Three tests were performed in acidic pH where pH was maintained at 6.5, 6.0 and 5.5. 96.25%, 90.54% and 78.6% degradation were observed in those acidic pH mediums respectively. Similarly, three experiments were carried out in basic pH mediums where pH values were 7.5, 8.0 & 8.5 and 97.25%, 91.42% & 81.24% of degradations were found from those pH mediums respectively (Fig: IIIA.1 (b)).

In case of *Pseudomonas otitidis* strain MCC10330, 99.32% degradation was found at the optimal pH value i.e. 7. Below that pH, degradation efficacy of the strain decreased gradually. 96.24% degradation was obtained at pH 6.5 whereas, 88.52% & 81.25% degradations were found at pH 6.0 and pH 5.5 respectively. Slight decrease in degradation percentage was found while increasing the pH value. 97.21% & 90.24% degradations were found at pH 7.5 and 8.0 respectively. 84.24% degradation was observed at pH 8.5 (Fig: IIIA.2 (b)).

IIIA.2.3 Effect of inoculums size:

Inoculums size played a pivotal role in the bioremediation of Phenol. For the convenience in calculation, inoculums size has been denoted by percentage. In case of the each strain, eight experiments were carried out with eight different ranges of the inoculums, ranging between 1% to 8% with an interval of 1%.

In case of *Brevibacillus formosus* strain NRRL NRS- 863, a moderate range of variation was obtained while changing the percentage of inoculums. Around 70% and 78% degradations were observed when the inoculums size of *Brevibacillus formosus* strain NRRL NRS- 863 were 1% and 2% respectively. 84.25% and 90.3% degradations were achieved when the inoculums size was increased up to 3% & 4% respectively. When 5% inoculums were added to the media, 97.42% degradation was observed and it was increased to almost 100% when 6% inoculums were added. But, in case of 7% & 8% inoculums, degradation percentage again decreased to around 98% and 92% respectively indicating 6% inoculums size to be the optimum inoculums size for this particular strain while degrading Phenol (Fig: IIIA.1 (c))

Almost similar profile was observed during the study of *Pseudomonas otitidis* strain MCC10330. Around 70%, 78% & 89% degradation of Phenol was achieved when 1%, 2% & 3% inoculums of the strain was added to the culture media respectively. Around 94% & 97% degradations were found when the inoculums size was increased up to 4% and 5% respectively. Best degradation was obtained at 6% inoculums size where 99.4% Phenol was degraded. Beyond that, when the inoculums size was increased up to 7% & 8%, a slight decrease in the degradation efficacy was found as 98.21% and 95.25% degradation of Phenol was observed against those inoculums size respectively (Fig: IIIA.2 (c)).

IIIA.2.4 Effect of incubation time:

One of the most important parameter was the incubation time. It was found that, up to a certain time period, the degradation efficacy of both the strains increased and after that, decreased.

While culturing *Brevibacillus formosus* strain NRRL NRS- 863, six (6) different times were selected for the study viz. 18 hr, 24 hr, 30 hr, 36 hr, 40 hr and 48 hr. A wide variation was found during this study. Only 52.54% degradation of Phenol was observed after 18 hour and after 24 hour, it increased only up to 68.6%. After 30 hours of incubation, 80.74% of degradation was achieved and after 36 and 40 hours of incubation, 90.33% & 99.18% degradations were obtained. But after 48 hours, degradation percentage decreased slightly as 97.48% degradation was observed. So, the optimal time for this strain was found to be 40 hours (Fig: IIIA.1 (d)).

Similarly in case of *Pseudomonas otitidis* strain MCC10330, the most suitable time was optimized at 40 hours. Below and after this time span, degradation efficacy of the strain decreased. During the initial stage of the study, approximately 62% & 78% degradations were obtained when the incubation time was 18 hours and 24 hours respectively. 88.34% degradation was observed after 30 hours and in turn, 94.24% degradation was found after 36 hours. At 40 hours, 99.34% degradation was resulted. And finally at 48 hours, degradation efficacy again slightly decreased up to 96.21% (Fig: IIIA.2 (d)).

IIIA.2.5 Effect of media volume:

Seven different volumes of media were utilized to check the effect of the media volume on the degradation efficacy of the strains. 200 mL, 400 mL, 500 mL, 600 mL, 800 mL, 1L and 1.6 L media volumes were chosen to conduct the study.

While studying *Brevibacillus formosus* strain NRRL NRS- 863, a very small range of variation was noticed in case of the outcome i.e. percent of degradation of Phenol. Around 97% & 98% degradations were achieved when the volume of the culture media was 200 mL and 400 mL respectively. The peak was found at 500 mL media volume where almost 100% degradation was achieved. Beyond that volume, the degradation efficacy of the strain was decreased. Around 97% & 93% degradations were obtained when the media volume was 600 mL & 800 mL respectively. And finally at the volume of 1L & 1.6 L, 92.25% & 91.57%

degradations of Phenol were observed. Though there was not much variation, 500 mL media volume was optimized (Fig: IIIA.1 (e)).

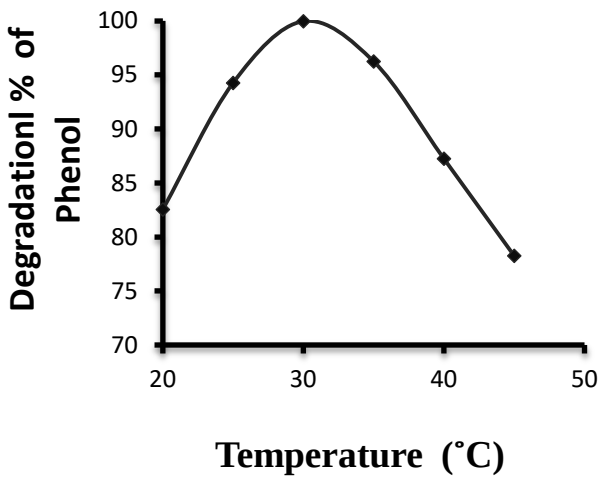
Also, during the study of *Pseudomonas otitidis* strain MCC10330, a small scale changes were found during the study of media volume. Around 98% degradations were achieved when the media volume were 200 mL & 400 mL. Optimum value of the media volume was observed at 500 mL, where 99.38% degradation was found. After that, the degradation efficacy of the strain decreased gradually and reached up to 79.24% when the media volume was 1.6L (Fig: IIIA.2 (e)).

IIIA.2.6 Effect of initial concentration of Phenol:

It was a vital parameter for both of the strains. In normal parameters, which were applied during the screening (as mentioned in IIA.1.4.2 & IIA.1.6), degradation percentage rapidly declined while increasing the initial conc. of Phenol. But when all the optimized parameters were applied together to treat higher conc. of Phenol, the scenario totally changed. Up to a certain concentration as well as in all the optimized parameters, the degradation percentage remained almost same as well as very fruitful although, a small decrease was observed. Beyond that concentration, degradation percentage decreased.

While studying *Brevibacillus formosus* strain NRRL NRS- 863, 98.25% & 99.12% degradations were achieved when the initial conc. of Phenol was 600 mg/L and 700 mg/L respectively. Maximum degradation was observed at 800mg/L initial conc. where almost complete degradation was observed. Approximately 98% and 94% degradations were gained at 900 mg/L and 1000 mg/L initial conc. respectively. So, 800 mg/L initial conc. of Phenol was optimized for *Brevibacillus formosus* strain NRRL NRS- 863 (Fig: IIIA.1 (f)).

During the study of *Pseudomonas otitidis* strain MCC10330, 96.24% & 97.45% degradations were found when the initial conc. of Phenol was 600 mg/L & 700 mg/L respectively and in turn, 98.74% & 99.12% of degradations were achieved at the initial conc, of 800 mg/L & 900 mg/L respectively. Ultimately at 1000 mg/L initial concentration, the peak was found where 99.43% degradation was obtained. But when the conc. of Phenol increased further, fall in the degradation percentage observed. At 1200 mg/L of initial concentration, 86.74% degradation was achieved (Fig: IIIA.2 (f)).



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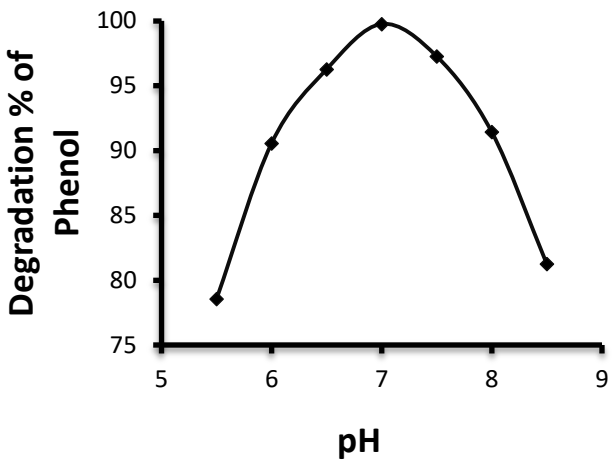

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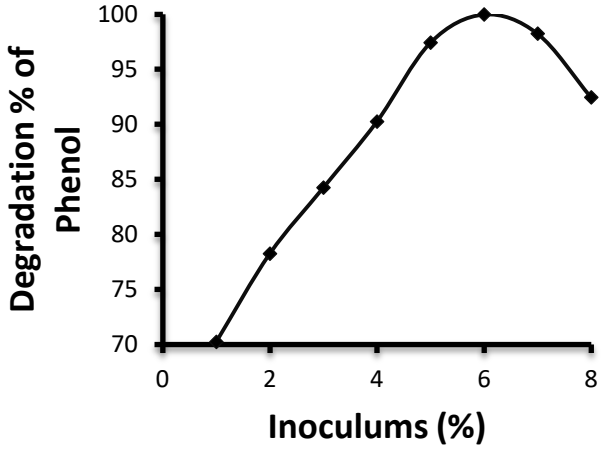

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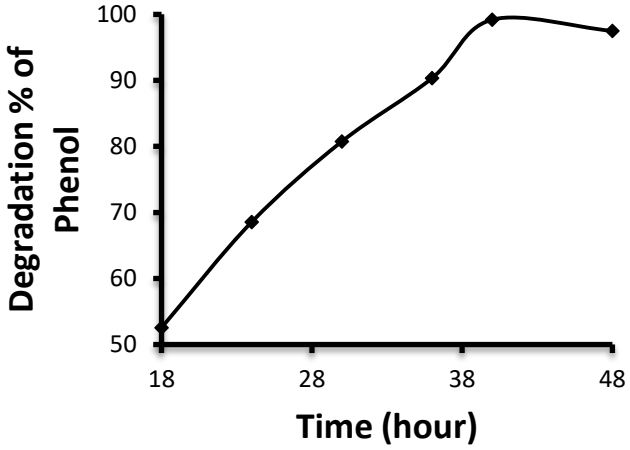

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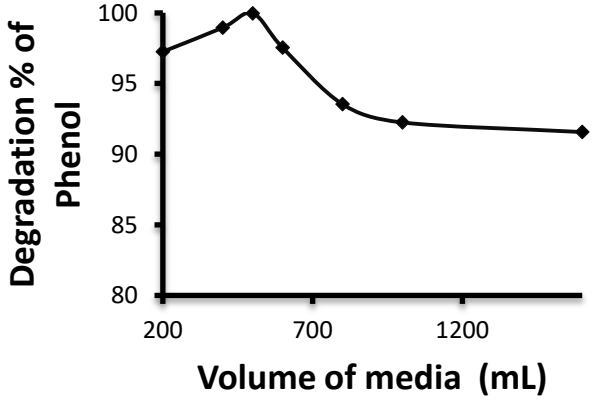

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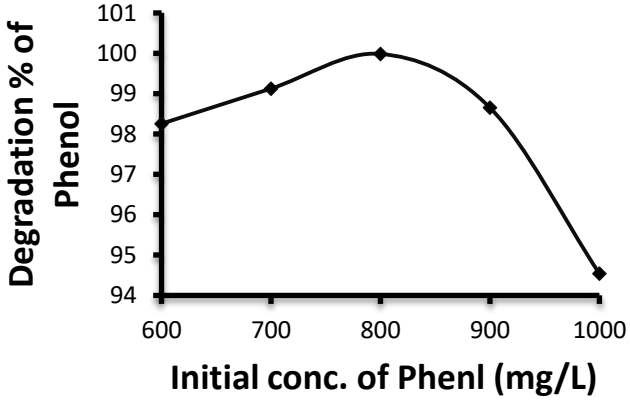
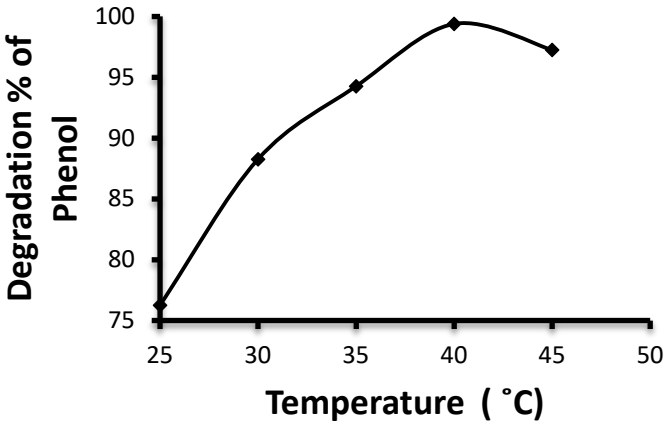


(f)

Fig IIIA.1: Effect in the Degradation efficacy of *Brevibacillus formosus* strain NRRL NRS- 863 in six different parameters; (a) Temperature (b) pH (c) Inoculums size (d) Time (e) Media volume & (f) Initial conc. of Phenol



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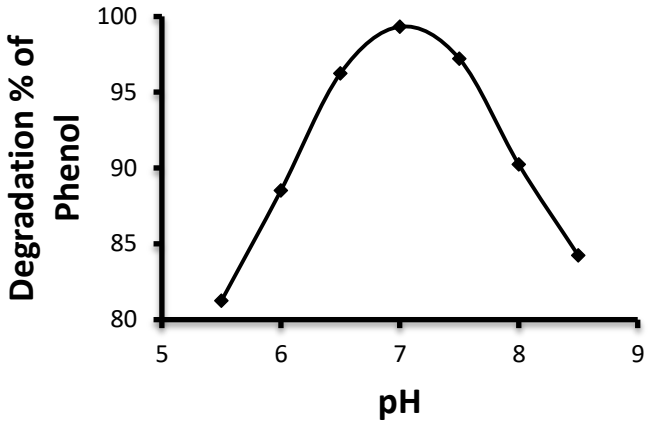

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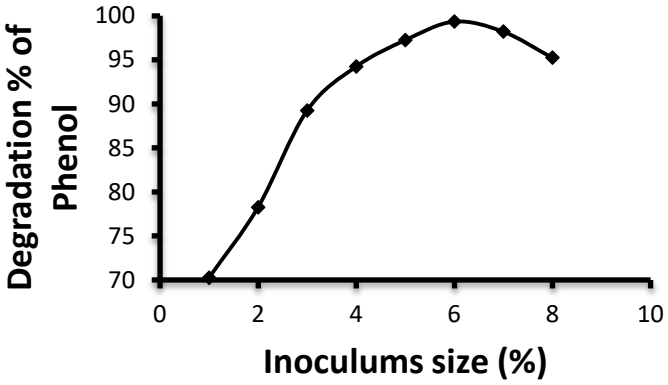
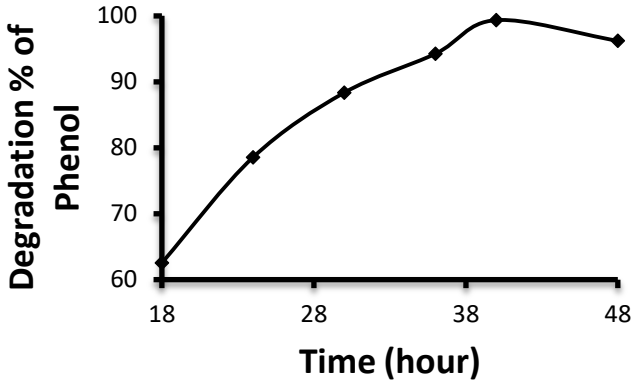
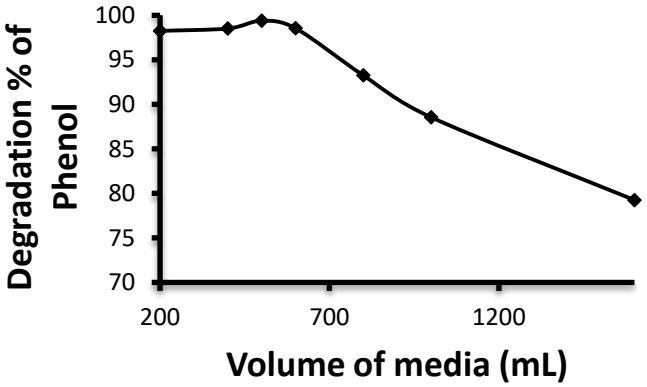


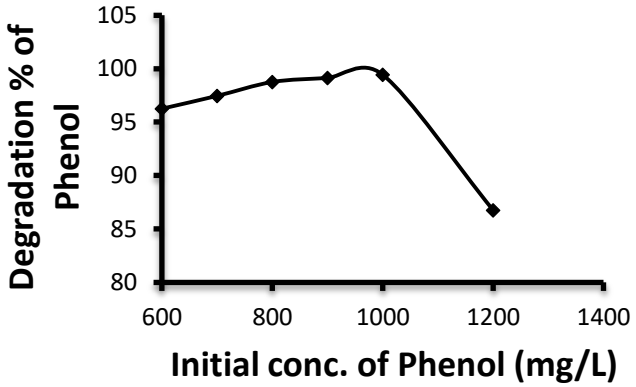
(c)



(d)



(e)



(f)

Fig IIIA.2: Effect in the degradation efficacy of *Pseudomonas otitidis* strain MCC10330 in six different parameters; (a) Temperature (b) pH (c) Inoculums size (d) Time (e) Media volume & (f) Initial conc. of Phenol

Sub chapter III B

Optimization of parameters of the 4- Chloro Phenol degrading strains

IIIB.1 Materials and Methods:

IIIB.1.1 Materials:

Bacillus timonensis strain 10403023 and *Bacillus cereus* strain K1 were isolated from the contaminated soil, collected from South Howrah state General Hospital by enrichment method as described in the chapter II, section IIA.1.5. A large volume of inoculums of the two above mentioned strains was already prepared via acclimatization process in Mineral Salt Medium (MSM) as described in chapter II, section IIA.1.8.

4- Chloro Phenol solution was prepared synthetically in the laboratory. Conc. of 4- Chloro Phenol stock solution was maintained as 10 g/L (or 10,000mg/L).

IIIB.1.2 Experimental set up: Same as mentioned in IIIA.1.2.

IIIB.1.3 Analytical Method: Also in case of the standard curve preparation along with the standard equation to deduct the residual 4- Chloro Phenol, known concentrations of 4- Chloro Phenol were utilized starting from 20 mg/L to 1000 mg/L. Rest of the procedure was same as that of the chapter II, section IIA.1.7.

IIIB.2 Results and Discussions:

Six parameters were considered for this study also. Moreover, the study was conducted for the two strains simultaneously. Some parameters found to be same and some to be different from each other.

IIIB.2.1 Effect of temperature:

Bacillus timonensis strain 10403023 was cultured in five different temperatures ranging between 25^{o}C to 45^{o}C with an interval of 5^{o}C to find out the most suitable one. 40^{o}C temperature was found to be the most favourable temperature for this particular strain while removing 4- Chloro Phenol. Complete degradation was achieved in this temperature when the initial conc. was 600 mg/L (higher than the initial conc. of 4- Chloro Phenol which was maintained during screening). Below that temperature, the degradation efficacy of the strain decreased. At 25^{o}C, 82.54% degradation was obtained and at 30^{o}C, 88.25% degradation was

achieved. At 35^{o}C, almost 95% degradation was found. But, when the temperature increased, a small decrease in the degradation percentage was occured. 90.21% degradation was achieved at 45^{o}C temperature (Fig: IIIB.1 (a)).

On the other hand, in case of *Bacillus cereus* strain K1, 25^{o}C temperature was found to be most suitable during degradation of the 4- Chloro Phenol. However, a small variation in the degradation percentage was observed during this study. 99.12% degradation was achieved in 25^{o}C temperature when the initial conc. of 4- Chloro Phenol was 600 mg/L. Here, the strain was cultured in six different temperatures from 20^{o}C to 45^{o}C with an interval of 5^{o}C. Below and above the optimal temperature, the degradation percentage decreased gradually. A slight decrease in degradation percentage was noted at 20^{o}C where almost 97% degradation was achieved. And beyond the optimal temperature (i.e. 25^{o}C) the degradation efficacy of the strain decreased slowly. At 30^{o}C and 35^{o}C, almost 97% and 96 % degradations were obtained respectively. When the temperature increased further up to 40^{o}C & 45^{o}C, 92.98% and 80.21% degradations were achieved by the strain respectively (Fig: IIIB.2 (a)).

IIIB.2.2 Effect of pH:

In case of *Bacillus timonensis* strain 10403023, almost 99.68percent degradation was achieved at pH 7.5 which was found to be the optimal pH for this strain. Three tests were performed in acidic pH where pH was maintained at 6.5 & 6.0 & 5.5. 90.45% and 88.54% & 84.25% degradations were observed in those acidic pH mediums respectively. In neutral pH (i.e. pH 7), around 96% degradation was observed. Finally, three experiments were carried out in basic pH mediums where pH values were 7.5, 8.0 & 8.5 and 99.68%, 97.92% & 96.57% of degradations were found from those pH mediums respectively (Fig: IIIB.1 (b)) indicating the optimal pH at 7.5.

In case of *Bacillus cereus* strain K1, 97.43% degradation was found at the optimal pH value i.e. 6.5. Below that pH, degradation efficacy of the strain decreased gradually. 93.29% degradation was obtained at pH 6.0 whereas, 88.2% degradation was found at pH 5.5. Slight decrease in degradation percentage was also found while increasing the pH value. 96.81% & 96.44% degradation was found at pH 7.0 & 7.5 respectively. 95.95% & 95.43% degradations were observed at pH 8.0 & 8.5 respectively (Fig: IIIB.2 (b)).

IIIB.2.3 Effect of inoculums size:

Inoculums size played a vital role also, in the bioremediation of 4- Chloro Phenol. In case of the each strain, eight experiments were carried out with eight different ranges of the inoculums, ranging between 1% to 8% with an interval of 1%.

In case of *Bacillus timonensis* strain 10403023, a moderate range of variation was obtained while changing the percentage of inoculums. 68.24% and 78.25% degradations were observed when the inoculums size of the strain was 1% and 2% respectively. 80.01% and 86.25% degradations were achieved when the inoculums size was increased up to 3% & 4% respectively. When 5% inoculums added to the media, 90.51% degradation was observed and it was increased up to 99.13%, when 6% inoculums were added. But, in case of 7% & 8% inoculums, degradation percentage again decreased to around 95% and 90% respectively indicating the 6% inoculums size to be the optimum inoculums size for this particular strain while degrading 4- Chloro Phenol (Fig: IIIB.1 (c)).

Almost similar profile was witnessed during the study of *Bacillus cereus* strain K1. 88.20%, 88.03% & 93.35% degradations of 4- Chloro Phenol was achieved when 1%, 2% & 3% inoculums of the strain were added to the culture media respectively. 93.28% & 97.44% degradations were found when the inoculums size was increased up to 4% and 5% respectively. At 6% inoculums size, 97.48% 4- Chloro Phenol was degraded. Beyond that, when the inoculums size was increased up to 7% & 8%, 98.02% and 99.25% degradations were observed against those inoculums size respectively (Fig: IIIB.2 (c)). After that, again degradation percentage decreased. So, optimal inoculums size was found to be 8% in case of *Bacillus cereus* strain K1.

IIIB.2.4 Effect of incubation time:

While culturing *Bacillus timonensis* strain 10403023, eight (8) different times were selected for the study viz. 6 hr, 12 hr, 18 hr, 24 hr, 30 hr, 36 hr, 42 hr & 48 hr. A vast variation was found during this study. Only around 40% degradation of 4- Chloro Phenol was observed after 6 hour and after 12 hour, it increased only up to ~ 49%. After 18 hours of incubation, 77.35% of degradation was achieved and after 24 and 30 hours of incubation, 99.65% & 90.24% degradations were obtained. It was further decreased at 36 hours, where degradation percentage was observed as 88.75%. Around 83% & 81% degradations were achieved at 42

hr and 48 hr respectively. So, the optimal time for this strain was found to be 24 hours (Fig: IIIB.1 (d)).

Similarly in case of *Bacillus cereus* strain K1, the most suitable time was optimized at 24 hours. Below and after this time span, degradation efficacy of the strain decreased. During the initial stage of the study, only 32.3% & 52.39% degradations were obtained when the incubation time was 6 hours and 12 hours respectively. 69.83% degradation was observed after 18 hours of incubation and in turn, ~ 100% degradation was found after 24 hours. But after 30 hours and 36 hours, 96.94% & 94.37% degradations were resulted showing a slight decline in the curve. And finally at 42 & 48 hours, degradation efficacy further decreased up to 94.30% & 93.61% respectively (Fig: IIIB.2 (d)).

IIIB.2.5 Effect of media volume:

Seven different volumes of media were utilized to check the effect of the media volume on the degradation efficacy of the strains (as mentioned in the IIIA.2.5).

While studying *Bacillus timonensis* strain 10403023, a very small range of variation was noticed in case of the outcome i.e. percent degradation of 4- Chloro Phenol. Around 97% & 98% degradations were achieved when the volume of the culture media was 200 mL and 400 mL respectively. The peak was found at 500 mL of the media volume where 99.69% degradation was achieved. Beyond that volume, the degradation efficacy of the strain was slightly decreased. Around 97% & 95% degradations were obtained when the media volume was 600 mL & 800 mL respectively. And finally at the volume of 1L & 1.6 L, 92.35% & 90.24% degradations of 4- Chloro Phenol were observed. Though there was not much variation, 500 mL media volume was optimized (Fig: IIIB.1 (e)).

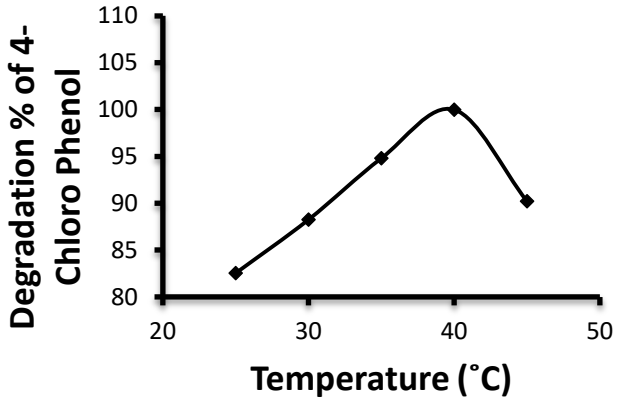
Also, during the study of *Bacillus cereus* strain K1, a small scale changes were observed during the study of media volume. 97.26% & 98.77% degradations were recorded when the media volume was 200 mL & 400 mL respectively. At 500 mL, 96.82% degradation was found indicating a slight decline in the curve. 96.58% degradation, which was almost same as that of the degradation gained at 500 mL volume, was noticed at 600 mL volume of the media. Then, the degradation efficacy of the strain continued to decrease gradually and reached up to 94.01% when the media volume was 1.6L (Fig: IIIB.2 (e)).

IIIB.2.6 Effect of initial concentration of 4- Chloro Phenol:

It was one of the most important parameter for both of the strains. Same phenomena (as described in IIIA.2.6) were noticed here also.

While studying *Bacillus timonensis* strain 10403023, around 95% & 97% degradations were achieved when the initial conc. of the 4-Chloro Phenol was maintained as 600 mg/L and 700 mg/L respectively. Maximum degradation was observed at 800mg/L initial conc. where 99.62% degradation was observed. A sudden fall in the curve was observed when the initial conc. further increased up to 900 mg/L & 1000 mg/L where around 84% & 72% degradations were found respectively. When the initial conc. further increased up to 1000 mg/L, 72% degradation was found approximately. The strain was found to remove maximum amount of 4- Chloro Phenol when the initial conc. was 800 mg/L (Fig: IIIB.1 (f)).

During the study of *Bacillus cereus* strain K1, 99.01% & 99.3% degradations were found when the initial conc. of 4- Chloro Phenol was 500 mg/L & 600 mg/L respectively and in turn, 98.32% & 97.49% of degradation was achieved at the initial conc, of 700 mg/L & 800 mg/L respectively. At 900 mg/L initial concentration, a sudden fall was occurred as 90.44% degradation was obtained there. At last at 1000 mg/L initial concentration, 86.4% degradation was gained. (Fig: IIIB.2 (f)). So it was found that *Bacillus cereus* strain K1 was able to remove maximum amount of 4- Chloro Phenol when the initial conc. will be 600 mg/L.



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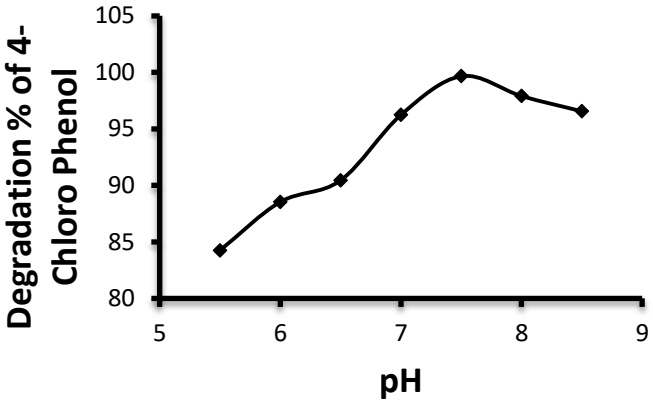

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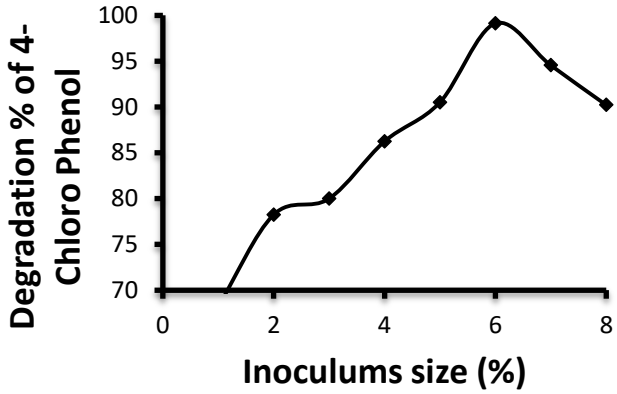

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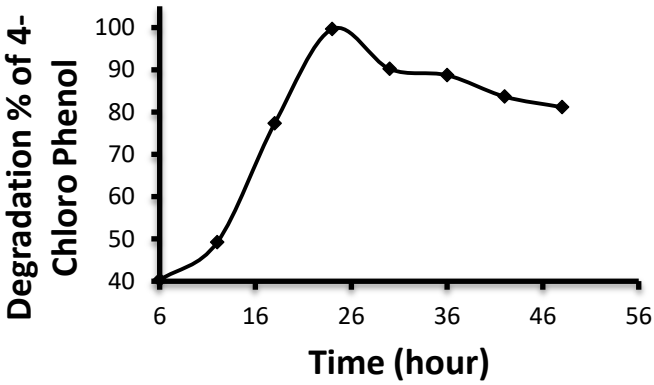

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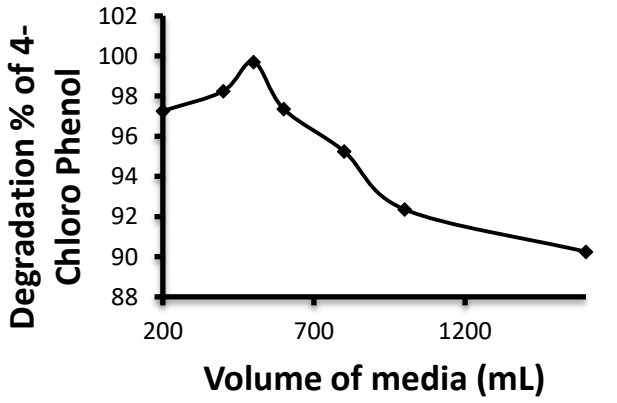

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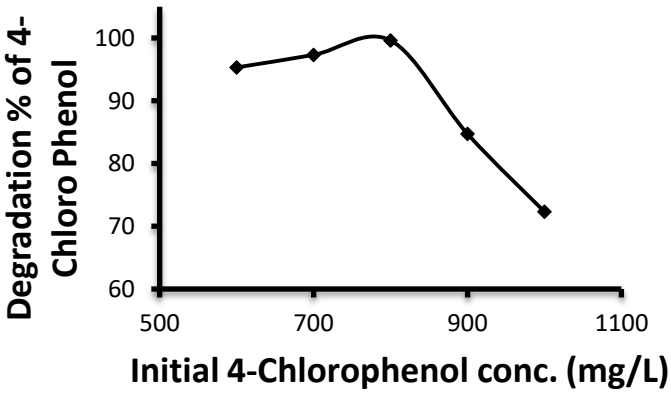
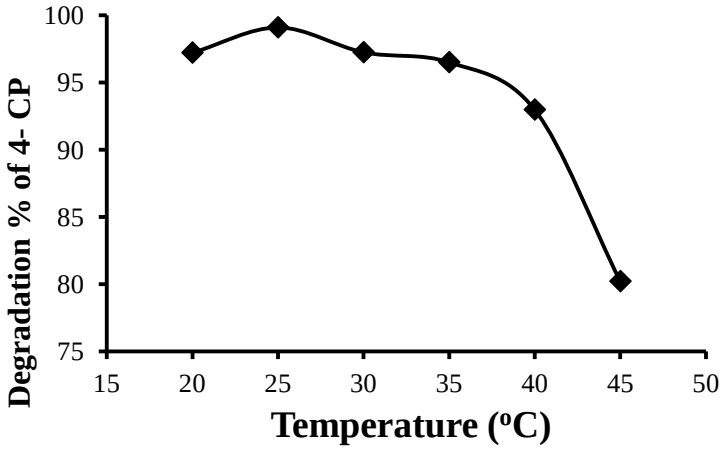


(f)

Fig IIIB.1: Effect in the degradation efficacy of *Bacillus timonensis* strain 10403023 in six different parameters; (a) Temperature (b) pH (c) Inoculums size (d) Time (e) Media volume & (f) Initial conc. of 4- Chloro Phenol



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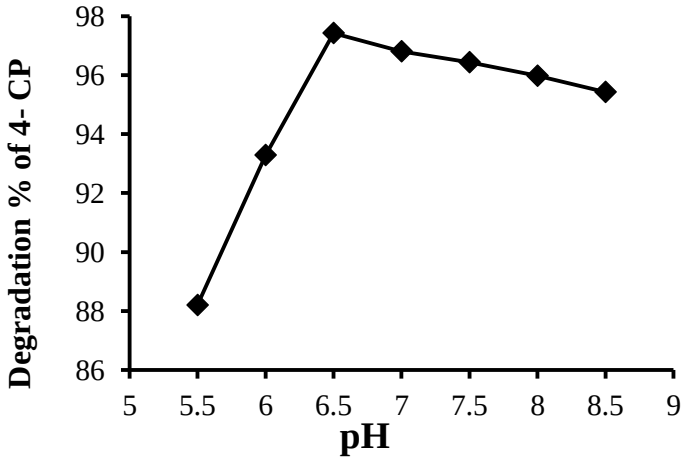

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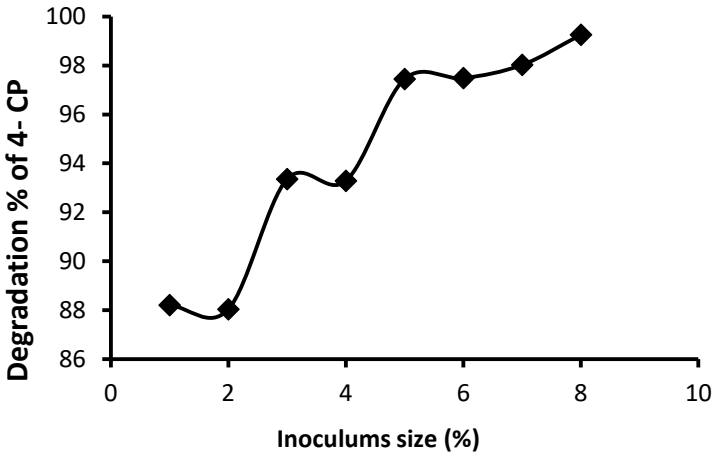

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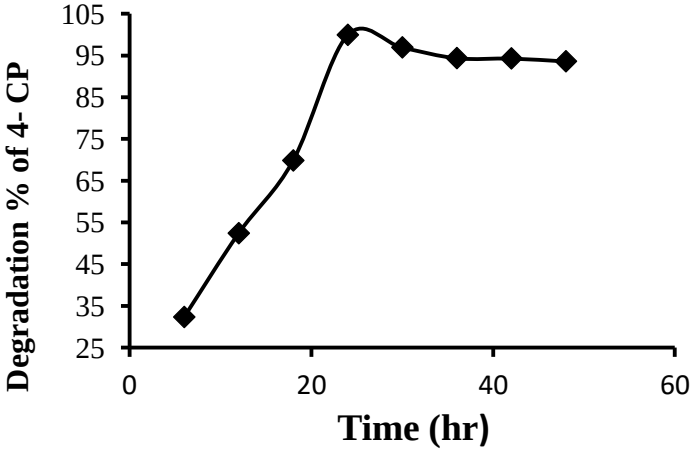

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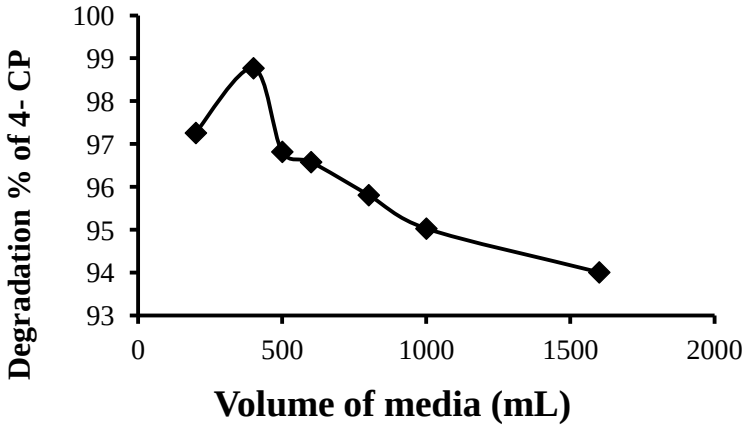

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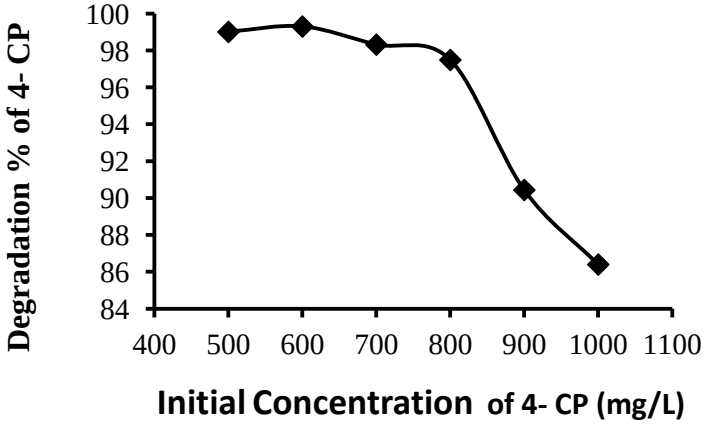


(f)

Fig IIIB.2: Effect in the degradation efficacy of *Bacillus cereus* strain K1 in six different parameters; (a) Temperature (b) pH (c) Inoculums size (d) Time (e) Media volume & (f) Initial conc. of 4- Chloro Phenol

Sub chapter III C

Optimization of parameters of the Catechol degrading strains

IIIC.1 Materials and Methods:

IIIC.1.1 Materials:

Bacillus Psudomucoides strain NBRC 101232 and *Bacillus paramycoides* strain MCCC 1A04098 were isolated from the contaminated soil, collected from South Howrah state General Hospital by enrichment method as described in the chapter II, section IIA.1.5. A large volume of inoculums of the two above mentioned strains was already prepared via acclimatization process in Mineral Salt Medium (MSM) as described in chapter II, section IIA.1.8.

Catechol solution was prepared synthetically in the laboratory. Conc. of Catechol stock solution was maintained as 10 g/L (or 10,000mg/L).

IIIC.1.2 Experimental set up: Same as mentioned in IIIA.1.2.

IIIC.1.3 Analytical Method: In case of reparation of the standard curve along with the standard equation to measure the residual Catechol, known concentrations of Catechol were used instead of Phenol or 4- Chloro Phenol ranging between 20 mg/L to 1000 mg/L. Rest of the procedures were same as mentioned in IIA.1.7.

IIIC.2 Results and Discussions:

Six parameters were considered for this study also. Moreover, the study was conducted for the two strains simultaneously. Some parameters found to be same and some to be different from each other.

IIIC.2.1 Effect of temperature:

Bacillus Psudomucoides strain NBRC 101232 was cultured in six different temperatures ranging between 20^{o}C to 45^{o}C with an interval of 5^{o}C to find out the most suitable one. 35^{o}C temperature was found to be the most favourable temperature for this particular strain while degrading Catechol. Complete degradation was achieved in this temperature when the initial conc. was 600 mg/L (higher than the initial conc. Catechol which was maintained during screening). Below that temperature, the degradation efficacy of the strain decreased. At 20^{o}C,

lowest degradation was obtained i.e. 56.35% and at 25^{o}C, 79.25% degradation was achieved. At 30^{o}C, almost 87% degradation was found. Similarly, when the temperature increased from the optimum temperature, decrease in the degradation percentage was occurred. 87.78% degradation was achieved at 40^{o}C temperature and in turn, 60.33% degradation was found at 45^{o}C (Fig: IIIC.1 (a)).

On the other hand, in case of *Bacillus paramycoides* strain MCCC 1A04098, 25^{o}C temperature was found to be most suitable during degradation of the Catechol. 98.21% degradation was achieved in 25^{o}C temperature when the initial conc. of Catechol was 600 mg/L. Here also, the strain was cultured in six different temperatures from 20^{o}C to 45^{o}C with an interval of 5^{o}C. Below and above the optimal temperature, the degradation percentage decreased gradually. A moderate decrease in degradation percentage was noted at 20^{o}C where almost 87% degradation was achieved. And beyond the optimal temperature (i.e. 25^{o}C) the degradation efficacy of the strain decreased rapidly. At 30^{o}C and 35^{o}C, almost 80% and 74 % degradation was obtained respectively. When the temperature increased further up to 40^{o}C & 45^{o}C, 70.45% and 65.39% degradations were achieved by the strain respectively (Fig: IIIC.2 (a)).

IIIC.2.2 Effect of pH:

In case of *Bacillus Psudomucoides* strain NBRC 101232, almost complete degradation was achieved at pH 5.5 which was found to be the optimal pH for this strain where complete degradation was obtained. Five tests were performed in acidic pH where pH was maintained at 4.0, 5.0, 5.5, 6.0 & 6.5. In the extreme acidic pH i.e. pH 4.0 & pH 5.0, 82.35% and 96.39% degradations were observed respectively. When pH value further increased above the optimal pH, degradation percentage continued to decrease rapidly. At pH 6.0, a sudden fall in the curve was recorded as the degradation percentage decreased to 78% approximately. Around 51%, 40% & 29% degradations were observed at the pH of 6.5, 7.0 & 7.5. Lowest degradation was achieved at pH 8.0 (Fig: IIIC.1 (b)).

In case of *Bacillus paramycoides* strain MCCC 1A04098, 98.21% degradation was found at the optimal pH value i.e. 5.5. Degradation efficacy of the strain decreased to 87.64% when the pH was maintained as 5.0. Besides that, degradation efficacy also decreased while increasing the pH. 84.20% degradation was obtained at pH 6.0 whereas, 80.21% degradation was found at pH 6.5. At the neutral pH, around 79% degradation was gained and in turn,

approximately 72%, 61% & 53% degradations were obtained respectively at the pH of 7.5, 8.0 & 8.5(Fig: IIIC.2 (b)).

IIIC.2.3 Effect of inoculums size:

Just like the previous experiments, in case of the each strain, eight experiments were carried out with eight different ranges of the inoculums, ranging between 1% to 8% with an interval of 1%.

In case of *Bacillus Psudomucoides* strain NBRC 101232, a wide range of variation was obtained while changing the percentage of inoculums. 21.02% and 34.77% degradations were observed when the inoculums size of the strain was 1% and 2% respectively. 41.22% and 64.25% degradations were achieved when the inoculums size was increased up to 3% & 4% respectively. When 5% inoculums added to the media, 83.08% degradation was observed and it was increased up to 100% almost, when 6% inoculums were added. But, in case of 7% & 8% inoculums, degradation percentage again decreased to around 92% and 88% respectively. Hence 6% inoculums size was the optimum inoculums size for this particular strain while degrading Catechol (Fig: IIIC.1 (c)).

Almost similar profile was witnessed during the study of *Bacillus paramycoides* strain MCCC 1A04098. Here also, a wide variation in the output was recorded. 21.52%, 42.87% & 50.74% degradation of Catechol was achieved when 1percent, 2% & 3% inoculums of the strain were added to the culture media respectively. 61.96% & 80.49% degradations were found when the inoculums size was increased up to 4% and 5% respectively. At 6% inoculums size, 98.29% Catechol was removed. Beyond that, when the inoculums size was increased up to 7% & 8%, 92.24% and 86.88% degradations were observed against those inoculums size respectively (Fig: IIIC.2 (c)). So, 6% inoculums size was found to be optimal for *Bacillus paramycoides* strain MCCC 1A04098.

IIIC.2.4 Effect of incubation time:

While culturing *Bacillus Psudomucoides* strain NBRC 101232, ten (10) different times were selected for the study viz. 16 hr, 24 hr, 32 hr, 40 hr, 48 hr, 56 hr, 64 hr, 72 hr, 80 hr & 96 hr. A wide range of variation was found during this study. Only around 12% degradation of Catechol was observed after 16 hour and after 24 hour, it increased only up to ~ 15%. After 32 hours of incubation, 21.52% of degradation was achieved and in turn, after 40 and 48 hours of incubation, 35.68% & 51.97% degradations were obtained. It was further increased at 56 hours, where degradation percentage was observed as 64.07%. Around 82% degradation was achieved at 64 hr. Finally the peak was observed at 72 hours where 99.25% of degradation was recorded. Beyond this time span, the degradation efficacy again decreased as 87.07% & 76.36% degradations were found at 80 hours and 96 hours respectively. So, the optimal time for this strain was found to be 72 hours (Fig: IIIC.1 (d)).

Similarly in case of *Bacillus paramycoides* strain MCCC 1A04098, the most suitable time was optimized at 72 hours. Below and after this time span, degradation efficacy of the strain decreased. During the initial stage of the study, only 27.24% & 55.24% degradations were obtained when the incubation time was 12 hours and 24 hours respectively. 63.87% degradation was observed after 36 hours of incubation and in turn, 80.36% degradation was found after 48 hours. After 60 hours and 72 hours, 91.45% & 98.52% degradations were resulted showing the peak of the curve at 72 hours. After that, at 80 & 96 hours, degradation efficacy further decreased up to 95.34% & 81.11% respectively (Fig: IIIC.2 (d)).

IIIC.2.5 Effect of media volume:

Like the previous studies, here also seven different volumes of the media were deployed to check the effect of media volume on the degradation efficacy of the strains (as mentioned in the IIIA.2.5).

While studying *Bacillus Psudomucoides* strain NBRC 101232, a moderate range of variation was noticed in case of the outcomes. Around 98% & 97% degradations were achieved when the volume of the culture media was 200 mL and 400 mL respectively. At 500 mL of the media volume, 95.34% degradation was achieved. Beyond that volume, the degradation efficacy of the strain increased again and 99.21% degradation was obtained when the media volume was 600 mL. At the volume of 800 mL & 1L, again the output decreased up to 92.34% & 82.14% respectively. Finally at 1.6 L of volume, a rapid decline was found as only

62.21% degradation obtained. 600 mL media volume was optimized for the further studies (Fig: IIIC.1 (e)).

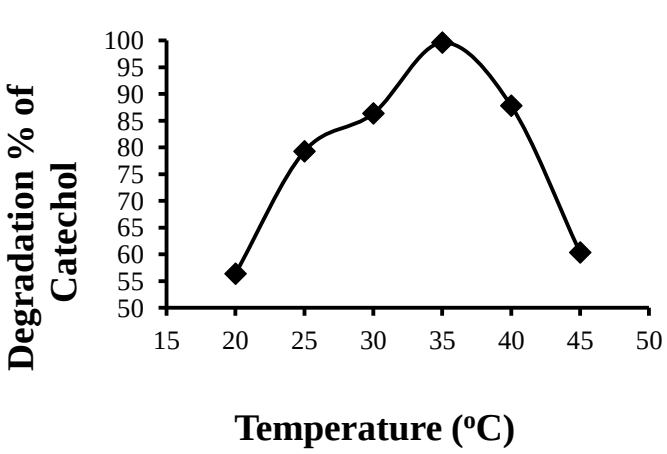
Also, during the study of *Bacillus paramycoides* strain MCCC 1A04098, a moderate changes were observed during the study of media volume. Around 96% & 97% degradations were recorded when the media volume was 200 mL & 400 mL respectively. At 500 mL, 98.54% degradation was found indicating a slight increase in the curve. 96.24% degradation was noticed at 600 mL volume of the media. Then, the degradation efficacy of the strain continued to decrease and reached up to 76.24% & 75.44% when the media volume was 1L & 1.6L respectively (Fig: IIIC.2 (e)).

IIIC.2.6 Effect of initial concentration of Catechol:

It was one of the most important parameters for both of the strains. Same phenomena (as described in IIIA.2.6) were noticed here also.

While studying *Bacillus Psudomucoides* strain NBRC 101232, around 99% & 88% degradations were achieved when the initial conc. of the Catechol was maintained as 600 mg/L and 700 mg/L respectively. Then the degradation efficacy of the strain continued to decrease. At 800mg/L initial conc. 74.25% degradation was observed. When the initial conc. of the Catechol further increased up to 900 mg/L & 1000 mg/L, around 61% & 58% degradations were found respectively which were not satisfactory. So, the strain was found to remove maximum amount of Catechol when the initial conc. was 600 mg/L (Fig: IIIC.1 (f)).

During the study of *Bacillus paramycoides* strain MCCC 1A04098, 98.25% & 90.41% degradations were found when the initial conc. of Catechol was maintained as 600 mg/L & 700 mg/L respectively and in turn, 81.02% & 71.36% of degradations were achieved at the initial conc., of 800 mg/L & 900 mg/L respectively. At 1000 mg/L initial concentration, 64.58% degradation was obtained. So it was found that *Bacillus paramycoides* strain MCCC 1A04098 was able to degrade maximum amount of Catechol when the initial conc. will be 600 mg/L. (Fig: IIIC.2 (f)).



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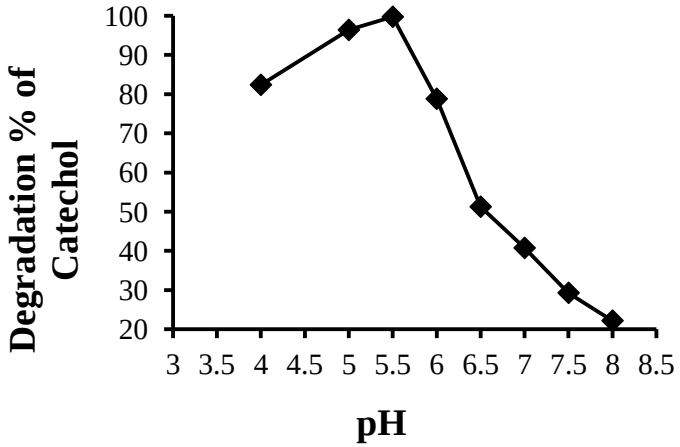

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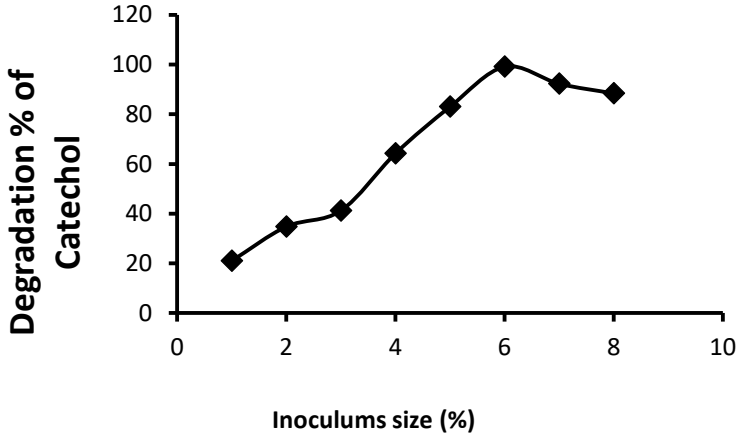

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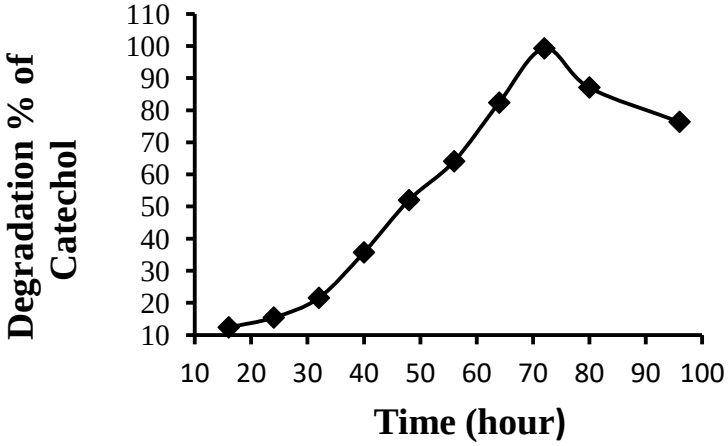

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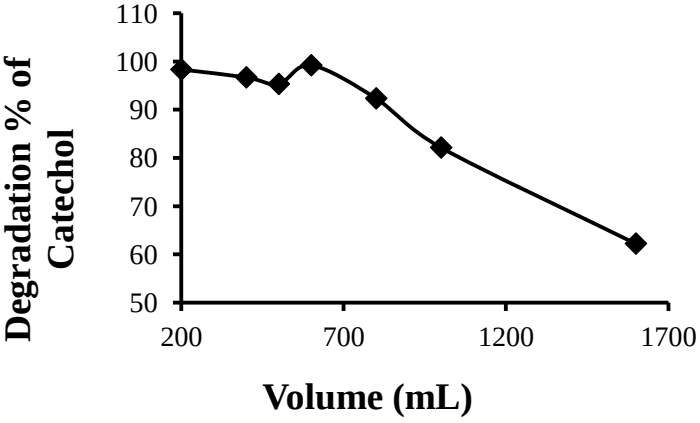

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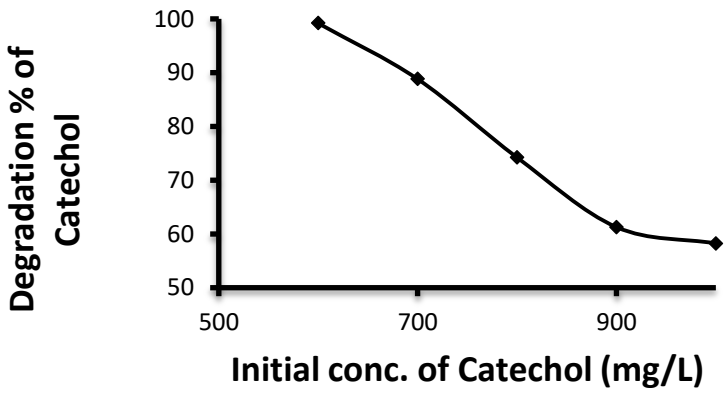
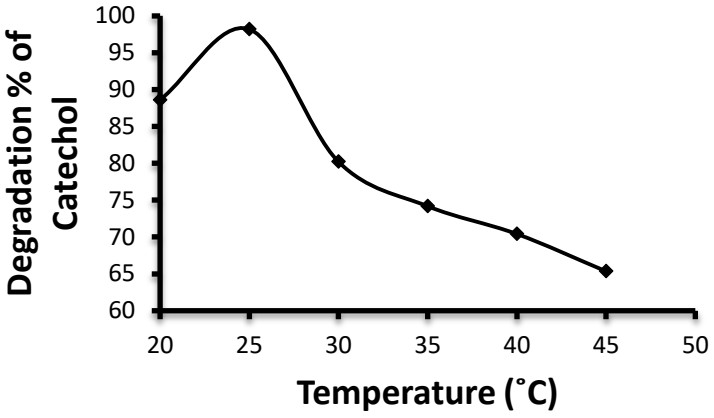


(f)

Fig IIIC.1: Effect in the degradation efficacy of *Bacillus Psudomucoides* strain NBRC 101232 in six different parameters; (a) Temperature (b) pH (c) Inoculums size (d) Time (e) Media volume & (f) Initial conc. of Catechol



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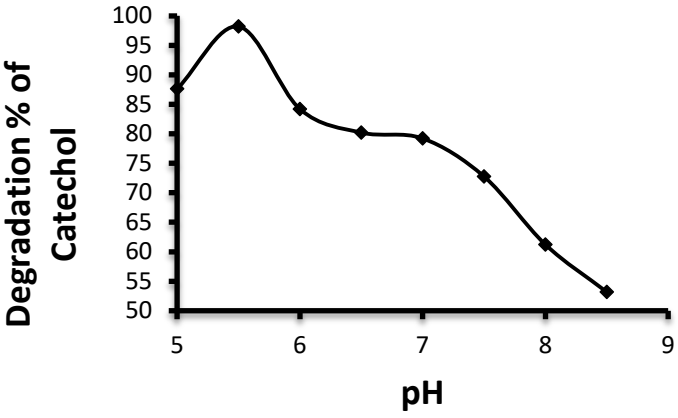

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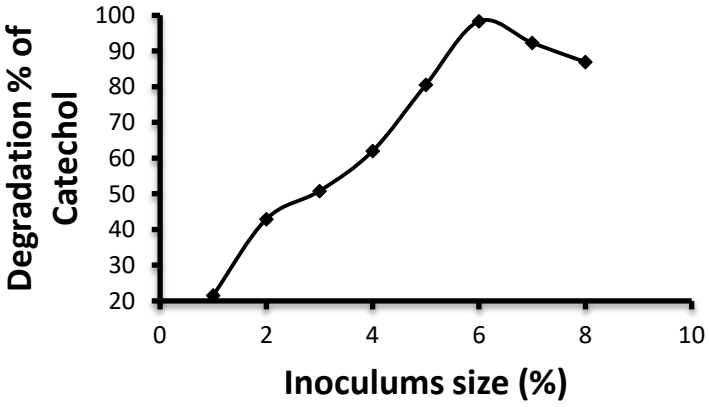

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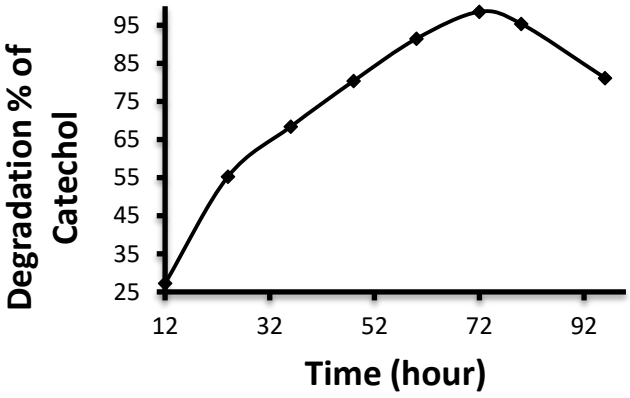

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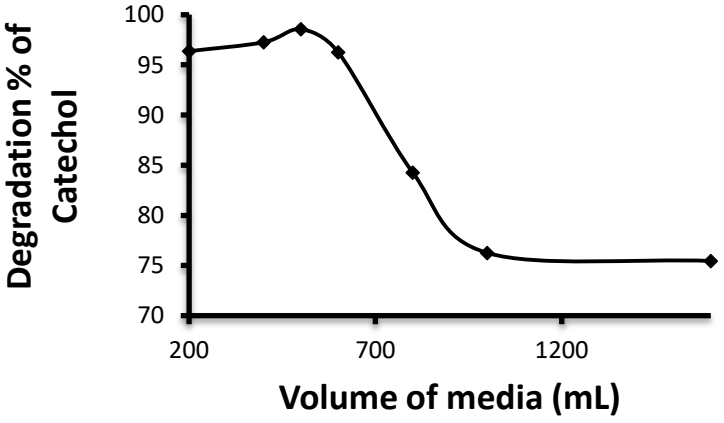

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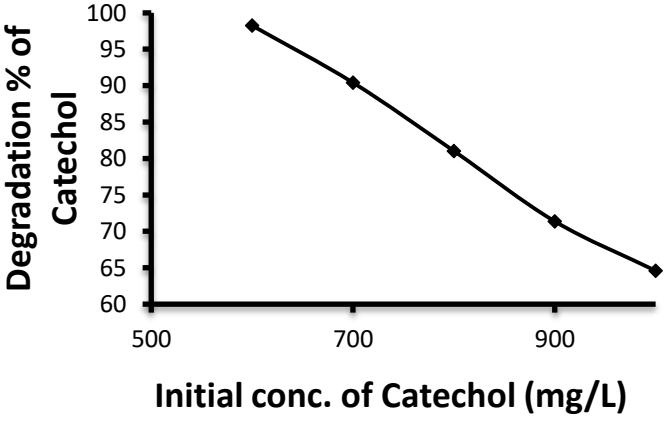


(f)

Fig IIIC.2: Effect in the degradation efficacy of *Bacillus paramycoides* strain MCCC 1A04098 in six different parameters; (a) Temperature (b) pH (c) Inoculums size (d) Time (e) Media volume & (f) Initial conc. of Catechol

Conclusions:

From the above study, it is clear the physico – chemical parameters are species specific. While optimizing the parameters of the two Phenol degrading strains i.e. *Brevibacillus formosus* strain NRRL NRS- 863 & *Pseudomonas otitidis* strain MCC10330, it was found that both of the strains seem to be neutrophilic as both of them was optimized particularly at pH 7. Both of them were found to mesophilic also as they were optimized at the temperature of 30^{o}C and 40^{o}C respectively. The scenario was changed during the optimization of the 4-Chloro Phenol degrading strains. *Bacillus timonensis* strain 10403023 was found to be optimized at 40^{o}C and 7.5 pH which thereby, indicate that it is mesophilic and slightly basophilic in nature respectively. On the other hand, *Bacillus cereus* strain K1 was found to be psychotropic as well as slightly acidophilic. In case of the Catechol degrading strains, both of them were acidophilic as 5.5 pH were their optimal pH value. *Bacillus Psudomucoides* strain NBRC 101232 was found to be mesophilic whereas, *Bacillus paramycoides* strain MCCC 1A04098 was found to be psychotropic species.

Table III.1: Optimized parameters for the six different strains at a glance

Name of the isolated strains	Optimized parameters in batch culture							
	Temperature (^{o}C)	pH	Time (hour)	Media volume (mL)	Inoculums size (percent)	Initial conc. of Phenol(mg/L)	Initial conc. of 4- Chloro Phenol (mg/L)	Initial conc. of Catechol (mg/L)
Brevibacillus formosus	30	7	40	500	6	800	Not done	Not done
Pseudomonas otitidis	40	7	40	500	6	1000	Not done	Not done
Bacillus timonensis	40	7.5	24	500	6	Not done	800	Not done
Bacillus cereus	25	6.5	24	400	8	Not done	600	Not done
Bacillus Psudomucoides	35	5.5	72	600	6	Not done	Not done	600
Bacillus paramycoides	25	5.5	72	500	6	Not done	Not done	600

In this chapter, the procedure and outputs of parameter optimization of the six isolated strains in batch reactor have been described in brief. However, no statistical analysis of those optimized parameters was deployed here. It has been described in the next chapter.

Chapter IV

Optimization of process parameters through Response Surface Methodology (RSM)

Introduction:

In the previous chapter (i.e. in Chapter III), it was displayed that how biodegradation of any substance by some bacteria or algae or fungi, depends upon some physico – chemical or biological parameters. Though, several studies were already conducted to identify the perfect values of the parameters, it was necessary to optimize those parameters statistically.

A set of statistical and mathematical methods called Response Surface Methodology (RSM) which can be used to create, enhance, and optimize various processes (Myers & Montgomery; 2002). The most widespread uses of the RSM occur in specific scenarios when a number of input factors may have an impact on a process's quality attribute or performance metric. The quality attribute is therefore referred to as Response. The researchers have control over the input variables, which are also referred to as independent variables (Careley et al., 2004). Response surface methodology encompasses three main methods: empirical statistical modelling to establish a suitable approximation relationship between the yield and the process variables, optimisation techniques to identify the values of the process variables that yield desired response values, and experimental strategy to explore the space of the process or independent variables (Carley et al., 2004).

The experimenter's capacity to create a workable estimation for the true response function (f) is a vital component in the successful application of RSM. Generally, it is suitable to use a low-order polynomial in a relatively limited part of the independent variable space. Either a first-order or a second-order model is employed frequently (Carley et al., 2004).

The first order model is found to be suitable when the experiment is limited within relatively a small range of independent variables.

In case of two independent variables, the first order model in terms of coded factors is:

$$Y = ß_0 + ß_1X_1 + ß_2X_2 \qquad (4.1)$$

Where, $ß_0$ is the intercept term, $ß_1$ & $ß_2$ is the linear main effect and Y= process response.

Equation no. 4.1 consists of the main effects of two variables only i.e. X_1 & X_2. If there is an interaction effect between these variables, then the equation will be as follows:

$$Y = ß_0 + ß_1X_1 + ß_2X_2 + ß_{12}X_1X_2 \qquad (4.2)$$

Addition of such interaction effects produce curvature into the response function (Carley et al., 2004).

Now, the curvature in the response surface often becomes so strong that a first - order model seems to be inadequate to validate. Then, second- order model is required there (Carley et al., 2004). When there are two variables, a second- order equation is:

$$Y = ß_0 + ß_1X_1 + ß_2X_2 + ß_{11}X_1^2 + ß_{22}X_2^2 + ß_{12}X_1X_2 \quad (4.3)$$

The second order model seems to be more flexible. In general, a second order model is

$$Y = ß_0 + \sum_{i=1} ß_i x_i + \sum_{i=1} ß_{ii} x_i^2 + \sum_{i=1} \sum_{j=1} ß_{ij} x_i x_j + C \quad (4.4)$$

Here, Y is the process response, i and j are the index number of the patterns, $ß_0$ is the intercept term, $ß_i$ is the linear main effect, $ß_{ii}$ is the quadratic effect, where as $ß_{ij}$ is the interactive effect. C is the random error between the predicted and experimental values.

Here in this study, parameters were to be optimized via Response Surface Methodology (RSM). Like that of the chapter III, the same parameters were taken into account for the study. Manually optimized parameters were justified here via statistical and mathematical models and particularly, the interaction effects of the six parameters were obtained here which were not possible to be achieved by only manual optimization.

Annadurai G et al., involved Response Surface Methodology in 2008 while degrading Phenol by utilising *Pseudomonas putia* ATCC 31800. Here, initial Phenol concentration was maintained as 0.2 g/L. Optimal pH value was found to be 7 and in case of optimal temperature, it was 30^oC.

Sivasubramanian S; and Namasivayam S Karthick R; deployed the RSM study in 2014 where Phenol was degraded by involving a mixture of *Lactobacillus plantarum*, *Candida utilis*, *Actinomycetes*, *Streptomyces albus* and *Aspergillus oryzae*. Here four parameters were considered by them viz. initial conc. of Phenol, temperature, pH and residence time. It was found that 90.5percent Phenol can be degraded by this consortium when the initial conc. of Phenol was 1008 mg/L, temperature was 35.5 ^oC, pH 7.

Sivasubramanian S; and Namasivayam S Karthick R; performed a similar experiment in 2015 where with the help of RSM, they degraded almost 99percent Phenol by using a microbial mixture (*Candida tropicalis*, *Aspergillus fumigatus*, *Candida albicans*, *Candida haemulonis* and *Streptomyces alboflavus*). Here, 1000 mg/L Phenol was degraded by the consortium at pH 7, 35^oC temperature and 96 hours of residence time.

Ahmed S.A.A and Gogina E S successfully finished an experiment in 2023 where Central Composite design (CCD) based RSM was involved to degrade Phenol via the electrosulfate process. The optimal parameters obtained from this study were as follows: pH 3.78, 15 minutes reaction time, 2 g/L persulfate dose, 0.6 ampere of applied current & 100 mg/L initial conc. of Phenol.

In the current study, six parameters were considered viz. pH, temperature, residence time, percent of inoculums (i.e. inoculums size), media volume and initial conc. of Phenolic substance.

Sub chapter IV A

Optimization of parameters for the Phenol degrading strains via Response Surface Methodology

IVA.1 Materials and Methods:

IVA.1.1 Materials:

Brevibacillus formosus strain NRRL NRS- 863 and *Pseudomonas otitidis* strain MCC10330 were isolated from the contaminated soil, collected from South Howrah state General Hospital by enrichment method as described in the chapter II, section IIA.1.5. A large volume of inoculums of the two above mentioned strains were already prepared via acclimatization process in Mineral Salt Medium (MSM) as described in chapter II, section IIA.1.8.

Phenol solution was prepared synthetically in the laboratory. Conc. of Phenol stock solution was maintained as 10 g/L (or 10,000mg/L).

IVA.1.2 Experimental set up:

Same as mentioned in the chapter III, section IIIA.1.2.

IVA.1.3 Analytical Method:

Details of this process has been mentioned in chapter II, section IIA.1.7.

IVA.1.4 Experimental design:

In the chapter III, it was found that, only the optimal values of initial conc. of Phenol & temperature was different in case of the two strains. Rest were same. Keeping the optimal values in mind, the ranges of the parameters were set in the software.

The popular second order Central Composite Design (CCD) was involved in the case of the experimental design. The CCD based RSM with six independent factors at three levels full factorial was applied using design expert 13.0.5.0. statistical tool.

In case of *Brevibacillus formosus* strain NRRL NRS- 863, Six (6) independent variables (Table IVA.1) are X1 (A): 600 – 1000 mg/L; X2 (B): 6 – 8; X3 (C): 25^{o} – 35^{o}C; X4 (D): 400 – 600 mL; X5 (E): 4 – 8% and X6 (F): 35 – 45 hour.

In case of *Pseudomonas otitidis* strain MCC10330, Six (6) independent variables (Table IVA.2) are Z1 (A): 800 – 1200 mg/L; Z2 (B): 6 – 8; Z3 (C): 35^{o} – 45^{o}C; Z4 (D): 400 – 600 ml; Z5 (E): 4 – 8% and Z6 (F): 35 – 45 hour.

Both in cases, these six independent variables were coded at three levels between -1 and +1 based on preliminary experimental outputs. The experiment designed with six factors, resulted into ten (10) replicates at the design centre to evaluate pure error and carried in

randomised order. In the design, the response can be related to choose factors by quadratic model. The nature of the process can be explained by the equation no. 4.4. ANOVA was performed to reveal the relationship in between process variables and response. Coefficient of determination R^2 indicated the quality of fit of the polynomial model. Adjusted R^2 and statistical importance was calculated by F test. The desired goal was to remove Phenol at minimum time limit.

Table IVA.1: Independent variables with coded levels for *Brevibacillus formosus*

Independent variable	Symbol	Coded levels		
		-1	0	+1
Initial conc. of Phenol (mg/L)	X1	600	800	1000
pH of bacterial media	X2	6	7	8
Temperature (^oC)	X3	25	30	35
Volume of bacterial media(mL)	X4	400	500	600
Inoculums percent (percent)	X5	4	6	8
Residence time(hr)	X6	35	40	45

Table IVA.2: Independent variables with coded levels for *Pseudomonas otitidis*

Independent variable	Symbol	Coded levels		
		-1	0	+1
Initial conc. of Phenol (mg/L)	Z1	800	1000	1200
pH of bacterial media	Z2	6	7	8
Temperature (^oC)	Z3	35	40	45
Volume of bacterial media(mL)	Z4	400	500	600
Inoculums percent (percent)	Z5	4	6	8
Residence time(hr)	Z6	35	40	45

IVA.2. Results and Discussions:

IVA.2.1. Fitting of the model and Statistical analysis:

For both the strains, total 86 experiments were done as per the design matrix and the only one response was percent of degradation of Phenol. Table IVA.3 & IVA.4 displayed both the predicted and experimental/actual values of percent of degradation of Phenol by *Brevibacillus formosus* & *Pseudomonas otitidis* respectively. Second order and linear polynomial equations (equation 4.5 & 4.6 for *Brevibacillus formosus* & *Pseudomonas otitidis* respectively) were fitted to the actual data to obtain the regression equation. To reveal the suitable model, sequential model sum of squares and model summary statistics were deducted (for *Brevibacillus formosus*, Table IVA.5 and for *Pseudomonas otitidis*, IVA.6 respectively). The Sequential P-value for the quadratic model is less than 0.0001 in both cases; Maximum predicted R^2 and adjusted R^2 values were 0.9085 and 0.9431 respectively for the percent of degradation of the Phenol in case of *Brevibacillus formosus*. In case of *Pseudomonas otitidis*, those values were 0.9498 and 0.9503 respectively for the percent of degradation of the Phenol.

Cubic model found to be aliased for both; here the sequential P- values were> 0.05 for both. So, finally the quadratic model was chosen for both strains for further determination of the percent of degradation of Phenol. The following equations clearly showed that:

percent of Phenol degradation by *Brevibacillus formosus* (Y): $97.31+ 1.27X1 – 0.0367X2 – 0.5944X3 – 0.5733X4+ 0.6621X5+0.2271X6 – 0.9891X1X2 – 0.36911X1X3 – 0.6344X1X4+ 0.1294X1X5+ 1.11X1X6+0.2553X2X3+ 0.3544X2X4+0.0563X2X5 – 0.4988X2X6+ 0.7187X3X4 – 0.6531X3X5 – 0.8081X3X6 – 0.0747X4X5 – 0.3397X4X6+0.3672X5X6 – 1.12X1^2 – 4.69X2^2– 5.14X3^2 – 4.69X4^2 – 2.64X5^2 – 5.64X6^2$ (4.5)

Negative coefficients for the model components X2, X3, X4, X1X2, X1X3, X1X4, X2X6, X3X5, X3X6,X4X5, X4X6, $X1^2$, $X2^2$, $X3^2$, $X4^2$, $X5^2$ and $X6^2$ exhibit negative impacts on the percent of degradation of Phenol while positive coefficients X1, X5, X6, , X1X5, X1X6, X2X3, X2X4, X2X5, X3X4 and X5X6 have positive impacts on the of degradation of Phenol (equation 4.5). Moreover, the coefficients, whose values are near 0, reveal lower relative intensity. X2, X2X5 and X4X5 belong to in this class which could not affect percent of degradation of the Phenol so much.

percent of Phenol degradation by *Pseudomonas otitidis* (Y): 97.60 + 0.6129Z1+ 0.2038Z2+ 0.0655Z3+ 0.0100Z4+1.42Z5+ 2.26Z6+ 0.8994Z1Z2+ 0.5609Z1Z3+ 0.1288Z1Z4 - 0.6909Z1Z5+ 0.2997Z1Z6+ 0.9881Z2Z3 - 0.6659Z2Z4+ 1.32Z2Z5 - 0.2119Z2Z6 - 0.0556Z3Z4+ 1.54Z3Z5 - 0.1147Z3Z6+ 0.1194Z4Z5+ 0.3963Z4Z6 - 0.2366Z5Z6 - $10.06Z1^2$ - $1.58Z2^2$ – $7.58Z3^2$ - $8.58Z4^2$ - $1.58Z5^2$ +$0.9372Z6^2$ (4.6)

Negative coefficients for the model components Z1Z5, Z2Z4, Z2Z6, Z3Z4, Z3Z6, Z5Z6, $Z1^2$, $Z2^2$, $Z3^2$ and$Z5^2$ reveal negative impacts on the percent of degradation of Phenol while positive coefficients Z1, Z2, Z3,Z4, Z5, Z6, Z1Z2, Z1Z3, Z1Z4, Z1Z6, Z2Z3, Z2Z5, Z3Z5, Z4Z, Z4Z6 and $Z6^2$ stands for positive impacts on the percent of degradation of Phenol (equation 4.6). Moreover, the coefficients, whose values are near 0, reveal lower relative intensity. Z3, Z4 and Z3Z4 were categorised in this class which could not affect percent of degradation of the Phenol so much.

Table IVA.3: Experimental design matrix for the degradation of Phenol by *Brevibacillus formosus* strain NRRL NRS- 863

Run order	Space type	X1:Initial Concentration of Phenol (mg/L)	X2: pH	X3: Temperature (°C)	X4: Medium Volume (mL)	X5: Inoculums (percent)	X6: Residence Time(hr)	Y: percent of Phenol degradation		
								Experimental value	Predicted value	Error
1	Factorial	600	6	35	600	4	45	70.23	69.18	1.05
2	Axial	800	7	30	500	4	40	92.35	94.01	-1.66
3	Factorial	1000	6	35	600	4	45	70.54	73.64	-3.10
4	Factorial	1000	6	25	400	8	35	75.32	77.00	-1.68
5	Centre	800	7	30	500	6	40	99.98	97.31	2.67
6	Factorial	1000	6	25	600	8	45	74.67	78.31	-3.64
7	Factorial	600	8	35	400	8	35	74.21	73.77	0.4432
8	Factorial	600	6	25	400	4	35	70.25	71.36	-1.11
9	Factorial	600	8	35	400	8	45	70.29	70.81	-0.5173
10	Factorial	1000	8	25	600	4	45	74.35	72.58	1.77
11	Factorial	600	8	25	600	4	45	70.31	70.60	-0.2927
12	Axial	800	8	30	500	6	40	90.25	92.59	-2.34
13	Factorial	1000	8	35	600	4	35	72.08	73.05	-0.9736
14	Factorial	600	6	25	400	8	35	70.16	72.44	-2.28
15	Axial	800	7	25	500	6	40	90.35	92.77	-2.42
16	Factorial	1000	8	35	400	8	35	71.25	72.90	-1.65
17	Centre	800	7	30	500	6	40	99.98	97.31	2.67
18	Centre	800	7	30	500	6	40	99.98	97.31	2.67
19	Factorial	600	6	25	600	4	45	70.32	69.61	0.7103
20	Factorial	600	6	35	600	4	35	70.35	72.97	-2.62
21	Axial	800	7	35	500	6	40	89.36	91.58	-2.22
22	Factorial	1000	8	35	600	8	45	70.81	73.27	-2.46
23	Factorial	600	6	35	400	8	35	72.36	70.95	1.41
24	Axial	1000	7	30	500	6	40	95.36	97.46	-2.10
25	Factorial	1000	6	35	600	8	35	74.35	72.88	1.47
26	Factorial	600	6	35	600	8	35	74.26	72.33	1.93
27	Factorial	1000	8	25	400	8	35	77.67	74.84	2.83
28	Factorial	1000	8	25	400	4	35	70.24	73.03	-2.79
29	Factorial	1000	8	35	400	4	35	71.52	72.49	-0.9740
30	Axial	600	7	30	500	6	40	92.38	94.93	-2.55
31	Centre	800	7	30	500	6	40	99.98	97.31	2.18
32	Factorial	600	6	25	400	8	45	73.24	74.71	-1.47
33	Factorial	600	6	35	400	8	45	70.25	69.99	0.2644
34	Factorial	600	8	25	600	4	35	72.36	73.16	-0.7972
35	Factorial	600	6	25	600	4	35	70.85	70.17	0.6808
36	Factorial	600	8	25	600	8	35	71.25	74.16	-2.91
37	Centre	800	7	30	500	6	40	99.49	97.31	2.18
38	Factorial	600	8	35	600	4	45	70.54	71.19	-0.6501
39	Factorial	1000	6	25	600	8	35	75.36	72.97	2.39
40	Factorial	600	6	25	600	8	45	72.3	71.85	0.4460

41	Factorial	600	8	25	400	8	35	76.58	74.23	2.35
42	Factorial	600	8	35	600	8	35	74.03	76.57	-2.54
43	Factorial	1000	6	35	400	8	35	71.55	74.04	-2.49
44	Factorial	1000	6	25	400	8	45	86.35	83.70	2.65
45	Centre	800	7	30	500	6	40	99.98	97.31	2.18
46	Factorial	600	8	25	400	4	45	71.25	71.74	-0.4893
47	Factorial	1000	6	25	600	4	35	72.36	71.68	0.6818
48	Centre	800	7	30	500	6	40	99.98	97.31	2.18
49	Factorial	600	6	35	600	8	45	70.58	70.01	0.5723
50	Factorial	1000	8	35	400	4	45	73.58	72.49	1.09
51	Factorial	600	8	35	400	4	35	77.03	73.88	3.15
52	Factorial	1000	6	35	400	4	45	76.38	75.85	0.5272
53	Factorial	1000	6	25	400	4	35	78.51	75.41	3.10
54	Factorial	1000	8	35	600	8	35	75.65	73.16	2.49
55	Factorial	1000	6	25	600	4	45	74.62	75.55	-0.9262
56	Axial	800	7	30	500	8	40	92.35	95.33	-2.98
57	Factorial	1000	6	35	400	4	35	74.38	73.86	0.5214
58	Factorial	600	6	35	400	4	45	70.65	68.86	1.79
59	Factorial	600	8	35	400	4	45	65.92	69.45	-3.53
60	Centre	800	7	30	500	6	40	99.49	97.31	2.18
61	Factorial	1000	8	35	400	8	45	76.58	74.37	2.21
62	Factorial	1000	8	25	600	4	35	70.35	70.71	-0.3598
63	Factorial	1000	8	35	600	4	45	75.36	71.69	3.67
64	Factorial	600	8	25	600	8	45	76.54	73.07	3.47
65	Axial	800	6	30	500	6	40	90.35	92.66	-2.31
66	Centre	800	7	30	500	6	40	99.98	97.31	2.18
67	Axial	800	7	30	600	6	40	90.25	92.05	-1.80
68	Axial	800	7	30	400	6	40	90.35	93.20	-2.85
69	Factorial	1000	8	25	400	8	45	78.35	79.54	-1.19
70	Factorial	1000	6	25	400	4	45	81.51	80.64	0.8722
71	Axial	800	7	30	500	6	35	89.35	91.45	-2.10
72	Factorial	600	6	25	400	4	45	72.35	72.16	0.1861
73	Factorial	600	6	25	600	8	35	71.54	70.94	0.5953
74	Centre	800	7	30	500	6	40	99.98	97.31	2.18
75	Factorial	600	8	25	400	4	35	74.32	72.94	1.38
76	Factorial	1000	6	35	600	8	45	76.28	74.99	1.29
77	Factorial	1000	8	25	600	8	35	71.25	72.23	-0.9778
78	Factorial	1000	6	35	400	8	45	77.52	77.50	0.0192
79	Factorial	600	8	35	600	4	35	79.35	76.98	2.37
80	Factorial	1000	6	35	600	4	35	72.35	73.00	-0.6506
81	Factorial	1000	8	25	400	4	45	74.52	76.26	-1.74
82	Axial	800	7	30	500	6	45	89.36	91.90	-2.54
83	Factorial	600	8	25	400	8	45	75.36	74.51	0.8527
84	Factorial	600	8	35	600	8	45	71.23	72.25	-1.02
85	Factorial	600	6	35	400	4	35	70.39	71.29	-0.8984
86	Factorial	1000	8	25	600	8	45	75.68	75.57	0.1104

Table IVA.4: Experimental design matrix for the degradation of Phenol by *Pseudomonas otitidis* strain MCC10330

Run order	Space type	Z1:Initial Concentration of Phenol (mg/L)	Z2: pH	Z3: Temperature (°C)	Z4: Medium Volume (mL)	Z5: Inoculums (percent)	Z6: Residence Time(hr)	Y: percent of Phenol degradation		
								Experimental value	Predicted value	Error
1	Factorial	800	8	35	400	8	35	69.24	68.63	0.6128
2	Factorial	800	8	35	600	8	35	65.27	66.62	-1.35
3	Factorial	1200	6	35	600	4	35	68.91	68.38	0.5301
4	Axial	1000	6	40	500	6	40	94.32	95.81	-1.49
5	Factorial	1200	6	45	600	4	45	73.25	71.27	1.98
6	Axial	1000	7	40	500	4	40	94.35	94.60	-0.2490
7	Factorial	1200	6	35	400	8	45	68.34	68.16	0.1771
8	Factorial	800	6	35	600	4	35	73.25	69.03	4.22
9	Factorial	1200	6	35	400	4	35	66.31	67.69	-1.38
10	Factorial	800	8	35	600	8	45	75.36	70.65	4.71
11	Factorial	1200	8	35	600	8	35	67.24	66.79	0.4458
12	Factorial	1200	8	45	400	8	35	77.51	74.93	2.58
13	Axial	1000	7	40	500	6	45	97.36	100.79	-3.43
14	Factorial	1200	8	45	400	4	35	70.35	67.53	2.82
15	Factorial	800	6	35	400	4	45	72.35	73.11	-0.7554
16	Factorial	800	8	35	400	8	45	71.36	71.08	0.2815
17	Factorial	800	6	35	600	8	45	72.37	73.13	-0.7646
18	Factorial	1200	8	35	600	4	35	62.35	65.06	-2.71
19	Factorial	800	6	35	600	4	45	74.36	74.86	-0.5048
20	Factorial	800	6	35	400	8	45	69.32	70.90	-1.58
21	Factorial	1200	6	45	400	8	45	74.36	70.39	3.97
22	Factorial	800	6	45	400	4	45	70.81	66.95	3.86
23	Factorial	1200	6	45	600	4	35	61.66	64.70	-3.04
24	Factorial	800	6	45	600	8	45	73.62	72.90	0.7213
25	Factorial	800	8	45	400	4	45	64.35	65.80	-1.45
26	Factorial	1200	6	35	400	8	35	67.24	63.67	3.57
27	Factorial	1200	8	45	400	8	45	75.51	78.12	-2.61
28	Factorial	800	6	45	400	4	35	61.39	63.16	-1.77
29	Factorial	800	6	45	600	8	35	67.25	68.47	-1.22
30	Factorial	1200	6	45	400	4	45	68.35	69.22	-0.8708
31	Factorial	1200	8	35	400	4	35	70.35	67.04	3.31
32	Axial	1000	7	40	400	6	40	86.32	89.01	-2.69
33	Centre	1000	7	40	500	6	40	99.28	97.60	1.68
34	Factorial	1200	8	45	600	4	35	63.38	65.34	-1.96
35	Factorial	1200	8	35	600	4	45	70.8	71.24	-0.4431
36	Axial	1000	7	35	500	6	40	88.32	89.96	-1.64
37	Factorial	800	6	45	400	8	45	70.36	70.88	-0.5243
38	Centre	1000	7	40	500	6	40	99.28	97.60	1.68
39	Factorial	1200	6	35	400	4	45	71.35	73.13	-1.78
40	Factorial	1200	6	45	600	8	45	70.36	72.92	-2.56

41	Factorial	1200	8	35	400	8	35	67.29	68.29	-1.00
42	Centre	1000	7	40	500	6	40	99.28	97.60	1.68
43	Axial	1000	8	40	500	6	40	94.35	96.22	-1.87
44	Axial	1000	7	40	600	6	40	88.36	89.03	-0.6711
45	Factorial	1200	8	45	400	4	45	70.35	71.67	-1.32
46	Factorial	800	6	35	400	8	35	69.34	67.60	1.74
47	Factorial	1200	6	45	600	8	35	70.36	67.30	3.06
48	Axial	800	7	40	500	6	40	86.35	86.92	-0.5732
49	Factorial	1200	8	45	600	8	35	71.24	73.21	-1.97
50	Factorial	1200	8	45	600	8	45	78.9	77.99	0.9105
51	Factorial	800	6	45	400	8	35	68.25	68.04	0.2057
52	Factorial	800	8	35	600	4	45	64.83	67.10	-2.27
53	Factorial	1200	8	45	600	4	45	74.62	71.06	3.56
54	Factorial	1200	8	35	400	4	45	73.65	71.63	2.02
55	Factorial	800	6	35	400	4	35	68.36	68.86	-0.5004
56	Centre	1000	7	40	500	6	40	99.28	97.60	1.68
57	Factorial	1200	8	35	600	8	45	74.36	72.03	2.33
58	Centre	1000	7	40	500	6	40	99.28	97.60	1.68
59	Factorial	1200	6	35	600	8	35	64.21	64.83	-0.6223
60	Factorial	800	6	45	600	4	45	67.36	68.49	-1.13
61	Factorial	1200	6	35	600	8	45	71.36	70.91	0.4452
62	Factorial	800	8	45	400	8	45	78.94	75.02	3.92
63	Factorial	800	8	35	600	4	35	60.49	62.12	-1.63
64	Centre	1000	7	40	500	6	40	99.28	97.60	1.68
65	Factorial	800	8	45	600	8	45	71.84	74.37	-2.53
66	Axial	1000	7	40	500	8	40	94.32	97.43	-3.11
67	Axial	1000	7	45	500	6	40	88.36	90.09	-1.73
68	Factorial	800	8	45	600	4	35	65.21	60.15	5.06
69	Factorial	800	6	35	600	8	35	65.24	68.25	-3.01
70	Factorial	800	8	35	400	4	35	62.32	64.61	-2.29
71	Factorial	800	8	35	400	4	45	71.36	68.01	3.35
72	Factorial	800	8	45	400	8	35	70.35	73.03	-2.68
73	Axial	1200	7	40	500	6	40	85.36	88.15	-2.79
74	Factorial	1200	6	45	400	8	35	64.21	66.35	-2.14
75	Factorial	1200	8	35	400	8	45	67.48	71.94	-4.46
76	Axial	1000	7	40	500	6	35	96.35	96.28	0.0692
77	Factorial	800	6	45	600	4	35	64.32	63.11	1.21
78	Factorial	1200	6	35	600	4	45	76.35	75.41	0.9413
79	Centre	1000	7	40	500	6	40	99.28	97.60	1.68
80	Centre	1000	7	40	500	6	40	99.28	97.60	1.68
81	Factorial	800	8	45	400	4	35	61.22	62.86	-1.64
82	Factorial	800	8	45	600	4	45	60.49	64.67	-4.18
83	Centre	1000	7	40	500	6	40	99.28	97.60	1.68
84	Centre	1000	7	40	500	6	40	99.28	97.60	1.68
85	Factorial	800	8	45	600	8	35	73.25	70.79	2.46
86	Factorial	1200	6	45	400	4	35	63.27	64.24	-0.9658

Table IVA.5: Adequacy of the models tested for Phenol degradation (for *Brevibacillus formosus* strain NRRL NRS- 863)

Sequential Model Sum of Squares						
Source	**Sum of Squares**	**df**	**Mean Square**	**F-value**	**p-value**	**Remarks**
Mean vs. Total	5.362E+05	1	5.362E+05			
Linear vs. Mean	183.38	6	30.56	0.2841	0.9429	
2FI vs. Linear	304.10	15	20.27	0.1583	0.9999	
Quadratic vs. 2FI	**7857.95**	**6**	**1309.66**	**225.46**	**< 0.0001**	**Suggested**
Cubic vs. Quadratic	114.03	26	4.39	0.6297	0.8850	Aliased
Residual	222.89	32	6.97			
Total	5.448E+05	86	6335.29			

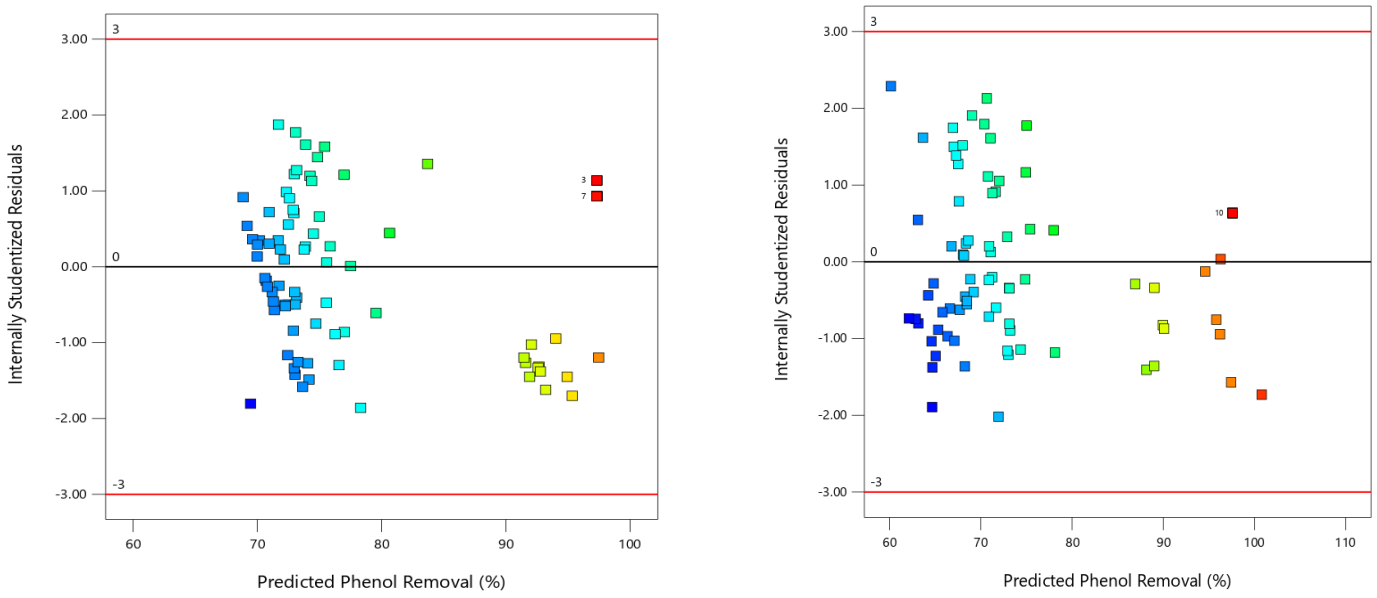
Model Summary Statistics						
Source	**Std. Dev.**	**R^2**	**Adjusted R^2**	**Predicted R^2**	**PRESS**	**Remarks**
Linear	10.37	0.0211	-0.0532	-0.0701	9290.66	
2FI	11.32	0.0561	-0.2536	-0.2817	11128.12	
Quadratic	**2.41**	**0.9612**	**0.9431**	**0.9085**	**794.46**	**Suggested**
Cubic	2.64	0.9743	0.9318	-2.7376	32451.57	Aliased

Table IVA.6: Adequacy of the models tested for Phenol degradation (for *Pseudomonas otitidis* strain MCC10330)

Sequential Model Sum of Squares						
Source	**Sum of Squares**	**df**	**Mean Square**	**F-value**	**p-value**	**Remarks**
Mean vs. Total	4.937E+05	1	4.937E+05			
Linear vs. Mean	496.07	6	82.68	0.5358	0.7795	
2FI vs. Linear	481.09	15	32.07	0.1753	0.9997	
Quadratic vs. 2FI	**11278.41**	**6**	**1879.73**	**253.53**	**< 0.0001**	**Suggested**
Cubic vs. Quadratic	167.38	26	6.44	0.7844	0.7352	Aliased
Residual	262.64	32	8.21			
Total	5.064E+05	86	5888.25			

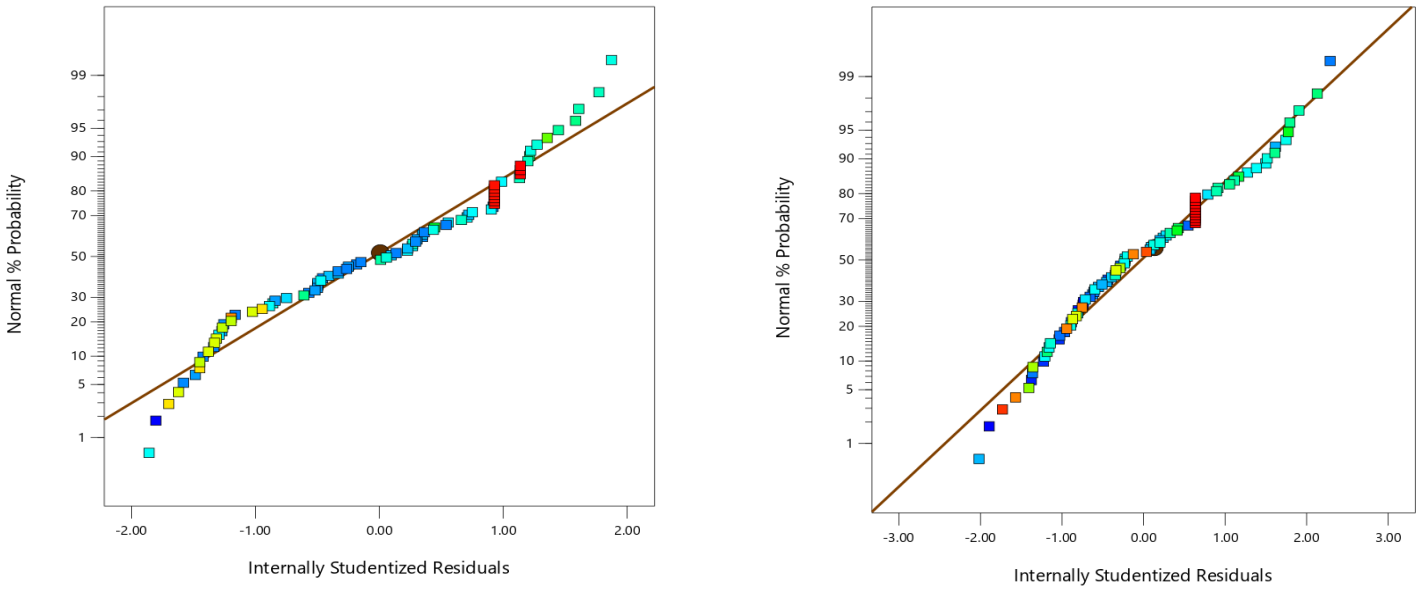
Model Summary Statistics						
Source	**Std. Dev.**	**R^2**	**Adjusted R^2**	**Predicted R^2**	**PRESS**	**Remarks**
Linear	12.42	0.0391	-0.0339	-0.0531	13359.02	
2FI	13.53	0.0770	-0.2258	-0.2592	15973.22	
Quadratic	**2.72**	**0.9661**	**0.9503**	**0.9498**	**1017.55**	**Suggested**
Cubic	2.86	0.9793	0.9450	-0.4375	18235.17	Aliased

In case of both strains, the constant variance assumptions were investigated by plotting internally studentized residual vs. predicted values of percent degradation of Phenol (Figure IVA.1 (a) & IVA.1 (b)). Studentized residuals were deducted by dividing the residuals by their standard deviations displaying a randomly scattered pattern within the detection limits -3 to +3 and so, prediction of model described in the equation no (4.5 & 4.6 respectively) for the percent degradation of Phenol is satisfactory. The normal probability plot of residuals (Figure IVA.2 (a) & IVA.2 (b)) for the percent degradation of Phenol showed a straight line pattern for the both strains, rather than S shaped followed by the points on the plot. As the residuals are distributed normally, transformation of response is not required. Relation between the predicted and experimental values of responses have been displayed in Figure IVA.3 (a) & IVA.3 (b) for *Brevibacillus formosus* strain NRRL NRS- 863 & *Pseudomonas otitidis* strain MCC10330 respectively. In both the figure, very little discrepancies were found by the straight trend line pointing a good relationship in between the predicted and experimental values.



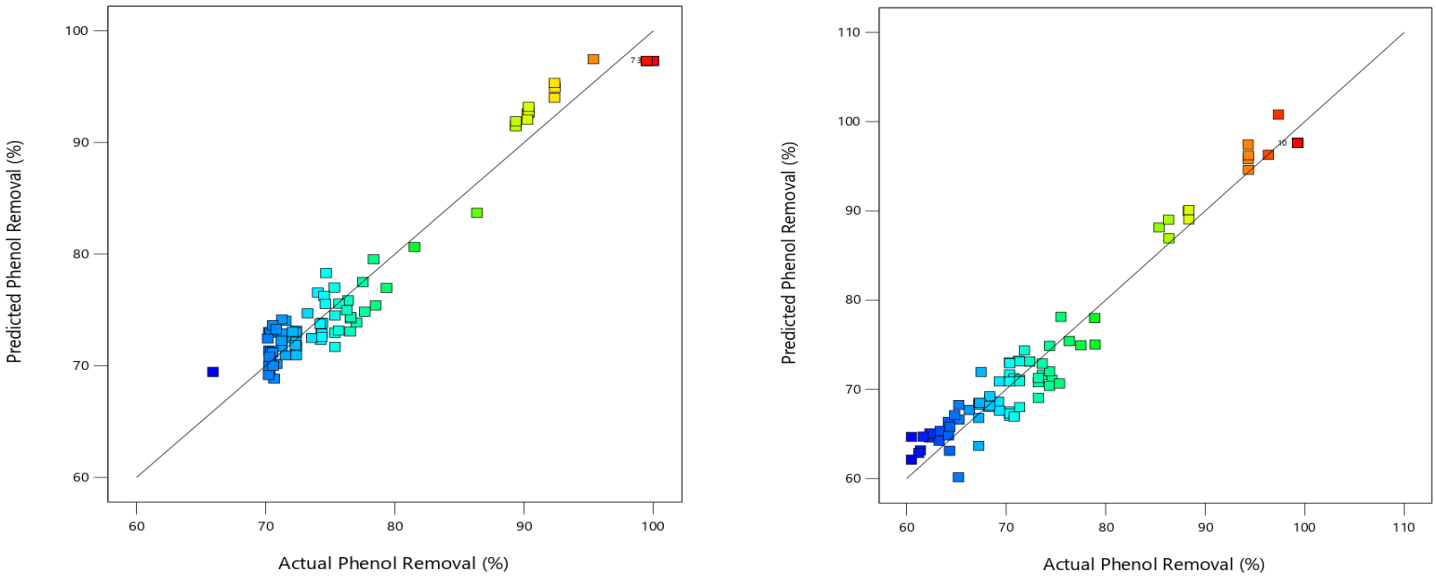
(a) (b)

Figure IVA.1: Internally studentized residuals vs. predicted values (a) *Brevibacillus formosus* (b) *Pseudomonas otitidis*



(a) (b)

Figure IVA.2: Internally studentized residuals vs. Normal percent probability (a) *Brevibacillus formosus* (b) *Pseudomonas otitidis*



(a) (b)

Figure IVA.3: Actual degradation data vs. predicted data (a) *Brevibacillus formosus* (b) *Pseudomonas otitidis*

IVA.2.2 ANOVA Test:

ANOVA is a measurable strategy which partitions total variation in a bunch of information into compartment parts connected with particular sources of variation for hypotheses testing of the factors (Rajkumar k et al., Sangeetha V et al.). Outcomes of ANOVA for percent of degradation of Phenol by both the strains have been given in Table IVA.7 & IVA.8 respectively. In the table no IVA.7 (ANOVA for percent degradation of Phenol by *Brevibacillus formosus*), The Model F-value of 53.21 implies the model is significant. There is only a 0.01percent chance that an F-value this large could occur due to noise. P-values less than 0.0500 indicate model terms are significant. In this case X1, X2, X3, X4, X5, X6, X1X2, X1X3, X1X4, X1X5, X1X6, X2X3, X2X4,X2X5, X2X6, X3X4, X3X5, X3X6, X4X5, X4X6, X5X6, $X2^2$, $X3^2$, $X4^2$, $X6^2$ are significant model terms. Values greater than 0.1000 indicate the model terms are not significant. Only the two terms i.e. $X1^2$ & $X5^2$ are not significant. The Lack of Fit F-value of 122.55 implies the Lack of Fit is significant. There is only a 0.01% chance that a Lack of Fit F-value this large could occur due to noise. If there are many insignificant model terms (not counting those required to support hierarchy), model reduction may improve the model.

Table IVA.7: ANOVA of the second order polynomial equation for the degradation of Phenol by *Brevibacillus formosus*

Source	Sum of Squares	df	Mean Square	F-value	p-value	Remarks
Model	8345.44	27	309.09	53.21	< 0.0001	significant
X1-Initial Phenol Concentration	105.94	1	105.94	18.24	< 0.0001	significant
X2-pH	0.0887	1	0.0887	0.0153	0.0021	significant
X3-Temperature	23.32	1	23.32	4.01	0.0298	significant
X4-volume of media	21.69	1	21.69	3.73	0.0382	significant
X5-Inoculums	28.93	1	28.93	4.98	0.0295	significant
X6-Residence Time	3.40	1	3.40	0.5861	0.0170	significant
X1X2	62.61	1	62.61	10.78	0.0017	significant
X1X3	8.72	1	8.72	1.50	0.0255	significant
X1X4	25.76	1	25.76	4.43	0.0396	significant
X1X5	1.07	1	1.07	0.1844	0.0092	significant
X1X6	78.41	1	78.41	13.50	0.0005	significant
X2X3	4.17	1	4.17	0.7182	0.0002	significant
X2X4	8.04	1	8.04	1.38	0.0043	significant
X2X5	0.2025	1	0.2025	0.0349	0.0225	significant
X2X6	15.92	1	15.92	2.74	0.0032	significant
X3X4	33.06	1	33.06	5.69	0.0203	significant
X3X5	7.98	1	7.98	1.37	0.0059	significant
X3X6	41.80	1	41.80	7.20	0.0095	significant
X4X5	0.3570	1	0.3570	0.0615	0.0051	significant
X4X6	7.38	1	7.38	1.27	0.0042	significant
X5X6	8.63	1	8.63	1.49	0.0279	significant
$X1^2$	3.00	1	3.00	0.5171	0.4750	Not significant
$X2^2$	52.57	1	52.57	9.05	0.0039	significant
$X3^2$	63.01	1	63.01	10.85	0.0017	significant
$X4^2$	52.57	1	52.57	9.05	0.0039	significant
$X5^2$	16.66	1	16.66	2.87	0.0957	Not significant
$X6^2$	75.88	1	75.88	13.06	0.0006	significant
Residual	336.92	58	5.81			
Lack of Fit	336.41	49	6.87	122.55	< 0.0001	
Pure Error	0.5042	9	0.0560			
Cor Total	8682.36	85				

In the table no IVA.8, the Model F-value of 61.22 implies the model is significant. There is only a 0.01% chance that an F-value this large could be occurred due to noise. P-values less than 0.0500 indicate model terms are significant. In this case Z1, Z2, Z3, Z4, Z5, Z6, Z1Z2, Z1Z3, Z1Z4, Z1Z5, Z1Z6, Z2Z3, Z2Z4, Z3Z4, Z3Z5, Z3Z6, Z4Z5, Z4Z6, $Z1^2$, $Z3^2$, $Z4^2$ are

significant model terms. On the other hand, Z2Z5, Z2Z6, Z5Z6, $Z2^2$, $Z5^2$ & $Z6^2$ are not significant model terms.

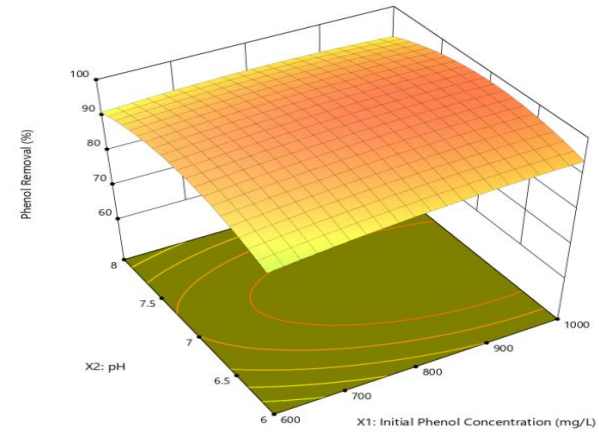
Table IVA.8: ANOVA of the second order polynomial equation for the degradation of Phenol by *Pseudomonas otitidis*

Source	Sum of Squares	df	Mean Square	F-value	p-value	Remarks
Model	12255.56	27	453.91	61.22	< 0.0001	Significant
Z1-Initial Phenol Concentration	24.79	1	24.79	3.34	0.0026	Significant
Z2-pH	2.74	1	2.74	0.3697	0.0055	Significant
Z3-Temperature	0.2828	1	0.2828	0.0381	0.0058	Significant
Z4-Medium Volume	0.0066	1	0.0066	0.0009	0.0063	Significant
Z5-Inoculums	132.54	1	132.54	17.88	< 0.0001	Significant
Z6-Residence Time	335.70	1	335.70	45.28	< 0.0001	Significant
Z1Z2	51.77	1	51.77	6.98	0.0106	Significant
Z1Z3	20.14	1	20.14	2.72	0.0047	Significant
Z1Z4	1.06	1	1.06	0.1431	0.0066	Significant
Z1Z5	30.55	1	30.55	4.12	0.0070	Significant
Z1Z6	5.75	1	5.75	0.7753	0.0122	Significant
Z2Z3	62.49	1	62.49	8.43	0.0052	Significant
Z2Z4	28.38	1	28.38	3.83	0.0252	Significant
Z2Z5	111.51	1	111.51	15.04	0.6533	Not Significant
Z2Z6	2.87	1	2.87	0.3875	0.5361	Not Significant
Z3Z4	0.1980	1	0.1980	0.0267	0.0027	Significant
Z3Z5	150.98	1	150.98	20.36	< 0.0001	Significant
Z3Z6	0.8418	1	0.8418	0.1135	0.0074	Significant
Z4Z5	0.9120	1	0.9120	0.1230	0.0271	Significant
Z4Z6	10.05	1	10.05	1.36	0.0391	Significant
Z5Z6	3.58	1	3.58	0.4831	0.4898	Not Significant
$Z1^2$	241.85	1	241.85	32.62	< 0.0001	Significant
$Z2^2$	5.98	1	5.98	0.8070	0.3727	Not Significant
$Z3^2$	137.15	1	137.15	18.50	< 0.0001	Significant
$Z4^2$	175.73	1	175.73	23.70	< 0.0001	Significant
$Z5^2$	5.98	1	5.98	0.8070	0.3727	Not Significant
$Z6^2$	2.10	1	2.10	0.2829	0.5968	Not Significant
Residual	430.02	58	7.41			
Lack of Fit	430.02	49	8.78			
Pure Error	0.0000	9	0.0000			
Cor Total	12685.59	85				

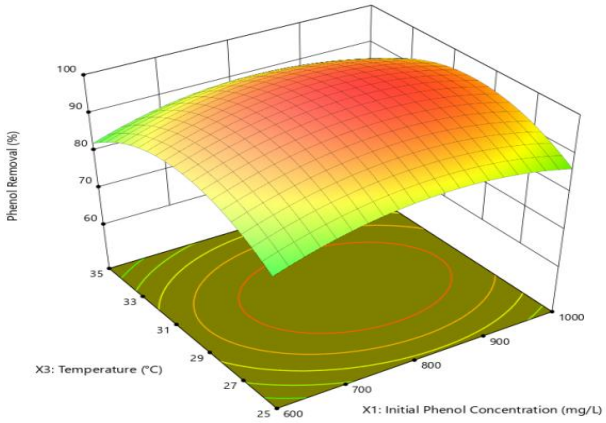
IVA.2.3 Effect of initial conc. of Phenol:

Effect of initial conc. of Phenol played a pivotal role in case of both the strains. In case of *Brevibacillus formosus* strain NRRL NRS- 863, the effect of initial conc. of the Phenol has been displayed in the contour plots. It was found when the initial conc. was 800 mg/L, 92 – 99% of degradation was achieved. But degradation percentage decreased as the initial conc. increased further. The p- value of this factor (X1) is less than 0.0001 which indicates the same (Table IVA. 7). From the 3D plots, it can be understood that the interaction effects between initial conc. of Phenol and media pH (X1X2), initial conc. of Phenol and temperature (X1X3), initial conc. of Phenol and media volume (X1X4), initial conc. of Phenol and inoculums size (X1X5) & initial conc. of Phenol and residence time (X1X6) all are significant as the values of all of these model terms < 0.05 (Table IVA.7). Figure IVA.4 clearly displayed the interaction effects.

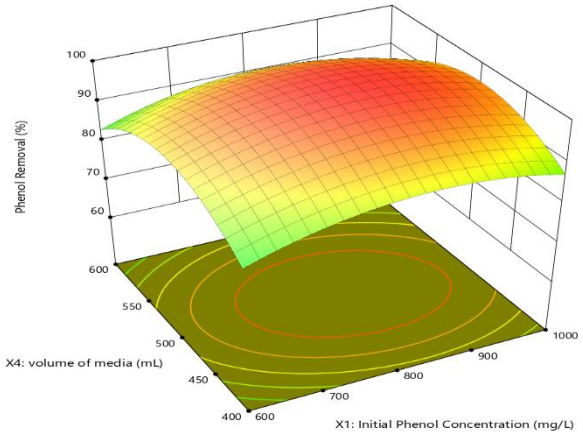
In case of *Pseudomonas otitidis* strain MCC10330, this model term has been denoted by Z1. Like that of previous strain, here also initial conc. of Phenol was an important factor as beyond a certain conc. an inhibitory effect arise. To understand the effect of initial conc. of Phenol, 3D and contour plots were showed in Figure IVA.5. It was observed that increase in initial conc. of Phenol up to 1000 mg/L; almost 100% degradation was obtained. Beyond this concentration, the degradation was decreased. The p- value of Z1 (0.0026) also indicates its significance on the model. From the 3D and contour plots, it can be showed that the interaction effects of initial conc. of Phenol vs. pH (Z1Z2), initial conc. vs. temperature (Z1Z3), initial conc. vs. media volume (Z1Z4), initial conc. vs. inoculums size (Z1Z5) and initial conc. vs. residence time (Z1Z6) possesses significant effects on percent of degradation of Phenol (Figure: IVA.5).



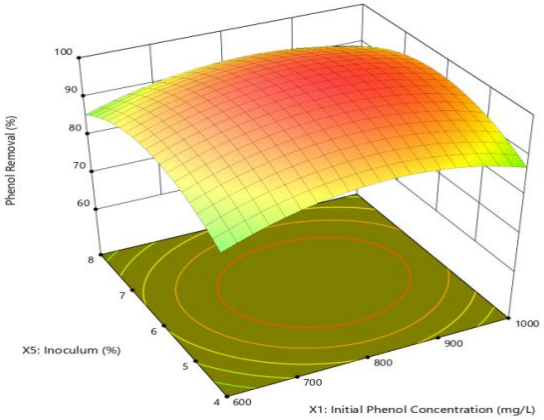
(a)



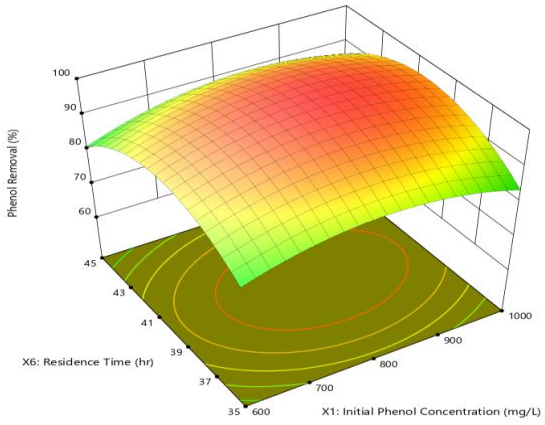
(b)



(c)

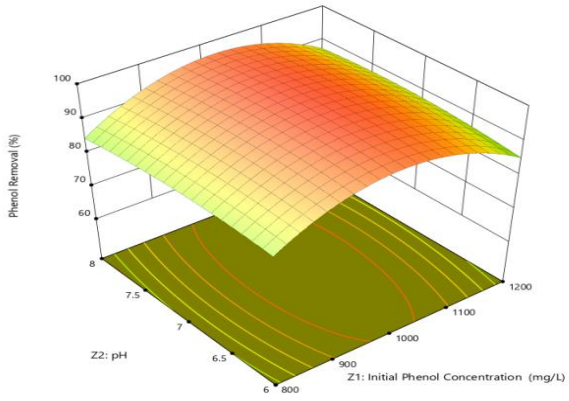


(d)



(e)

Figure IVA.4: **3- Dimensional surfaces and 2-Dimensional plots of the interaction effects of: (a) initial conc. of Phenol and pH of media (X1X2), (b) initial conc. of Phenol and temperature (X1X3), (c) initial conc. of Phenol and media vol. (X1X4), (d) initial conc. of Phenol and inoculums percent (X1X5), (e) initial conc. of Phenol and residence time (X1X6) on the percent of degradation of Phenol in case of *Brevibacillus formosus***



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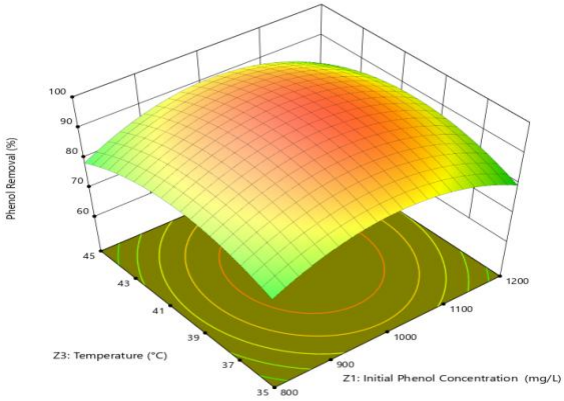

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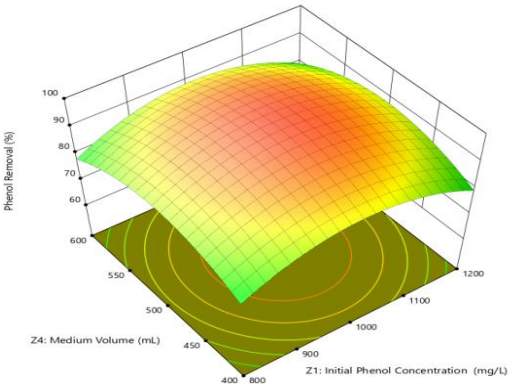
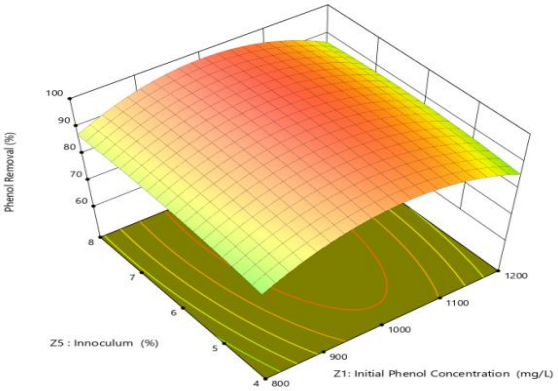
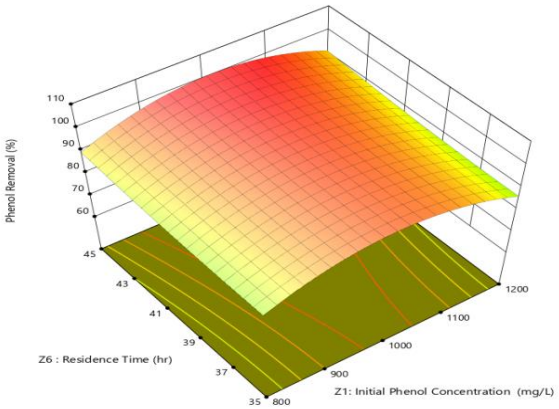


(c)



(d)



(e)

Figure IVA.5: 3- Dimensional surfaces and 2-Dimensional plots of the interaction effects of: (a) initial conc. of Phenol and pH of media (Z1Z2), (b) initial conc. of Phenol and temperature (Z1Z3), (c) initial conc. of Phenol and media vol. (Z1Z4), (d) initial conc. of Phenol and inoculums percent (Z1Z5), (e) initial conc. of Phenol and residence time (Z1Z6) on the percent of degradation of Phenol in case of *Pseudomonas otitidis*

IVA.2.4 Effect of pH:

In case of *Brevibacillus formosus,* pH (X2) left a significant impression on the response i.e. percent of degradation of Phenol. Degradation percent of Phenol was achieved maximum when the pH of the media was 7 i.e. the optimal pH value seems to be neutral. Both in acidic and basic pH, the degradation percent decreased. The p- value of this term was found to be 0.0021 indicating its significant role on the response. From the 3D and contour plots, it was concluded that the interaction effects between pH and temperature (X2X3), pH and volume of the media (X2X4), pH and inoculums size (X2X5) & pH and residence time (X2X6), all exhibit significant effects on the response as all of them are convex shaped (Figure:IVA.6). All of their p- values found to be < 0.05 also (Table IVA.7) which clearly indicated the significance of those model terms.

Also in case of *Pseudomonas otitidis,* Effect of pH (Z2) i.e. concentration of H^+ ions revealed a significant role in percent degradation of Phenol. P- value of the Z2 (0.0055) revealed the same. Percent of degradation of Phenol was maximum (more than 95percent) when the pH value of the culture media was 7. Percent degradation was decreased in both the situations while the pH value of the media increased and decreased indicating that maximum degradation was occurred at particularly neutral condition. Acidic as well as basic condition is not favourable for the strain to remove Phenol. From the 3D and contour plots, it can be showed that the interaction effects of: pH of the media vs. temperature (Z2Z3) and pH of the media vs. volume of the media (Z2Z4) had significant effects on percent degradation of Phenol as the plots are convex shaped (Figure: IVA.7 (a) & (b)). But in case of interaction effects between pH of the media vs. inoculums size (Z2Z5) and pH of the media vs. residence time (Z2Z6) did not exhibit significant effects on the degradation percent of Phenol. Plots are almost flat there (Figure: IVA.7 (c) & (d)).

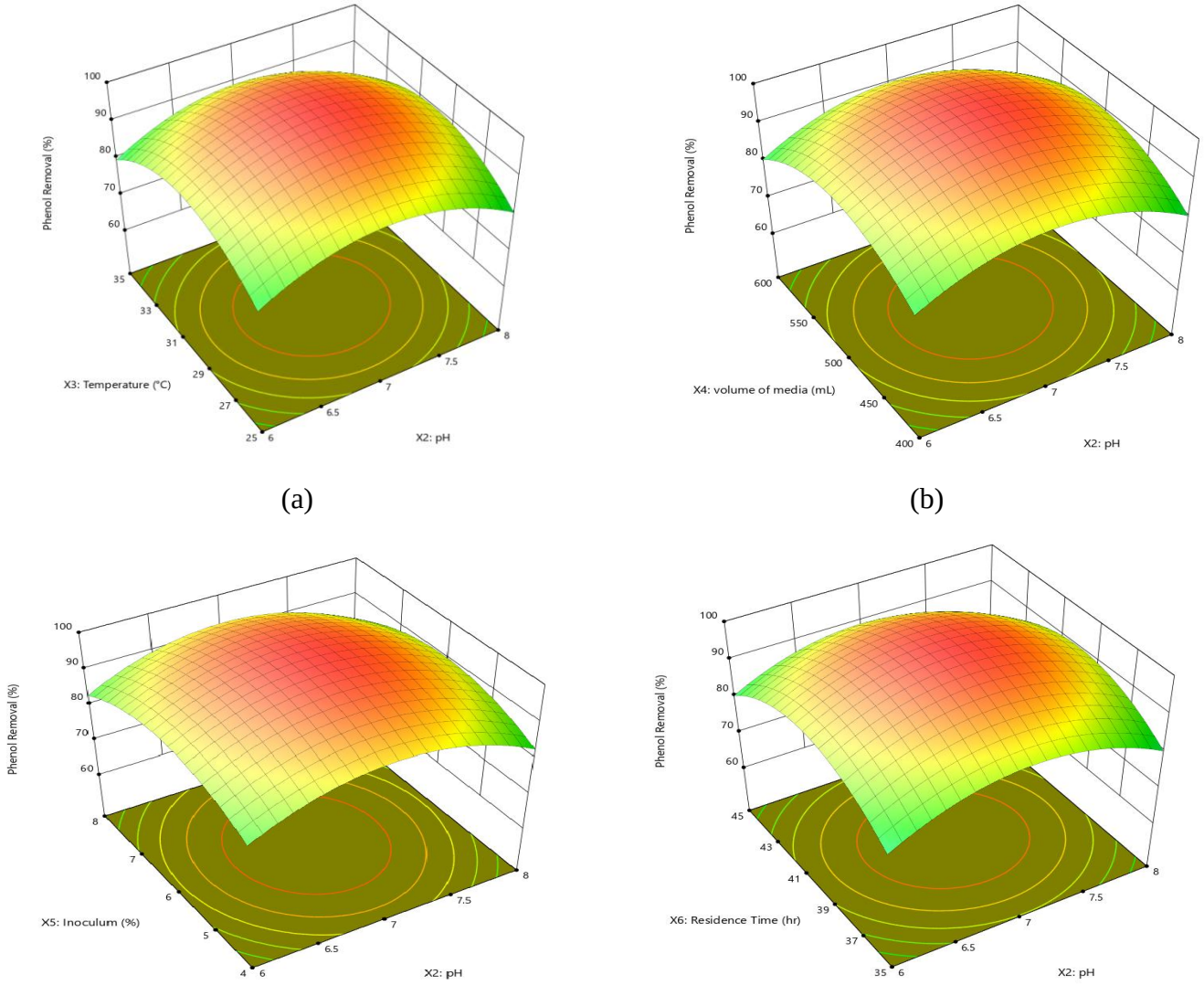


Figure IVA.6: **3-Dimensional surfaces and 2-Dimensional plots of the interaction effects of: (a) pH of the media and temperature (X2X3), (b) pH of the media and media vol. (X2X4), (c) pH of media and inoculums percent (X2X5), (d) pH of media and residence time (X2X6) on the percent of degradation of Phenol in case of *Brevibacillus formosus***

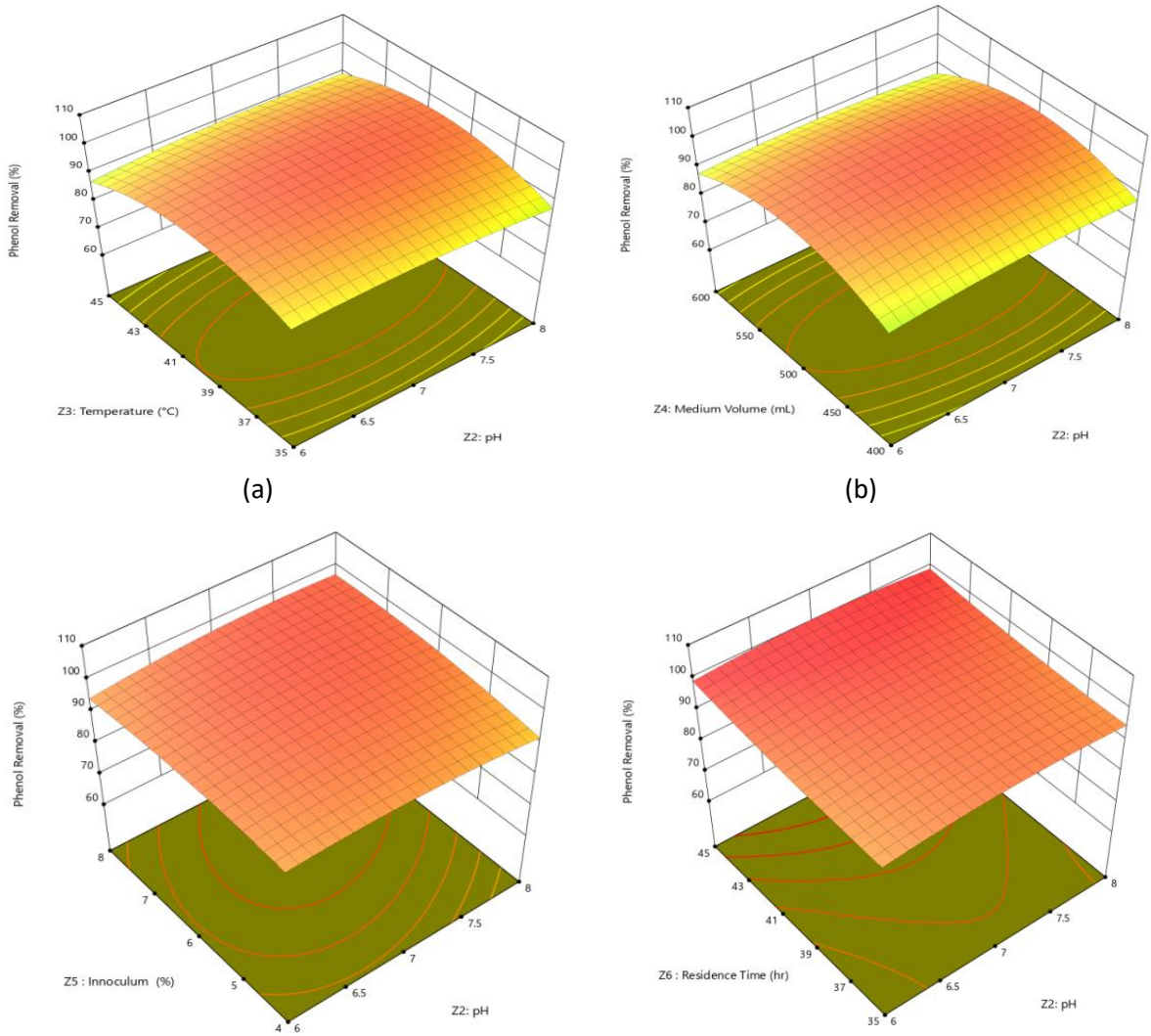
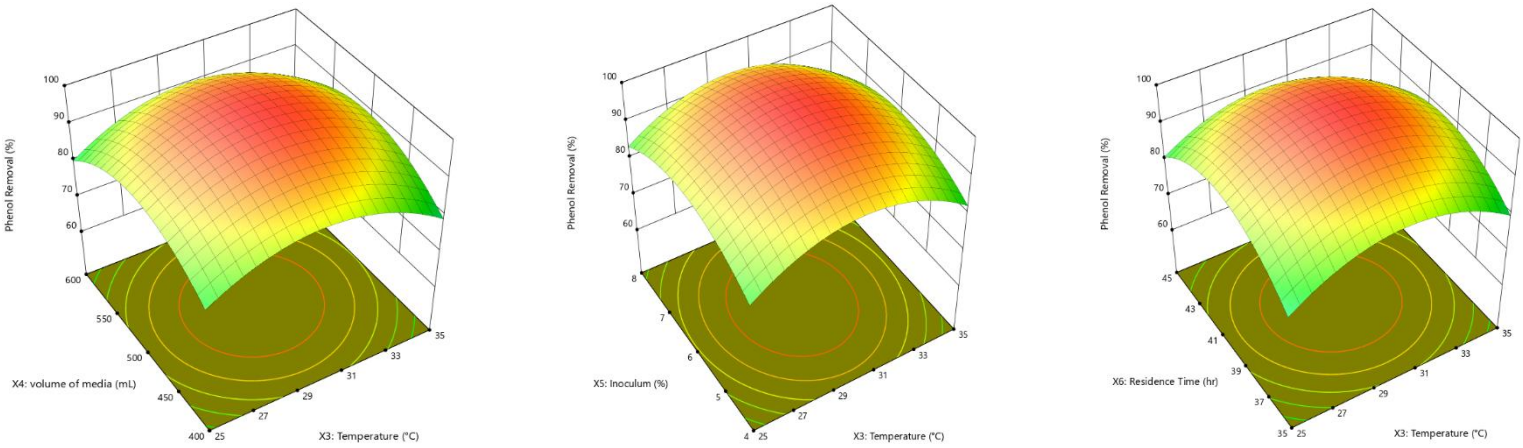


Figure IA.7: **3-Dimensional surfaces and 2-Dimensional plots of the interaction effects of: (a) pH of the media and temperature (Z2Z3), (b) pH of the media and media vol. (Z2Z4), (c) pH of media and inoculums percent (Z2Z5), (d) pH of media and residence time (Z2Z6) on the percent of degradation of Phenol in case of *Pseudomonas otitidis***

IVA.2.5 Effect of temperature:

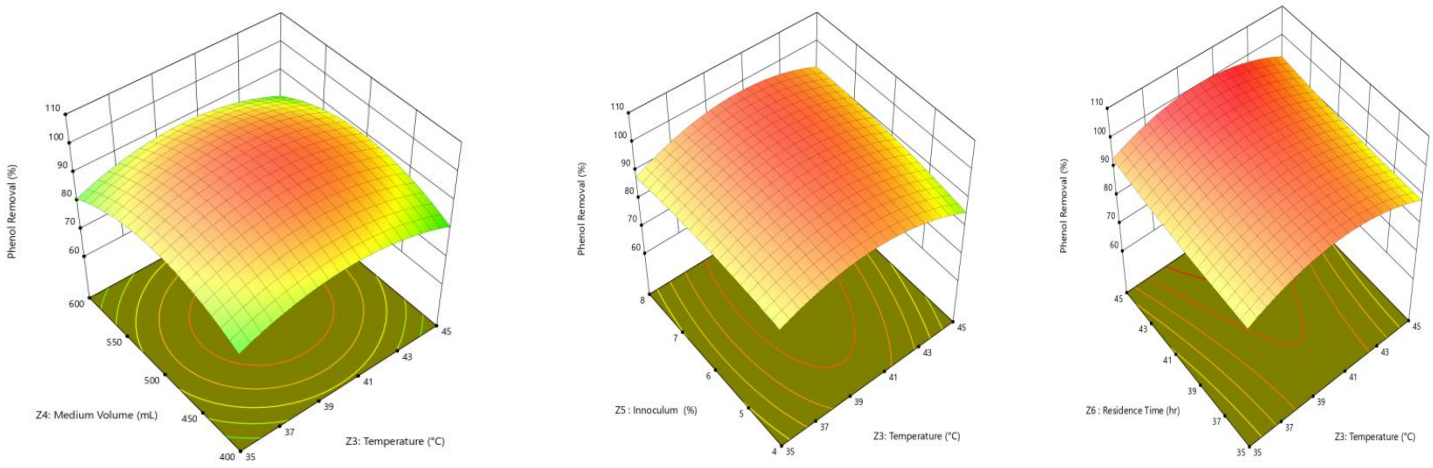
Temperature (X3) was found to be very significant on the degradation percent of Phenol by *Brevibacillus formosus.* Here, the p- value of this term was 0.0298 (Table IVA.7) which indicates the same. Temperature was optimized at 30^{o}C where maximum percent of degradation of Phenol was obtained. But when the temperature decreased up to 25^{o}C, the degradation percent also decreased. Same phenomenon was observed when the temperature increased up to 35^{o}C. The interaction effects of temperature and media volume (X3X4), temperature and inoculums size (X3X5) & temperature and residence time (X3X6) all found to be significant. The umbrella shaped 3D and contour plots (Figure: IVA. 8) also indicate the same.

Also, temperature was found to be very important factor during the study with *Pseudomonas otitidis* (p- value 0.0058 in the Table IVA.8). Degradation percent of Phenol was increased rapidly while temperature increased to 40^{o}C. Maximum degradation was also obtained at 40^{o}C temperature. But below and above this temperature, degradation percent decreased. The interaction effects of temperature vs. media volume (Z3Z4), temperature vs. inoculums percent (Z3Z5) and temperature vs. residence time (Z3Z6) all exhibited significant effect on the percent of degradation of Phenol. The convex appearances of the 3D surfaces and 2D plots (Figure IVA.9) revealed significant effects on the response i.e. percent degradation of Phenol.



(a) (b) (c)

Figure IVA.8: **3-Dimensional surfaces and 2-Dimensional plots of the interaction effects of: (a) temperature and media vol. (X3X4), (b) temperature and inoculums percent (X3X5), (c) temperature and residence time (X3X6) on the percent of degradation of Phenol in case of *Brevibacillus formosus***



(a) (b) (c)

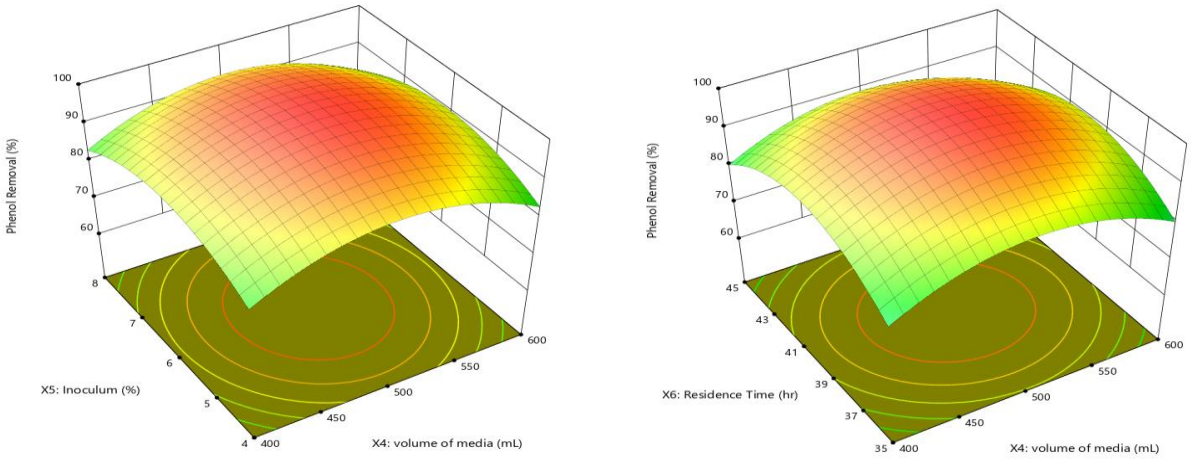
Figure IVA.9: **3-Dimensional surfaces and 2-Dimensional plots of the interaction effects of: (a) temperature and media vol. (Z3Z4), (b) temperature and inoculums percent (Z3Z5), (c) temperature and residence time (Z3Z6) on the percent of degradation of Phenol in case of *Pseudomonas otitidis***

VA.2.6 Effect of media volume:

In case of *Brevibacillus formosus*, the term media volume has been denoted by X4. The p-value of the X4 was 0.0382 (Table IVA.7) which is less than 0.05 and thereby, it can be deducted that the media volume has significant effect on the response i.e. percent degradation of Phenol. It was observed that, maximum percent of degradation was occurred when the media volume was 500 ml. 90 – 99% degradation was achieved in this media volume. But when the volume was increased or decreased, degradation percent of Phenol decreased in both cases. In case of the interaction effects of media volume vs. inoculums size (X4X5) & media volume vs. residence time (X4X6), both played significant roles on the response. P-Values of these model terms were found to be 0.0051 & 0.0042 respectively (Table IVA.7) indicating their significance. Furthermore, the 3D surfaces and 2D plots (Figure IVA.10) also indicate the same.

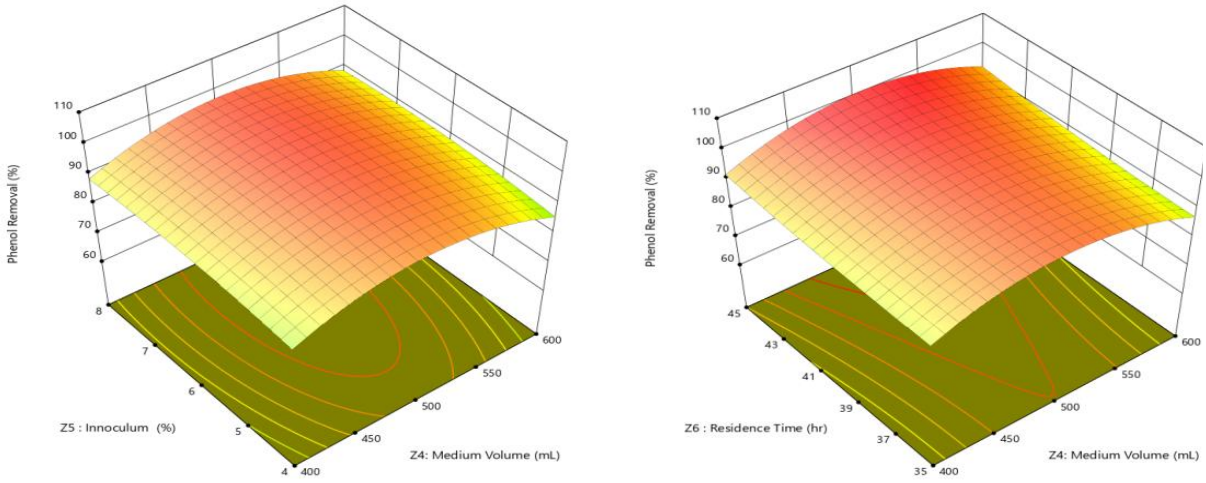
Also in case of *Pseudomonas otitidis*, volume of the culture (Z4) media played a vital role on degradation percent of Phenol. P- value of the Z4 is 0.0063 (Table 4) which stands for an evidence of its significance. Percent of degradation of Phenol increased with the increasing volume of the culture media. Up to 500 ml volume, degradation percent increased steadily. But beyond that, when the volume increased up to 600 and even 700 ml, the degradation

percent remained almost constant. In case of interaction effects of media volume vs. inoculums percent (Z4Z5) and media volume vs. residence time (Z4Z6) left very significant roles on degradation percent of Phenol. P- values of both the model terms were < 0.005 (Table IVA.8). Moreover, convex shaped curves (Figure: IVA.11) supported the same.



(a) (b)

Figure IVA.10: **3-Dimensional surfaces and 2-Dimensional plots of the interaction effects of: (a) media vol. and inoculums percent (X4X5) & (b) media vol. and residence time (X4X6) on the percent of degradation of Phenol in case of *Brevibacillus formosus***



(a) (b)

Figure IVA.11: **3-Dimensional surfaces and 2-Dimensional plots of the interaction effects of: (a) media vol. and inoculums percent (Z4Z5) & (b) media vol. and residence time (Z4Z6) on the percent of degradation of Phenol in case of *Pseudomonas otitidis***

IVA.2.7 Effect of inoculums size & residence time:

In case of *Brevibacillus formosus* strain NRRL NRS- 863, both of these factors were vital. P-values of these two factors (X5 & X6 respectively) were < 0.05 (Table IVA.7). Inoculums size was optimized at 6percent. Maximum degradation of Phenol was achieved when 6percent *Brevibacillus formosus* was added to the culture media. But below that size, at 4percent, degradation percent decreased steadily and even up to 70% in some cases. Decrease in the response was also noticed when the inoculums size was increased up to 8%. In case of residence time, 40 hours time period was found to be optimum. When the time period increased or decreased up to 35 hours and 45 hours respectively, degradation percent decreased in both cases. It was recorded that almost 100percent degradation of Phenol was occurred at 6percent inoculums size of *Brevibacillus formosus* within 40 hours, when the initial conc. of Phenol was 800 mg/L. Moreover, the interaction effect of these two factors i.e. inoculums size vs. residence time (X5X6) found to be significant also as the p- value of this model term was 0.0279 (Table IVA.7). The convex shaped 3D curve also (Figure IVA.12) supported the same.

Inoculums size and residence time both showed important effects on percent of degradation of Phenol in case of *Pseudomonas otitidis* strain MCC10330 also. Degradation percent increased with increased inoculums percent. Up to 6% inoculums, degradation percent increased simultaneously. After that, degradation percent suspended even after increasing the inoculums size. Similarly in case of residence time, degradation percent increased with increased residence time up to 40 hour. Beyond that, no big changes occurred rather negative effects arose. Almost complete degradation of 1000 mg/L Phenol was achieved within 40 hours of time and 6percent inoculums. P- values of both of these model terms were also below 0.005 which implied their significant roles on the response individually. But, the interaction effect of these two factors (Z5Z6) revealed insignificant effect on the degradation percent of Phenol. Figure: IVA.13 showed a flat shaped curve which denotes its insignificance. Also the p- value of this model term was obtained to be 0.4898 (Table IVA.8) which is > 0.05 indicating the insignificant effect of the interaction.

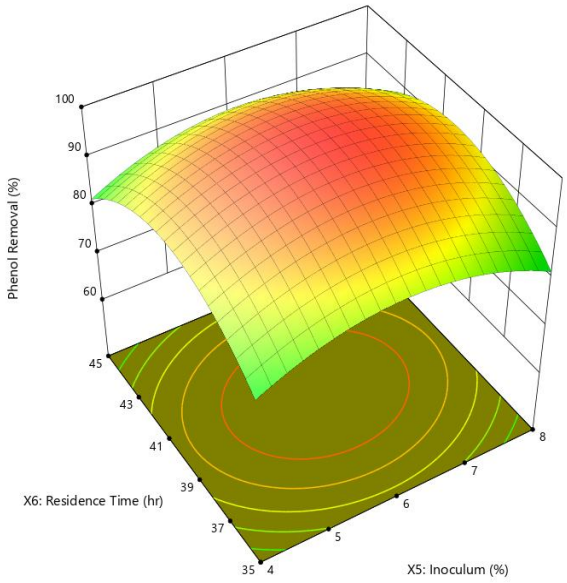


Figure IVA.12: **3-Dimensional surface and 2-Dimensional plot of the interaction effects of inoculums percent and residence time (X5X6) on percent of degradation of Phenol in case of *Brevibacillus formosus***

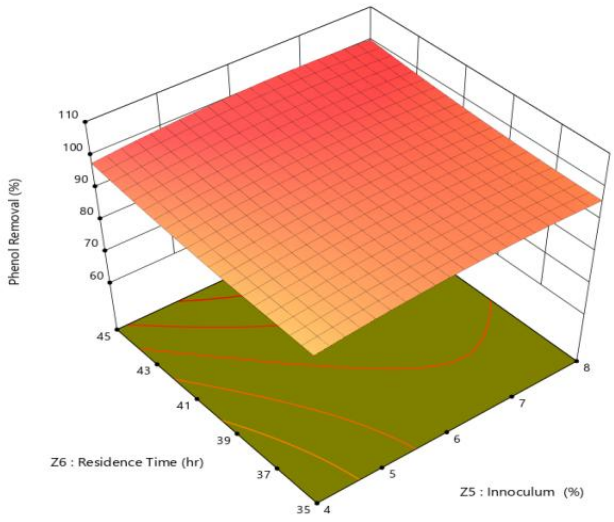


Figure IVA.13: **3-Dimensional surface and 2-Dimensional plot of the interaction effects of inoculums percent and residence time (Z5Z6) on percent of degradation of Phenol in case of *Pseudomonas otitidis***

IVA.2.8 Optimization of the operating parameters:

The Response Surface Methodology (RSM) was involved to deduct the optimum conditions for the six independent variables to achieve maximum percent of degradation of Phenol by the two isolated strains. Equation 4.5 was defined as objective function for the percent of degradation of Phenol in case of *Brevibacillus formosus* and the independent factors in their ranges were model constraints. Thus in case of *Brevibacillus formosus*, the following optimum conditions were achieved for the maximum percent of degradation of Phenol: 800 mg/L initial conc. of Phenol, 7 pH of the bacterial culture, 30^{o}C temperature, 500 mL volume of the culture media, 6percent inoculums size of the strain (i.e. *Brevibacillus formosus* strain NRRL NRS- 863) and 40 hour of residence time. 99.98% degradation of the Phenol was obtained by involving these six favourable parameters. The obtained optimal operating conditions were performed nine more times to validate the predicted values as well to minimise the error and only 2.3% error was obtained which confirmed the acceptance of the model as the values are within 95% CI.

Similarly in case of *Pseudomonas otitidis* strain MCC10330, equation 4.6 was defined as objective function for the percent of degradation of Phenol and the independent factors in their ranges were model constraints. The following optimum conditions were determined for the maximum percent of degradation of Phenol: 1000 mg/L initial conc. of Phenol, 7 pH of the bacterial culture, 40^{o}C temperature, 500 mL volume of the culture media, 6percent inoculums size of the strain (i.e. *Pseudomonas otitidis* strain MCC10330) and 40 hours of residence time. 99.28% degradation of the Phenol was obtained by involving these six favourable situations. The obtained optimal operating conditions were performed nine more times to validate the predicted values as well to minimise the error and 99.28% degradation of Phenol was achieved in each of experiments, obtaining only 1.6% of error and thereby, indicating the reliability of this model.

Sub chapter IV B

Optimization of parameters for the 4-Chloro Phenol degrading strains via Response Surface Methodology

IVB.1 Materials and Methods:

IVB.1.1 Materials:

Bacillus timonensis strain 10403023 and *Bacillus cereus* strain K1were isolated from the contaminated soil, collected from South Howrah state General Hospital by enrichment method as described in the chapter II, section IIA.1.5. A large volume of inoculums of the two above mentioned strains were already prepared via acclimatization process in Mineral Salt Medium (MSM) as described in chapter II, section IIA.1.8.

4- Chloro Phenol solution was prepared synthetically in the laboratory. Conc. of the stock solution was maintained as 10 g/L.

IVB.1.2 Experimental set up:

Same as mentioned in the chapter III, section IIIA.1.2.

IVB.1.3 Analytical Method:

Details of this process has been mentioned in chapter II, section IIA.1.7.

IVB.1.4 Experimental design:

In case of *Bacillus timonensis* strain 10403023, Six (6) independent variables (Table IVB.1) are X1 (A): 600 – 1000 mg/L; X2 (B): 7 – 8; X3 (C): 35^{o} – 45^{o}C; X4 (D): 400 – 600 ml; X5 (E): 4 – 8% and X6 (F): 18 – 30 hour.

In case of *Bacillus cereus* strain K1, Six (6) independent variables (Table IVB.2) are Z1 (A): 400 – 800 mg/L; Z2 (B): 6 – 7; Z3 (C): 20^{o} – 30^{o}C; Z4 (D): 300 – 500 ml; Z5 (E): 7 – 9% and Z6 (F): 18 – 30 hour.

Rest of the portion is same as described in sub chapter IVA, section IVA.1.4.

Table IVB.1: Independent variables with coded levels for *Bacillus timonensis*

Independent variable	Symbol	Coded levels		
		-1	0	+1
Initial conc. of 4- Chloro Phenol (mg/L)	X1	600	800	1000
pH of bacterial media	X2	7	7.5	8
Temperature (^{o}C)	X3	35	40	45
Volume of bacterial media(mL)	X4	400	500	600
Inoculums percent (percent)	X5	4	6	8
Residence time(hr)	X6	18	24	30

Table IVB.2: Independent variables with coded levels for *Bacillus cereus*

Independent variable	Symbol	Coded levels		
		-1	0	+1
Initial conc. of 4- Chloro Phenol (mg/L)	Z1	400	600	800
pH of bacterial media	Z2	6	6.5	7
Temperature (^{o}C)	Z3	20	25	30
Volume of bacterial media(mL)	Z4	300	400	500
Inoculums percent (percent)	Z5	7	8	9
Residence time(hr)	Z6	18	24	30

IVB.2. Results and Discussions:

IVB.2.1. Fitting of the model and Statistical analysis:

For both the strains, total 86 experiments were done as per the design matrix and the only one response was percent of degradation of 4- Chloro Phenol. Table IVB.3 & IVB.4 displayed both the predicted and experimental/actual values of percent of degradation of 4- Chloro Phenol by *Bacillus timonensis* & *Bacillus cereus* respectively. Second order and linear polynomial equations (equation 4.7 & 4.8 for *Bacillus timonensis* & *Bacillus cereus* respectively) were fitted to the actual data to obtain the regression equation. To reveal the suitable model, sequential model sum of squares and model summary statistics were deducted (for *Bacillus timonensis,* Table IVB.5 and for *Bacillus cereus,* IVB.6 respectively). The Sequential P-value for the quadratic model is less than 0.0001 in both cases; Maximum predicted R^2 and adjusted R^2 values were 0.9731 and 0.9505 respectively for the percent of degradation of the 4- Chloro Phenol in case of *Bacillus timonensis*. In case of & *Bacillus cereus,* those values were 0.9597 and 0.9443 respectively for the percent of degradation of

the 4- Chloro Phenol. Cubic model found to be aliased for both; here the sequential P- values were> 0.05 for both. So, finally the quadratic model was chosen for both strains for further determination of the percent of degradation of 4- Chloro Phenol. The following equations clearly displayed that:

Percentage of 4- Chloro Phenol degradation by *Bacillus timonensis* (Y): 96.54– 0.3703X1– 0.0082X2+ 0.1108X3– 1.17X4– 0.4632X5+ 0.6692X6+ 1.02X1X2+ 0.3816X1X3– 0.0766X1X4– 0.3750X1X5+ 0.5522X1X6+ 0.3131X2X3+ 0.8381X2X4– 0.9816X2X5– 0.1950X2X6+ 0.4363X3X4– 0.0072X3X5+ 0.4906X3X6+ 0.2934X4X5+ 1.26X4X6+1.91X5X6– $4.77X1^2$ – $14.72X2^2$ – $3.38X3^2$ + $2.67X4^2$ + $0.2099X5^2$ +$4.20X6^2$

(4.7)

Negative coefficients for the model components X1, X2, X4, X5, X1X4, X1X5, X2X5, X2X6, X3X5, $X1^2$, $X2^2$ and $X3^2$ reveal negative impacts on the percent of degradation of 4- Chloro Phenol while positive coefficients X3, X6, X1X2, X1X3, X1X6, X2X3, X2X4, X3X4, X3X6, X4X5, X4X6, X5X6, $X4^2$, $X5^2$ and $X6^2$ stands for positive impacts on the percent of degradation of 4- Chloro Phenol (Equation no 4.7). Moreover, the coefficients, whose values are near 0, reveal lower relative intensity. X2, X1X4 and X3X5 were categorised in this class which could not affect percent of degradation of the 4- Chloro Phenol so much.

percent of 4- Chloro Phenol degradation by *Bacillus cereus* (Y): 98.28- 0.0565Z1- 0.7950Z2- 0.0233Z3- 0.0227Z4- 0.0279Z5- 0.7759Z6+ 0.2178Z1Z2+ 0.2322Z1Z3- 0.1397Z1Z4- 0.2381Z1Z5+ 0.2503Z1Z6+ 0.2000Z2Z3+ 0.0756Z2Z4- 0.4366Z2Z5- 0.5106Z2Z6- 0.5688Z3Z4- 0.5247Z3Z5+ 0.5862Z3Z6- 0.3516Z4Z5+ 0.1631Z4Z6- 1.32Z5Z6- $0.3181Z1^2$ + $1.60Z2^2$ – $5.50Z3^2$ + $1.21Z4^2$ + $1.89Z5^2$ -$3.66Z6^2$ (4.8)

Negative coefficients for the model components Z1, Z2, Z3, Z4, Z5, Z6, Z1Z4, Z1Z5, Z2Z5, Z2Z6, Z3Z4, Z3Z5, Z4Z5, Z5Z6, $Z1^2$, $Z3^2$ and $Z6^2$ reveal negative impacts on the percent of degradation of 4- Chloro Phenol while positive coefficients Z1Z2, Z1Z3, Z1Z6, Z2Z3, Z2Z4, Z3Z6, Z4Z6, $Z2^2$, $Z4^2$ and $Z5^2$ stands for positive impacts on the of degradation of 4- Chloro Phenol. Moreover, the coefficients, whose values are near 0, reveal lower relative intensity. Z1, Z3, Z4, Z5 and Z2Z4 were categorised in this class which could not affect percent of degradation of the 4- Chloro Phenol so much.

Table IVB.3: Experimental design matrix for the degradation of 4- Chloro Phenol by *Bacillus timonensis* strain 10403023

Run order	Space type	X1:Initial Concentration of 4-Chloro Phenol (mg/L)	X2: pH	X3: Temperature (°C)	X4: Medium Volume(mL)	X5:Inoculums (percent)	X6: Residence Time(hr)	Y: percent of 4-Chlorophenol degradation		
								Experimental value	Predicted value	Error
1	Factorial	1000	8	35	600	8	18	88.73	84.46	4.27
2	Factorial	1000	7	45	600	8	30	94.11	93.34	0.7700
3	Factorial	1000	8	45	400	4	18	93.99	95.89	-1.90
4	Factorial	600	7	35	600	4	18	92.36	89.94	2.42
5	Factorial	1000	7	35	400	8	18	92.6	89.94	2.66
6	Axial	1000	7.5	40	500	6	24	88.32	91.40	-3.08
7	Factorial	600	8	35	600	4	30	85.58	88.84	-3.26
8	Factorial	600	8	35	400	4	18	94.95	95.78	-0.8303
9	Factorial	600	8	45	600	8	18	87.32	85.89	1.43
10	Factorial	1000	8	35	400	4	18	92.97	96.12	-3.15
11	Axial	800	8	40	500	6	24	88.32	91.75	-3.43
12	Centre	800	7.5	40	500	6	24	99.99	96.54	3.44
13	Axial	800	7.5	40	500	4	24	94.32	97.21	-2.89
14	Factorial	1000	8	35	600	8	30	93.17	91.86	1.31
15	Factorial	600	8	45	600	4	30	91.16	90.80	0.3631
16	Factorial	1000	7	45	600	8	18	82.25	83.19	-0.9428
17	Factorial	600	7	35	400	8	18	95.83	95.18	0.6472
18	Centre	800	7.5	40	500	6	24	99.99	96.54	3.40
19	Factorial	1000	7	35	400	8	30	95.99	93.09	2.90
20	Factorial	600	7	45	400	8	18	89.83	92.15	-2.32
21	Factorial	600	7	45	600	8	18	85.52	87.22	-1.70
22	Factorial	1000	8	45	400	8	30	90.41	92.17	-1.76
23	Axial	800	7.5	40	500	6	30	98.32	101.41	-3.09
24	Factorial	1000	7	45	600	4	18	87.98	86.15	1.83
25	Axial	800	7.5	35	500	6	24	89.24	93.05	-3.81
26	Factorial	1000	7	45	400	4	18	92.96	92.56	0.3999
27	Factorial	600	8	35	400	4	30	93.21	88.31	4.90
28	Factorial	600	8	35	600	8	18	83.16	85.93	-2.77
29	Centre	800	7.5	40	500	6	24	99.99	96.54	3.41
30	Factorial	600	7	45	400	4	18	96.97	94.78	2.19
31	Axial	600	7.5	40	500	6	24	88.36	92.14	-3.78
32	Factorial	1000	7	35	400	4	18	95.97	94.04	1.93
33	Factorial	600	7	35	400	8	30	97.97	96.13	1.84
34	Factorial	1000	8	45	600	4	18	90.35	92.83	-2.48
35	Centre	800	7.5	40	500	6	24	99.99	96.54	3.45
36	Factorial	1000	7	45	600	4	30	88.27	88.66	-0.3914
37	Factorial	600	7	45	400	8	30	95.66	95.06	0.6022
38	Factorial	600	8	35	400	8	30	89.03	89.42	-0.3874
39	Factorial	600	7	45	400	4	30	87.66	90.05	-2.39

40	Factorial	600	8	45	600	4	18	88.24	91.28	-3.04
41	Factorial	1000	7	35	600	4	30	86.97	86.43	0.5351
42	Factorial	600	8	45	400	4	30	89.71	88.52	1.19
43	Factorial	600	7	45	600	4	30	86.32	88.98	-2.66
44	Factorial	1000	7	45	400	4	30	92.07	90.04	2.03
45	Factorial	1000	8	45	400	8	18	90.51	87.83	2.68
46	Axial	800	7.5	40	400	6	24	96.32	100.39	-4.07
47	Factorial	1000	7	35	600	8	18	78.21	82.96	-4.75
48	Factorial	600	8	35	600	8	30	90.41	91.13	-0.7152
49	Factorial	1000	8	35	400	8	18	88.32	88.09	0.2341
50	Factorial	1000	7	35	600	4	18	82.37	85.89	-3.52
51	Factorial	1000	8	35	400	8	30	85.32	90.46	-5.14
52	Axial	800	7.5	40	600	6	24	95.24	98.04	-2.80
53	Factorial	1000	8	45	600	8	18	88.64	85.95	2.69
54	Factorial	600	8	35	400	8	18	89.51	89.25	0.2610
55	Axial	800	7.5	40	500	8	24	92.31	96.29	-3.98
56	Factorial	600	7	45	600	4	18	92.34	88.68	3.66
57	Factorial	600	7	35	600	8	30	94.83	94.49	0.3434
58	Factorial	1000	8	45	400	4	30	94.66	92.59	2.07
59	Centre	800	7.5	40	500	6	24	99.99	96.54	3.45
60	Factorial	1000	8	45	600	8	30	92.29	95.31	-3.02
61	Factorial	600	7	35	600	8	18	88.03	88.51	-0.4806
62	Centre	800	7.5	40	500	6	24	99.99	96.54	3.43
63	Factorial	600	8	45	400	4	18	92.99	94.03	-1.04
64	Centre	800	7.5	40	500	6	24	99.99	96.54	3.43
65	Factorial	600	8	45	400	8	30	91.16	89.60	1.56
66	Factorial	600	8	35	600	4	18	94.16	91.29	2.87
67	Factorial	600	7	35	400	4	18	97.98	97.79	0.1921
68	Factorial	600	7	35	600	4	30	90.11	88.28	1.83
69	Factorial	1000	7	35	400	4	30	87.74	89.56	-1.82
70	Factorial	600	7	45	600	8	30	97.66	95.16	2.50
71	Factorial	1000	8	35	600	4	18	93.4	91.32	2.08
72	Factorial	1000	7	45	400	8	30	91.83	93.55	-1.72
73	Axial	800	7.5	40	500	6	18	96.29	100.07	-3.78
74	Axial	800	7	40	500	6	24	88.32	91.76	-3.44
75	Factorial	1000	8	35	600	4	30	90.55	91.08	-0.5335
76	Centre	800	7.5	40	500	6	24	99.99	96.54	3.44
77	Factorial	1000	8	45	600	4	30	95.99	94.56	1.43
78	Factorial	600	8	45	400	8	18	88.03	87.47	0.5645
79	Factorial	1000	8	35	400	4	30	93.62	90.86	2.76
80	Factorial	600	7	35	400	4	30	86.32	91.10	-4.78
81	Factorial	600	8	45	600	8	30	93.83	93.05	0.7807
82	Axial	800	7.5	45	500	6	24	90.21	93.27	-3.06
83	Centre	800	7.5	40	500	6	24	99.99	96.54	3.44
84	Factorial	1000	7	35	600	8	30	93.1	91.14	1.96
85	Factorial	1000	7	45	400	8	18	88.1	88.43	-0.3262
86	Centre	800	7.5	40	500	6	24	99.99	96.54	3.45

Table IVB.4: Experimental design matrix for the degradation of 4- Chloro Phenol by *Bacillus cereus* strain K1

Run order	Space type	Z1:Initial Concentration of 4- Chloro Phenol (mg/L)	Z2: pH	Z3: Temperature (°C)	Z4: Medium Volume (mL)	Z5: Inoculums (percent)	Z6: Residence Time(hr)	Y: percent of 4-Chlorophenol degradation		
								Experimental value	Predicted value	Error
1	Centre	600	6.5	25	400	8	24	99.98	98.28	1.70
2	Axial	600	6.5	25	400	9	24	98.38	100.14	-1.76
3	Factorial	800	7	20	300	7	30	90.37	91.33	-0.9627
4	Factorial	800	7	20	500	7	18	96.32	93.60	2.72
5	Factorial	800	6	20	300	9	18	94.75	96.81	-2.06
6	Factorial	400	6	20	500	7	30	94.35	95.19	-0.8427
7	Axial	600	6.5	25	500	8	24	99.24	99.46	-0.2207
8	Factorial	400	7	30	300	9	30	88.25	90.02	-1.77
9	Factorial	800	6	30	300	9	30	92.31	93.91	-1.60
10	Axial	600	6.5	30	400	8	24	91.31	92.75	-1.44
11	Factorial	400	7	20	300	9	30	90.27	88.87	1.40
12	Factorial	800	7	20	500	7	30	96.32	93.33	2.99
13	Factorial	800	6	20	300	7	30	97.13	93.19	3.94
14	Factorial	800	7	20	300	9	30	86.13	89.03	-2.90
15	Factorial	400	7	20	300	9	18	93.52	96.09	-2.57
16	Factorial	400	7	20	500	7	18	96.35	94.04	2.31
17	Axial	600	6.5	20	400	8	24	90.83	92.80	-1.97
18	Factorial	800	6	30	300	7	18	90.21	93.10	-2.89
19	Factorial	800	7	30	300	7	18	92.75	94.09	-1.34
20	Centre	600	6.5	25	400	8	24	99.98	98.28	1.70
21	Axial	600	6	25	400	8	24	98.52	100.67	-2.15
22	Factorial	800	6	20	500	9	30	90.05	92.92	-2.87
23	Factorial	400	7	20	500	7	30	88.32	92.77	-4.45
24	Factorial	800	7	30	500	9	30	89.2	89.42	-0.2235
25	Factorial	800	6	20	300	9	30	90.24	92.63	-2.39
26	Factorial	400	6	20	500	7	18	90.21	94.42	-4.21
27	Axial	600	6.5	25	400	8	18	93.27	95.39	-2.12
28	Centre	600	6.5	25	400	8	24	99.98	98.28	1.70
29	Factorial	400	7	30	500	9	30	88.26	88.89	-0.6316
30	Centre	600	6.5	25	400	8	24	99.98	98.28	1.70
31	Factorial	400	7	20	300	7	18	89.35	92.14	-2.79
32	Factorial	800	6	20	300	7	18	96.35	92.07	4.28
33	Factorial	800	6	30	300	7	30	95.13	96.56	-1.43
34	Factorial	800	6	20	500	7	30	91.13	94.88	-3.75
35	Centre	600	6.5	25	400	8	24	99.98	98.28	1.70
36	Factorial	400	6	20	500	9	30	93.25	94.19	-0.9357
37	Axial	600	6.5	25	400	7	24	98.54	100.19	-1.65
38	Factorial	800	7	30	500	7	30	92.38	95.23	-2.85
39	Centre	600	6.5	25	400	8	24	99.98	98.28	1.70

40	Factorial	800	7	30	500	7	18	92.35	93.15	-0.8049
41	Factorial	400	6	30	500	9	18	91.8	94.44	-2.64
42	Factorial	800	7	20	500	9	30	91.13	89.62	1.51
43	Centre	600	6.5	25	400	8	24	99.98	98.28	1.70
44	Factorial	400	7	30	300	7	30	98.35	93.47	4.88
45	Factorial	400	6	20	300	7	18	95.51	92.83	2.68
46	Factorial	400	6	30	500	7	30	98.35	95.37	2.98
47	Factorial	400	6	30	300	9	30	92.35	93.69	-1.34
48	Factorial	400	7	20	500	9	18	98.35	96.58	1.77
49	Factorial	800	7	20	300	9	18	97.35	95.25	2.10
50	Factorial	400	7	30	500	9	18	87.51	93.11	-5.60
51	Factorial	400	6	20	300	9	18	96.35	98.52	-2.17
52	Factorial	400	7	20	500	9	30	94.75	90.02	4.73
53	Factorial	800	6	30	500	7	30	98.68	95.98	2.70
54	Factorial	800	7	30	300	9	30	91.73	91.11	0.6185
55	Axial	600	7	25	400	8	24	97.83	99.08	-1.25
56	Factorial	800	7	20	300	7	18	90.13	92.26	-2.13
57	Factorial	400	6	30	300	9	18	99.98	96.52	3.46
58	Factorial	400	6	30	300	7	30	90.11	95.39	-5.28
59	Factorial	800	6	30	500	9	30	96.35	91.92	4.43
60	Factorial	800	7	30	500	9	18	90.25	92.64	-2.39
61	Axial	600	6.5	25	400	8	30	92.56	93.84	-1.28
62	Axial	400	6.5	25	400	8	24	95.32	98.01	-2.69
63	Factorial	800	7	20	500	9	18	93.69	95.19	-1.50
64	Centre	600	6.5	25	400	8	24	99.98	98.28	1.70
65	Factorial	400	7	30	300	9	18	98.66	94.90	3.76
66	Factorial	400	6	20	300	9	30	95.5	93.34	2.16
67	Factorial	800	6	30	500	9	18	92.36	93.10	-0.7391
68	Factorial	400	6	30	300	7	18	90.21	92.93	-2.72
69	Factorial	400	7	20	300	7	30	88.75	90.22	-1.47
70	Factorial	400	6	30	500	7	18	96.32	92.25	4.07
71	Centre	600	6.5	25	400	8	24	99.98	98.28	1.70
72	Factorial	800	7	30	300	9	18	97.34	94.98	2.36
73	Factorial	400	6	20	500	9	18	99.98	98.71	1.27
74	Axial	800	6.5	25	400	8	24	97.19	97.90	-0.7119
75	Factorial	800	6	20	500	7	18	91.13	93.11	-1.98
76	Factorial	400	6	30	500	9	30	92.75	92.26	0.4897
77	Factorial	400	7	30	300	7	18	92.3	93.05	-0.7471
78	Factorial	800	6	30	500	7	18	90.75	91.86	-1.11
79	Factorial	400	7	30	500	7	30	90.35	93.74	-3.39
80	Centre	600	6.5	25	400	8	24	99.98	98.28	1.70
81	Factorial	800	7	30	300	7	30	98.44	95.51	2.93
82	Axial	600	6.5	25	300	8	24	96.32	99.51	-3.19
83	Factorial	800	6	30	300	9	18	98.32	95.74	2.58
84	Factorial	400	6	20	300	7	30	97.55	92.94	4.61
85	Factorial	400	7	30	500	7	18	98.37	92.67	5.70
86	Factorial	800	6	20	500	9	18	99.91	96.44	3.47

Table IVB.5: Adequacy of the models tested for 4- Chloro Phenol degradation (for *Bacillus timonensis* strain 10403023)

Sequential Model Sum of Squares						
Source	**Sum of Squares**	**df**	**Mean Square**	**F-value**	**p-value**	**Remarks**
Mean vs. Total	7.287E+05	1	7.287E+05			
Linear vs. Mean	144.68	6	24.11	1.05	0.3968	
2FI vs. Linear	587.85	15	39.19	2.06	0.0240	
Quadratic vs. 2FI	**619.88**	**6**	**103.31**	**10.02**	**< 0.0001**	**Suggested**
Cubic vs. Quadratic	223.05	26	8.58	0.7317	0.7912	Aliased
Residual	375.21	32	11.73			
Total	7.307E+05	86	8496.51			

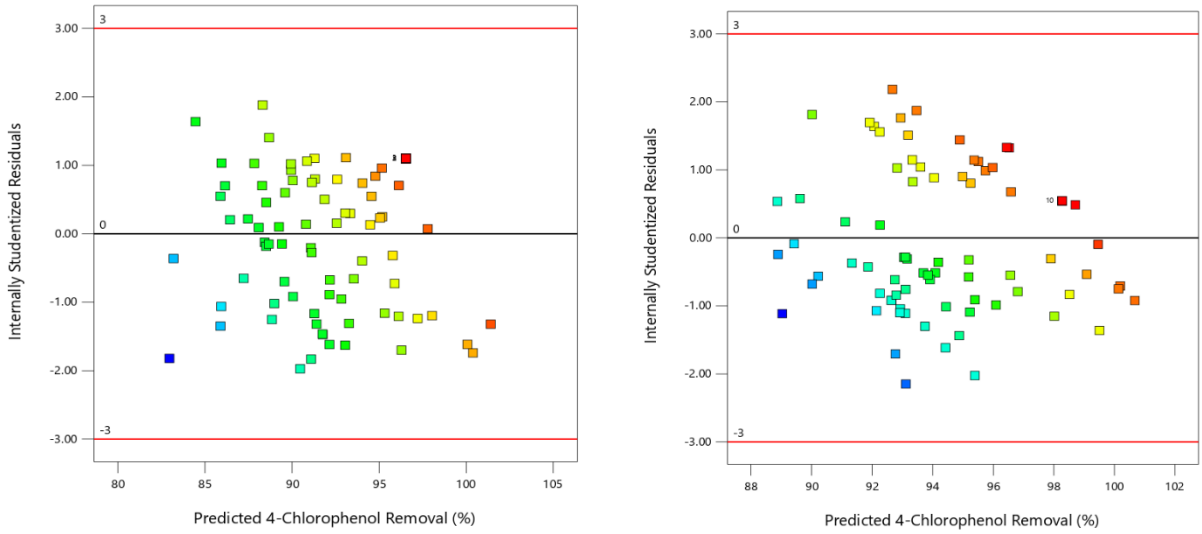
Model Summary Statistics						
Source	**Std. Dev.**	**R^2**	**Adjusted R^2**	**Predicted R^2**	**PRESS**	**Remarks**
Linear	4.78	0.0742	0.0039	-0.0647	2076.83	
2FI	4.36	0.3755	0.1706	0.0785	1797.50	
Quadratic	**3.21**	**0.9633**	**0.9505**	**0.9731**	**1418.00**	**Suggested**
Cubic	3.42	0.8077	0.4891	-34.8895	70008.61	Aliased

Table IVB.6: Adequacy of the models tested for 4- Chloro Phenol degradation (for *Bacillus cereus* strain K1)

Sequential Model Sum of Squares						
Source	**Sum of Squares**	**df**	**Mean Square**	**F-value**	**p-value**	**Remarks**
Mean vs. Total	7.697E+05	1	7.697E+05			
Linear vs. Mean	81.78	6	13.63	0.8712	0.5200	
2FI vs. Linear	229.21	15	15.28	0.9714	0.4946	
Quadratic vs. 2FI	**408.15**	**6**	**68.02**	**6.59**	**< 0.0001**	**Suggested**
Cubic vs. Quadratic	323.06	26	12.43	1.44	0.1611	Aliased
Residual	275.55	32	8.61			
Total	7.710E+05	86	8965.52			

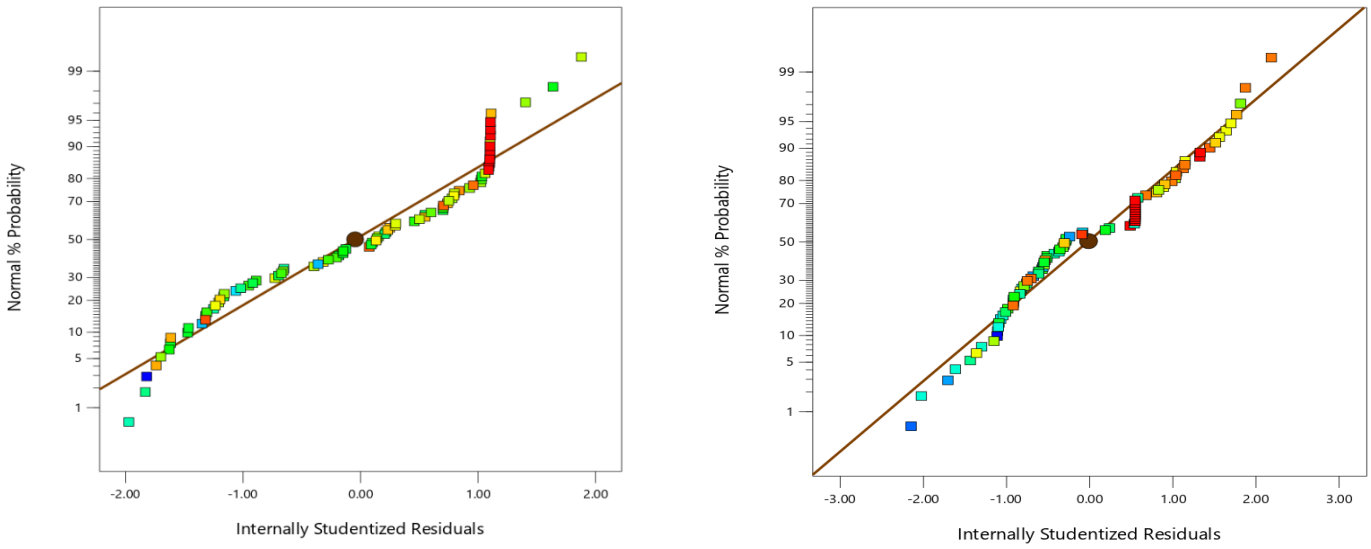
Model Summary Statistics						
Source	**Std. Dev.**	**R^2**	**Adjusted R^2**	**Predicted R^2**	**PRESS**	**Remarks**
Linear	3.96	0.0621	-0.0092	-0.1000	1449.53	
2FI	3.97	0.2360	-0.0147	-0.3536	1783.73	
Quadratic	**3.21**	**0.5457**	**0.9443**	**0.9597**	**1396.38**	**Suggested**
Cubic	2.93	0.7909	0.4446	13.2462	18773.04	Aliased

In case of both strains, the constant variance assumptions were investigated by plotting internally studentized residual vs. predicted values of percent degradation of 4- Chloro Phenol (Figure IVB.1 (a) & IVB.1 (b)). Studentized residuals were deducted by dividing the residuals by their standard deviations displaying a randomly scattered pattern within the detection limits -3 to +3 and so, prediction of model described in the equation no (4.7 & 4.8 respectively) for the percent degradation of 4- Chloro Phenol is satisfactory. The normal probability plot of residuals (Figure IVB.2 (a) & IVB.2 (b)) for the percentage degradation of 4- Chloro Phenol showed a straight line pattern for the both strains, rather than S shaped followed by the points on the plot. As the residuals are distributed normally, transformation of response is not required. Relation between the predicted and experimental values of responses have been displayed in Figure IVB.3 (a) & IVB.3 (b) for *Bacillus timonensis* strain 10403023& *Bacillus cereus* strain K1 respectively. In both the figure, very little discrepancies were found by the straight trend line pointing a good relationship in between the predicted and experimental values.



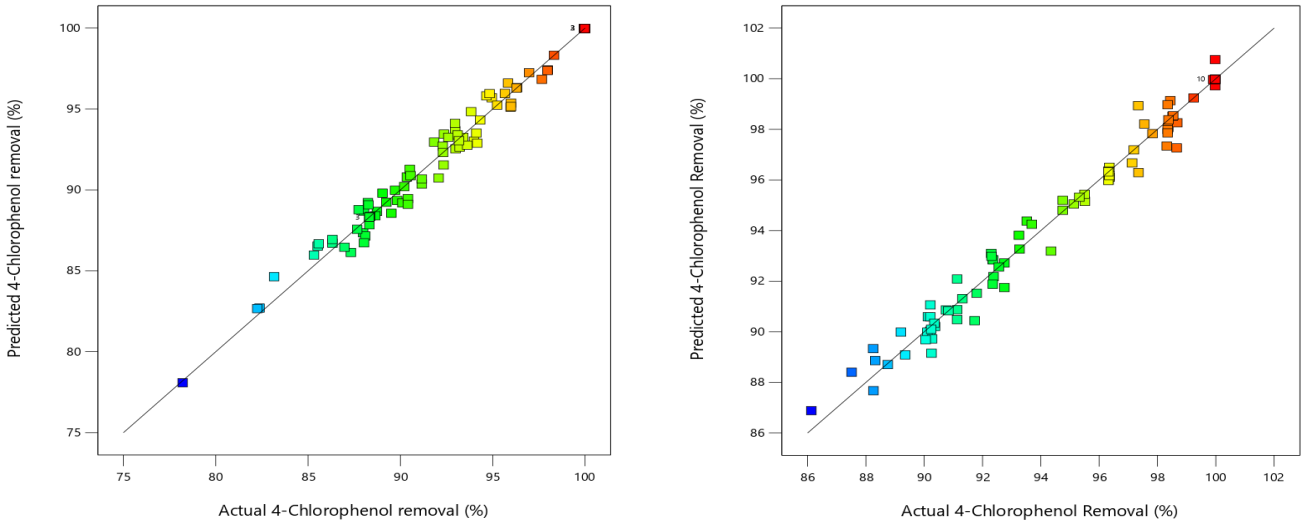
(a) (b)

Figure IVB.1: Internally studentized residuals vs. predicted values (a) *Bacillus timonensis* (b) *Bacillus cereus*



(a) (b)

Figure IVB.2: Internally studentized residuals vs. Normal percent probability (a) *Bacillus timonensis* (b) *Bacillus cereus*



(a) (b)

Figure IVB.3: Actual experimental degradation data vs. predicted data (a) *Bacillus timonensis* (b) *Bacillus cereus*

IVB.2.2 ANOVA Test:

Outputs of ANOVA for percent of degradation of 4- Chloro Phenol by the two strains have been given in Table IVB.7 & IVB.8 respectively. In the table IVB.7 (ANOVA for percent degradation of 4- Chloro Phenol by *Bacillus timonensis*), the Model F-value of 4.86 implies the model is significant. There is only a 0.01% chance that an F-value this large could occur due to noise. P-values less than 0.0500 indicate model terms are significant. In this case X1, X2, X3, X4, X5, X6, X1X2, X1X3, X1X4, X1X5, X1X6, X2X3, X2X4, X2X5, X2X6, X3X4, X3X5, X3X6 , X5X6, $X1^2$, $X2^2$, $X3^2$,and $X6^2$ are significant model terms. Values greater than 0.1000 indicate the model terms are not significant. So, X4X5 and X4X6 are not significant model terms. If there are many insignificant model terms (not counting those required to support hierarchy), model reduction may improve the model.

Table IVB.7: ANOVA of the second order polynomial equation for the degradation of 4- Chloro Phenol by *Bacillus timonensis*

Source	Sum of Squares	df	Mean Square	F-value	p-value	Remarks
Model	1352.41	27	50.09	4.86	< 0.0001	significant
X1-Initial Concentration	9.05	1	9.05	0.8774	0.0228	significant
X2-pH	0.0044	1	0.0044	0.0004	0.0136	significant
X3-Temperature	0.8096	1	0.8096	0.0785	0.0103	significant
X4-Medium Volume	91.10	1	91.10	8.83	0.0043	significant
X5-Innoculum	14.16	1	14.16	1.37	0.0461	significant
X6-Residance time	29.56	1	29.56	2.87	0.0258	significant
X1X2	66.71	1	66.71	6.47	0.0137	significant
X1X3	9.32	1	9.32	0.9033	0.0258	significant
X1X4	0.3752	1	0.3752	0.0364	0.0294	significant
X1X5	9.00	1	9.00	0.8725	0.0341	significant
X1X6	19.51	1	19.51	1.89	0.0143	significant
X2X3	6.28	1	6.28	0.6083	0.0386	significant
X2X4	44.96	1	44.96	4.36	0.0012	significant
X2X5	61.66	1	61.66	5.98	0.0175	significant
X2X6	2.43	1	2.43	0.2359	0.0290	significant
X3X4	12.18	1	12.18	1.18	0.0287	significant
X3X5	0.0033	1	0.0033	0.0003	0.0486	significant
X3X6	15.41	1	15.41	1.49	0.0227	significant
X4X5	5.51	1	5.51	0.5343	0.6278	Not significant
X4X6	101.10	1	101.10	9.80	0.4227	Not significant
X5X6	233.40	1	233.40	22.63	< 0.0001	significant
$X1^2$	54.23	1	54.23	5.26	0.0255	significant
$X2^2$	54.69	1	54.69	5.30	0.0249	significant
$X3^2$	27.29	1	27.29	2.65	0.0193	significant
$X4^2$	17.09	1	17.09	1.66	0.2032	Not significant
$X5^2$	0.1052	1	0.1052	0.0102	0.9199	Not significant
$X6^2$	42.13	1	42.13	4.08	0.0479	significant
Residual	598.26	58	10.31			
Lack of Fit	598.26	49	12.21	41622.92	< 0.0001	significant
Pure Error	0.0026	9	0.0003			
Cor Total	1950.67	85				

Outputs of ANOVA for percent of degradation of 4- Chloro Phenol (by *Bacillus cereus*) have been given in Table IVB.8. In that table, the Model F-value of 2.58 implies the model is significant. There is only a 0.13% chance that an F-value this large could occur due to noise. Here, Z1, Z2, Z3, Z4, Z5, Z6, Z1Z3, Z1Z4, Z1Z5, Z1Z6, Z2Z3, Z2Z6, Z3Z4, Z3Z5, Z3Z6,

Z4Z6, Z5Z6, $Z1^2$, $Z2^2$, $Z3^2$, $Z6^2$ are significant model terms whereas, Z1Z2, Z2Z4, Z2Z5, Z4Z5, $Z4^2$ and $Z5^2$ are not significant model terms.

Table IVB.8: ANOVA of the second order polynomial equation for the degradation of 4- Chloro Phenol by *Bacillus cereus*

Source	Sum of Squares	df	Mean Square	F-value	p-value	Remarks
Model	719.14	27	26.63	2.58	0.0013	significant
Z1-Initial Concentration of 4- Chloro Phenol	0.2108	1	0.2108	0.0204	0.0069	significant
Z2-pH	41.71	1	41.71	4.04	0.0190	significant
Z3-Temperature	0.0359	1	0.0359	0.0035	0.0032	significant
Z4-Medium Volume	0.0341	1	0.0341	0.0033	0.0244	significant
Z5-Inoculum	0.0513	1	0.0513	0.0050	0.0240	significant
Z6-Residence Time	39.73	1	39.73	3.85	0.0546	significant
Z1Z2	3.04	1	3.04	0.2942	0.5896	Not significant
Z1Z3	3.45	1	3.45	0.3343	0.0054	significant
Z1Z4	1.25	1	1.25	0.1210	0.0092	significant
Z1Z5	3.63	1	3.63	0.3516	0.0155	significant
Z1Z6	4.01	1	4.01	0.3885	0.0255	significant
Z2Z3	2.56	1	2.56	0.2480	0.0203	significant
Z2Z4	0.3660	1	0.3660	0.0355	0.8513	Not significant
Z2Z5	12.20	1	12.20	1.18	0.6815	Not significant
Z2Z6	16.69	1	16.69	1.62	0.0086	significant
Z3Z4	20.70	1	20.70	2.01	0.0120	significant
Z3Z5	17.62	1	17.62	1.71	0.0265	significant
Z3Z6	22.00	1	22.00	2.13	0.0097	significant
Z4Z5	7.91	1	7.91	0.7664	0.8249	Not significant
Z4Z6	1.70	1	1.70	0.1650	0.0061	significant
Z5Z6	112.10	1	112.10	10.86	0.0017	significant
$Z1^2$	0.2417	1	0.2417	0.0234	0.0089	significant
$Z2^2$	6.13	1	6.13	0.5938	0.0041	significant
$Z3^2$	72.33	1	72.33	7.01	0.0004	significant
$Z4^2$	3.48	1	3.48	0.3371	0.5638	Not significant
$Z5^2$	8.50	1	8.50	0.8239	0.6278	Not significant
$Z6^2$	31.96	1	31.96	3.10	0.0037	significant
Residual	598.61	58	10.32			
Lack of Fit	598.61	49	12.22			
Pure Error	0.0000	9	0.0000			
Cor Total	1317.76	85				

IVB.2.3 Effect of initial concentration of 4- Chloro Phenol:

In case of *Bacillus timonensis*, initial conc. of 4- Chloro Phenol (X1) is an important factor as beyond a certain conc. an inhibitory effect arise. To understand the effect of initial conc. of 4- Chloro Phenol, 3D and contour plots were showed in Figure IVB.4. It was observed that at the initial conc. of 800 mg/L 4- Chloro Phenol, almost 100percent degradation was obtained. Beyond this concentration, the degradation was decreased. From the 3D and contour plots, it can be showed that the interaction effect between initial conc. of 4- Chloro Phenol and pH (X1X2) initial conc. vs. temperature (X1X3), initial conc. and media volume (X1X4), initial conc. vs. inoculums size (X1X5) and initial conc. vs. residence time (X1X6) was found to exhibit significant effects on percent of degradation of 4- Chloro Phenol (Figure: IVB.4). But out all these interaction effects, initial conc. of 4- Chloro Phenol vs. temperature (X1X3) and initial conc. of 4- Chloro Phenol vs. residence time (X1X6) were found to be most effective as the plot is almost umbrella shaped (Figure: IVB.4 (b) & (e)). P- values of these two model terms (0.0258 & 0.143 respectively) also supported the same (Table IVB.7).

Also in case of *Bacillus cereus*, initial conc. of 4- Chloro Phenol (Z1) played an important role. The p- value of the Z1 was found to be 0.0013 (Table IVB.8). To understand the effect of initial conc. of 4- Chloro Phenol, 3D and contour plots were showed in Figure IVB.5. It was found when the in initial conc. of 4- Chloro Phenol was increased up to 600 mg/L; almost 100% degradation was obtained. Beyond this concentration, the degradation was decreased. From the 3D and contour plots, it can be showed that the interaction effect between initial conc. of 4- Chloro Phenol and pH (Z1Z2) is not significant as the plot is concave shaped (Figure: IVB.5 (a)). On the other hand, the interaction effects in between initial conc. vs. temperature (Z1Z3), initial conc. and media volume (Z1Z4), initial conc. vs. inoculums size (Z1Z5) and initial conc. vs. residence time (Z1Z6) was found to exhibit significant effects on percent of degradation of 4- Chloro Phenol (Figure: IVB.5 (b), (c), (d) & (e)). But out all these interaction effects, initial conc. of 4- Chloro Phenol vs. temperature was found to be most effective as the plot is so much convex shaped (Figure: IVB.5 (b)).

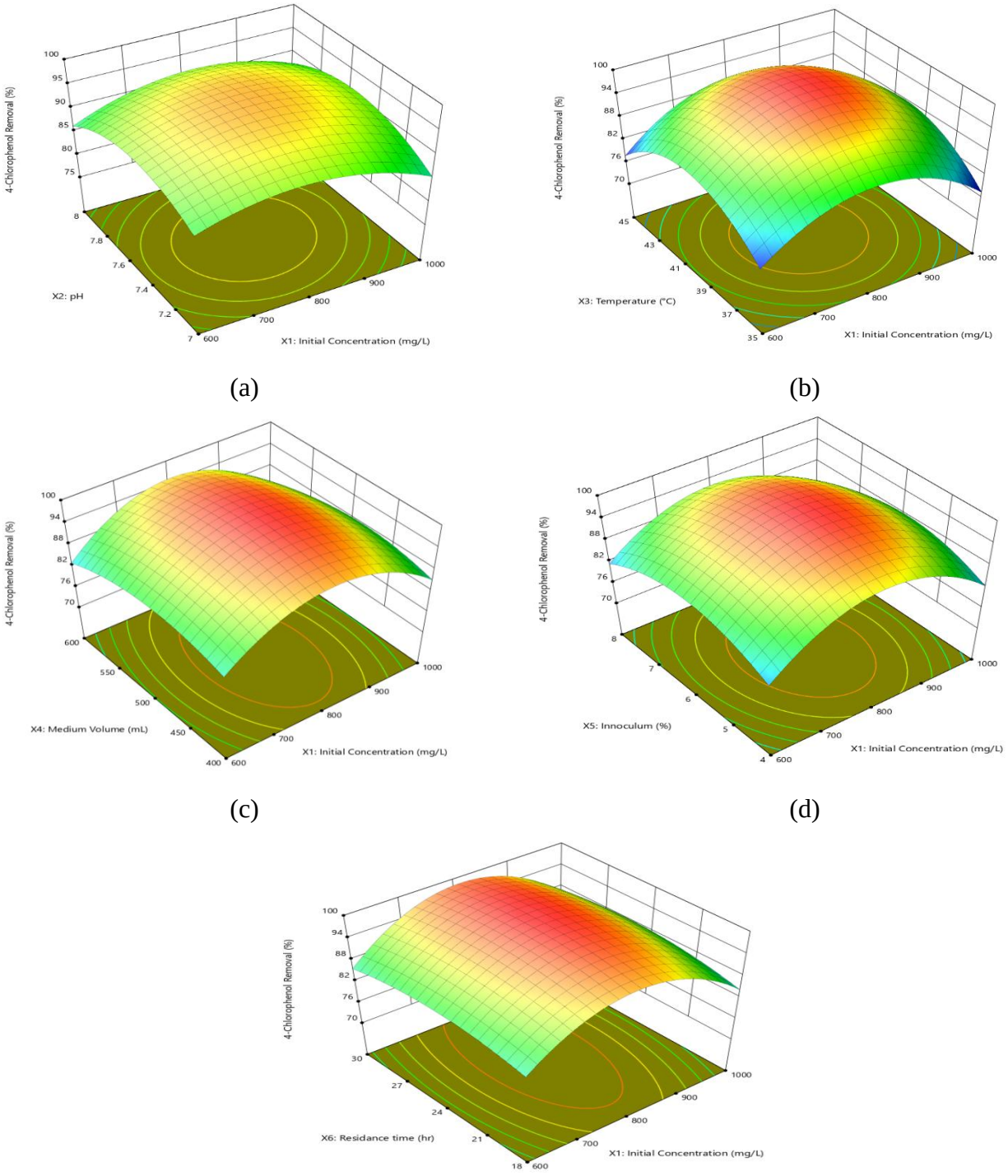


Figure IVB.4: **3- Dimensional surfaces and 2-Dimensional plots of the interaction effects of: (a) initial conc. of 4- Chloro Phenol and pH of media (X1X2), (b) initial conc. of 4- Chloro Phenol and temperature (X1X3), (c) initial conc. of 4- Chloro Phenol and media vol. (X1X4), (d) initial conc. of 4- Chloro Phenol and inoculums percent (X1X5), (e) initial conc. of 4- Chloro Phenol and residence time (X1X6) on the percent of degradation of 4- Chloro Phenol in case of *Bacillus timonensis***

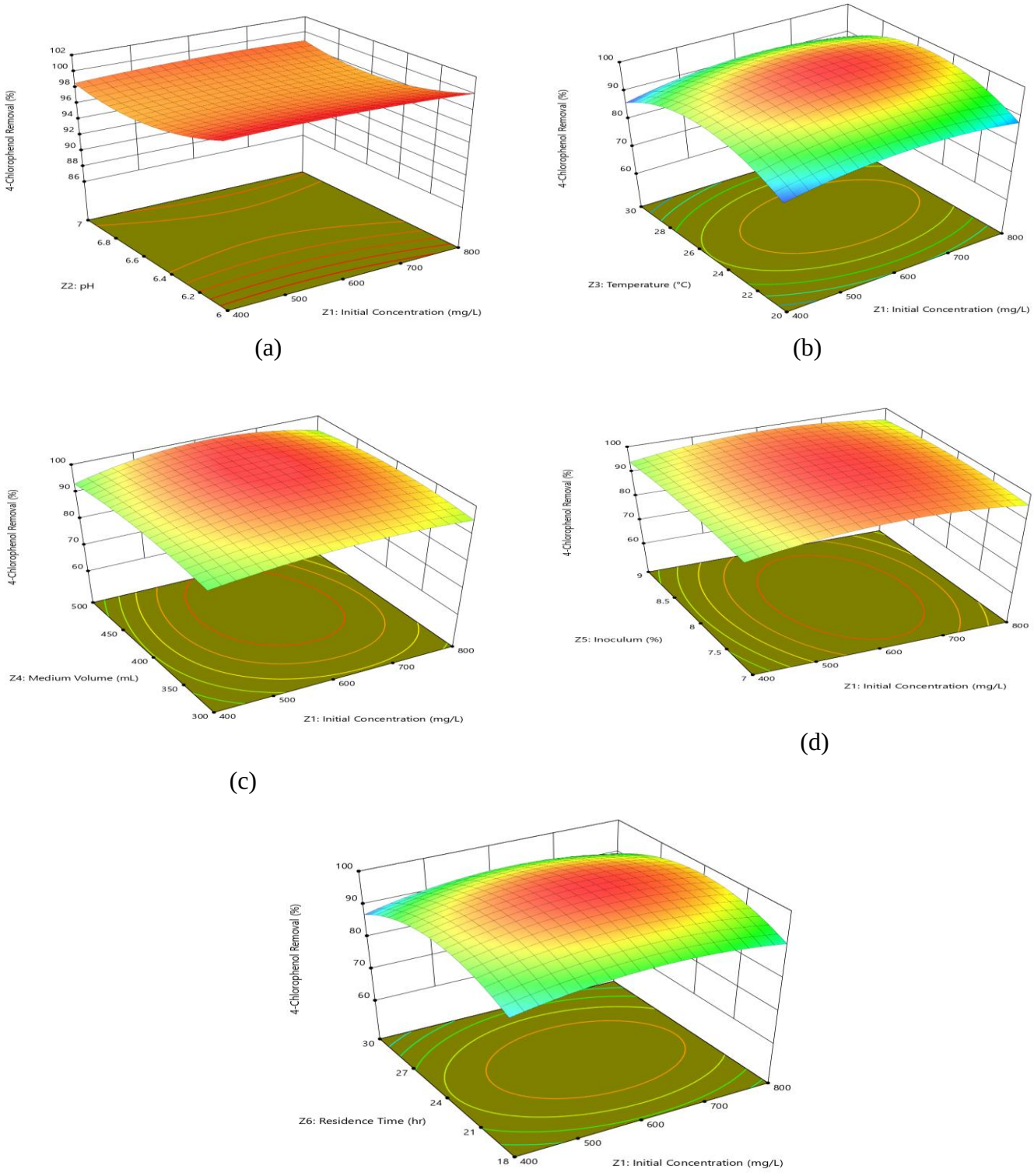


Figure IVB.5: **3- Dimensional surfaces and 2-Dimensional plots of the interaction effects of: (a) initial conc. of 4- Chloro Phenol and pH of media (Z1Z2), (b) initial conc. of 4- Chloro Phenol and temperature (Z1Z3), (c) initial conc. of 4- Chloro Phenol and media vol. (Z1Z4), (d) initial conc. of 4- Chloro Phenol and inoculums percent (Z1Z5), (e) initial conc. of 4- Chloro Phenol and residence time (Z1Z6) on the percent of degradation of 4- Chloro Phenol in case *of Bacillus cereus***

IVB.2.4 Effect of pH of the media:

In case of *Bacillus timonensis*, effect of pH (X2) i.e. concentration of H^+ revealed a significant role in percent degradation of 4- Chloro Phenol. Percent of degradation of 4- Chloro Phenol was maximum (88 – 99%) when the pH value of the culture media was not more than 7.5. Percent degradation was decreased in both situations while the pH value of the media increased and decreased indicating that maximum degradation was occurred at slightly basic condition. More basic as well as neutral condition is not favourable for the strain to remove 4- Chloro Phenol. From the 3D and contour plots, it can be showed that the interaction effects between pH of the media vs. temperature (X2X3), pH of the media vs. media volume (X2X4), pH of the media vs. inoculums percent (X2X5) & pH of the media vs. residence time (X2X6) all had significant effects on percent degradation of 4- Chloro Phenol as the plots are convex shaped (Figure: IVB.6 (a), (b), (c) & (d)). Moreover, table no IVB.7 clearly displayed that p- values of these model terms are < 0.05 indicating their significance on the response.

On the other hand, in case of *Bacillus cereus*, Effect of pH (Z2) revealed a significant role on percent degradation of 4- Chloro Phenol. The p- value of this model term (Z2) was found to be 0.0190 (Table IVB.8) which clearly indicates that it is very much significant. Percent of degradation of 4- Chloro Phenol was maximum (above 90percent in most cases) when the pH value of the culture media was not more than 6.5. Percent degradation was decreased in both situations while the pH value of the media increased and decreased indicating that maximum degradation was occurred at slightly acidic condition. More acidic as well as neutral condition is not favourable for the strain to remove 4- Chloro Phenol. From the 3D and contour plots, it can be showed that the interaction effects between pH of the media vs. temperature (Z2Z3) and pH of the media vs. residence time (Z2Z6) had significant effects on percent degradation of 4- Chloro Phenol as the plots are convex shaped (Figure: IVB.7 (a) & (d)). But in case of interaction effects between pH of the media vs. media volume (Z2Z4) and pH of the media vs. inoculums percent (Z2Z5) did not show much effect on the degradation percent of 4- Chloro Phenol. Plots are almost flat there (Figure: IVB.7 (b) & (c))

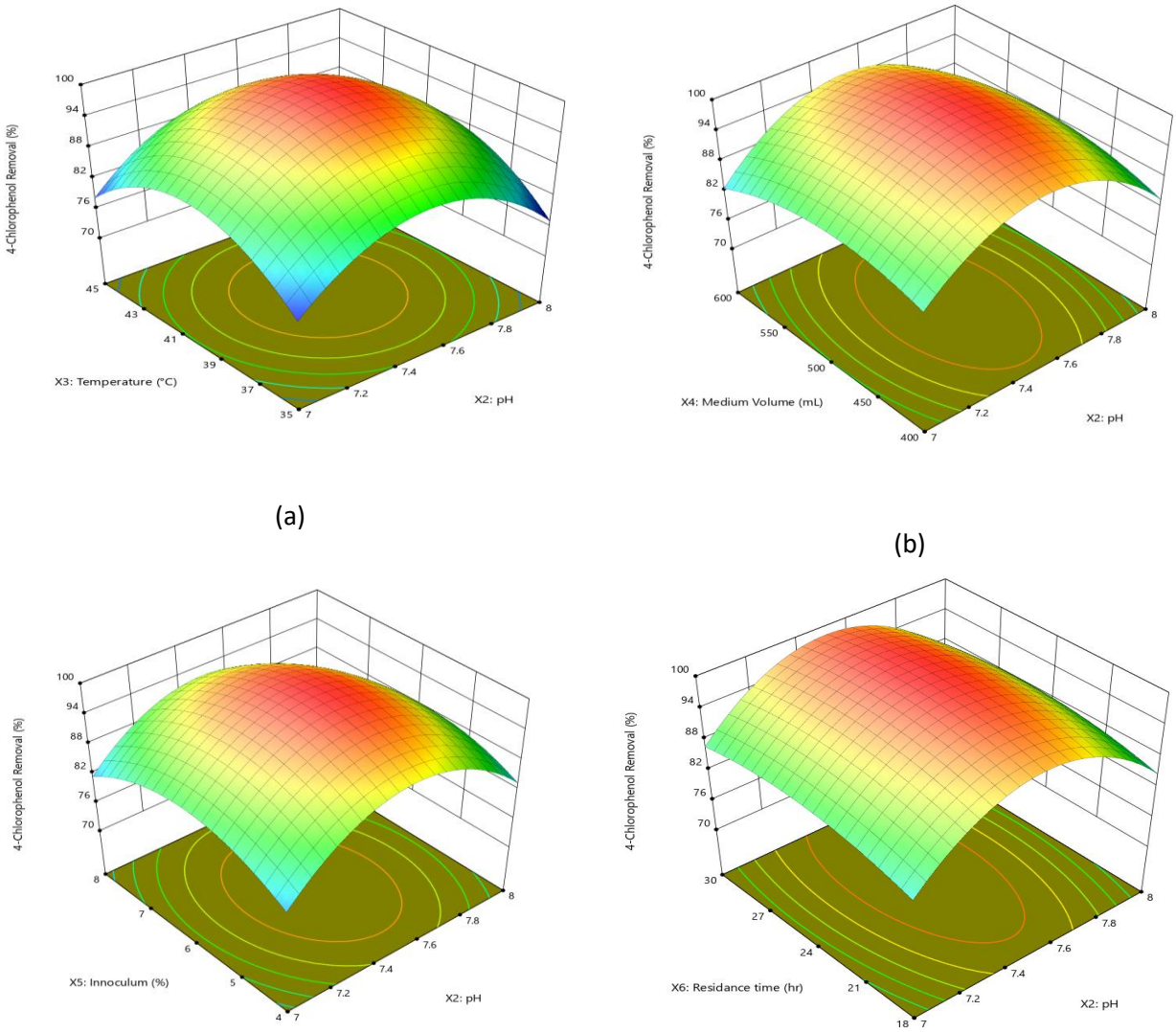


Figure IVB.6: **3- Dimensional surfaces and 2- dimensional plots of the interaction effects of: (a) pH of the media & temperature (X2X3), (b) pH of the media and media vol. (X2X4), (c) pH of the media and inoculums size (X2X5), (d) pH of the media and residence time on the percent of degradation of 4- Chloro Phenol in case of *Bacillus timonensis***

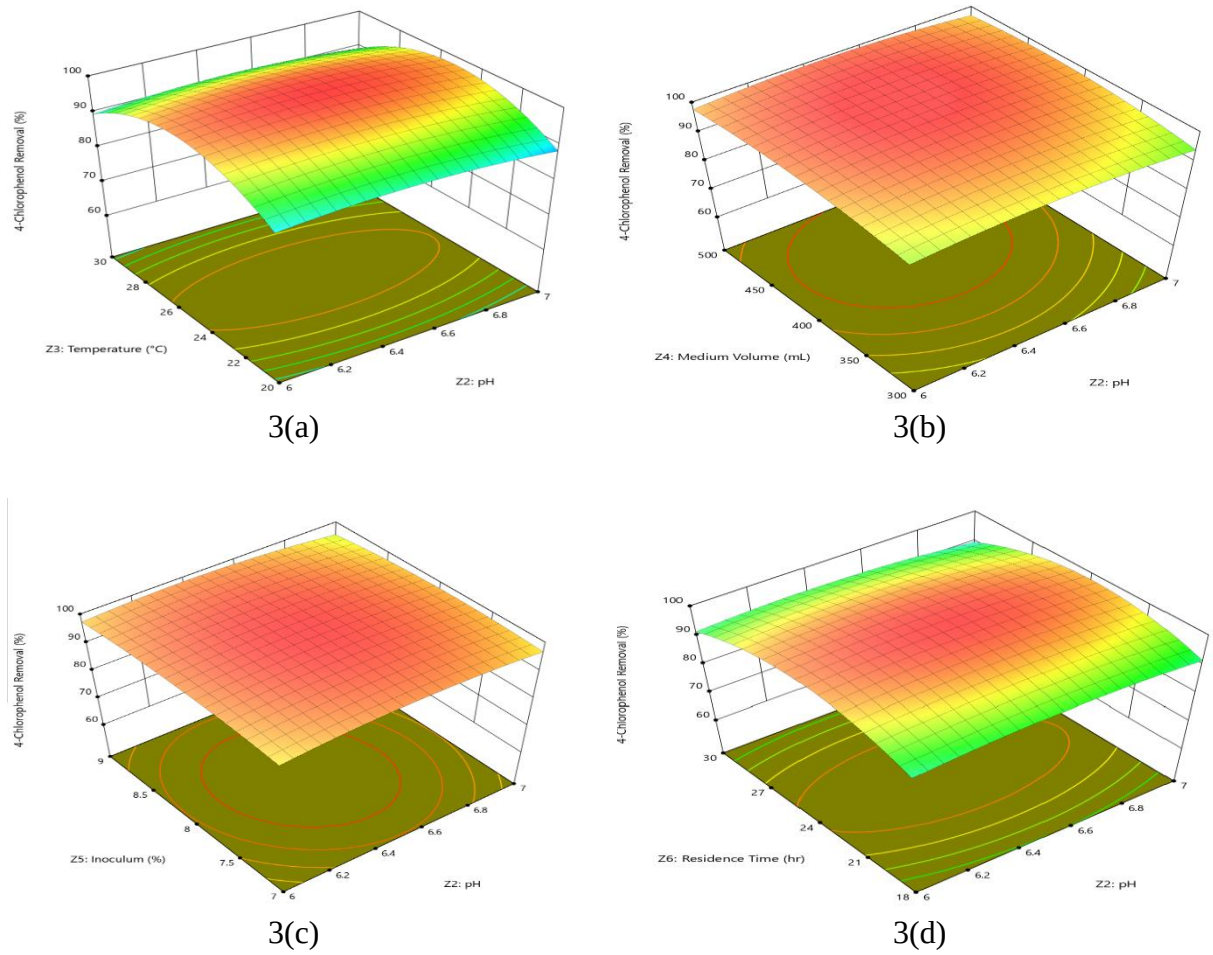
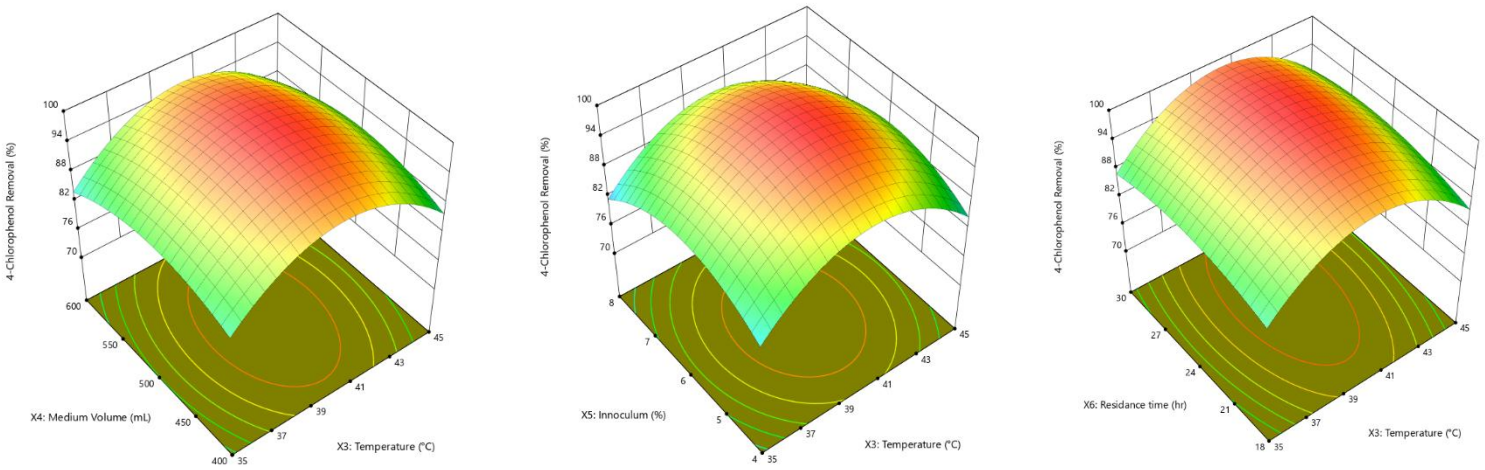


Figure IVB.7: **3-Dimensional surfaces and 2-Dimensional plots of the interaction effects of: (a) pH of the media and temperature (Z2Z3), (b) pH of the media and media vol. (Z2Z4), (c) pH of media and inoculums percent (Z2Z5), (d) pH of media and residence time (Z2Z6) on the percent of degradation of 4- Chloro Phenol in case of *Bacillus cereus***

IVB.2.5 Effect of temperature:

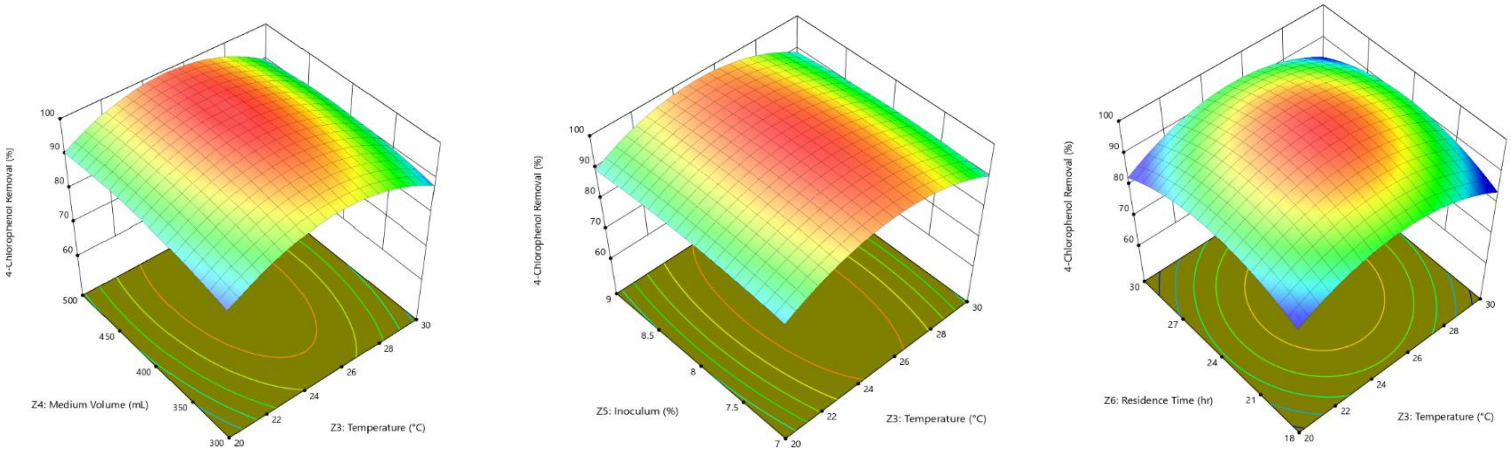
While studying *Bacillus timonensis*, it was revealed that the temperature (X3) had significant role on the response. The p- value of the X3 was found to be 0.0103 which clearly pointed out the significance of the temperature. Degradation percentage of the 4- Chloro Phenol was increased rapidly while increasing the temperature up to 40^{o}C. When the temperature further increased up to 45^{o}C, the degradation percent decreased. Similar phenomenon was recorded while decreasing the temperature up to 35^{o}C. So, temperature found to be optimal at 40^{o}C. While studying the interaction effects, it was found that, the interaction effects between temperature and media volume (X3X4), temperature and inoculums size (X3X5) & temperature and residence time (X3X6) all possessed significant effects as their p- values were found to be 0.0287, 0.0486 & 0.0227 respectively. Furthermore, the convex appearances of their 3D and contour plots also supported their significance (Figure IVB.8).

In case of *Bacillus cereus* also, Temperature was found to be very important factor during the experiments. Degradation percent of 4- Chloro Phenol was increased rapidly while temperature decreased below 30^{o}C. Maximum degradation was obtained at 25^{o}C temperature. But below that temperature, degradation percent decreased. The interaction effects between temperature vs. media volume (Z3Z4), temperature vs. inoculums percent (Z3Z5) and temperature vs. residence time (Z3Z6) all showed significant effect on the percent of degradation of 4- Chloro Phenol. Out of these, the interaction effect between temperatures vs. residence time (Z3Z6) possessed highest effect on the degradation percent of 4- Chloro Phenol. Figure: IVB.9 showed an umbrella shaped convex appearance and also very low p- value (0.0097) of Z3Z6, obtained in the ANOVA (Table IVB.8) supported the same.



(a) (b) (c)

Figure IVB.8: **3-Dimensional surfaces and 2-Dimensional plots of the interaction effects of: (a) temperature and media vol. (X3X4), (b) temperature and inoculums percent (X3X5), (c) temperature and residence time (X3X6) on the percent of degradation of 4-Chloro Phenol in case of *Bacillus timonensis***



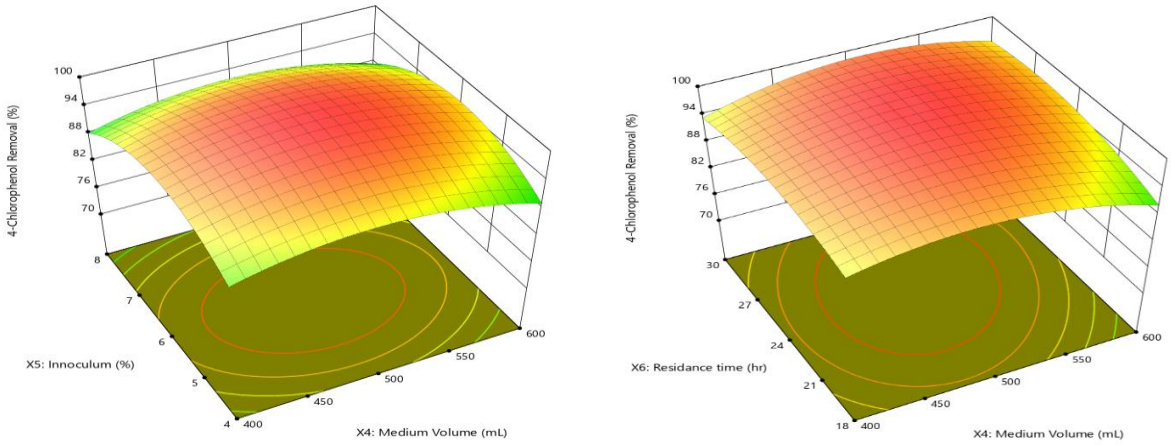
(a) (b) (c)

Figure IVB.9: **3-Dimensional surfaces and 2-Dimensional plots of the interaction effects of: (a) temperature and media vol. (Z3Z4), (b) temperature and inoculums percent (Z3Z5), (c) temperature and residence time (Z3Z6) on the percent of degradation of 4-Chloro Phenol in case of *Bacillus cereus***

IVB.2.6 Effect of media volume:

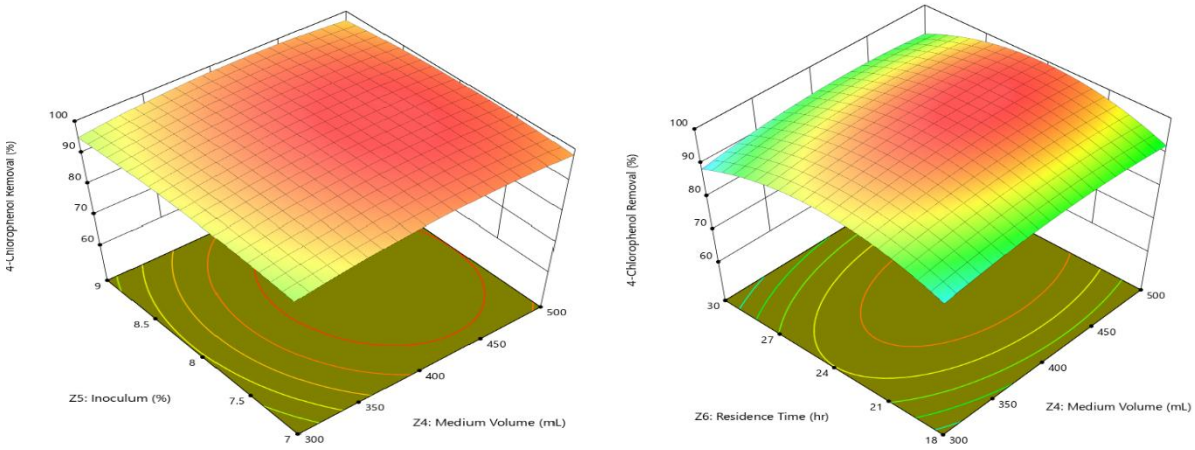
In case of *Bacillus timonensis*, the p- value of the media volume (X4) found to be 0.0043 (Table IVB.7) which clearly indicated that X4 had played a significant role on the degradation percentage of 4- Chloro Phenol. Up to 500 mL media volume, the degradation percent increased simultaneously. But beyond that volume, not so much changes were noticed in case of degradation percentage. But in case of the interaction effects between media volume & inoculums size (X4X5) and media volume & residence time (X4X6), both them were found to be insignificant. 3D curves are almost flat surfaced there (Figure IVB.10).

Besides that, in case of *Bacillus cereus*, Volume of the culture media played a pivotal role on degradation percent of 4- Chloro Phenol. Percent of degradation of 4- Chloro Phenol increased with the increasing volume of the culture media. Up to 400 ml volume, degradation percent increased steadily. But beyond that, when the volume increased up to 500 and even 700 ml, the degradation percent remained as constant. In case of interaction effects between media volume vs. inoculums percent (Z4Z5), it did not exhibit significant effect on the degradation percent. Almost flat shaped curve appeared there (Figure: IVB.11 (a)). Perhaps the reason behind that was total inoculums amount remained constant with increasing media volume. On the other hand, interaction effect between media volume vs. residence time (Z4Z6) left very significant role on the degradation percent. Convex shaped curve (Figure: IVB.11 (b)) supported the same.



(a) (b)

Figure IVB.10: **3-Dimensional surfaces and 2-Dimensional plots of the interaction effects of: (a) media vol. and inoculums percent (X4X5) & (b) media vol. and residence time (X4X6) on the percent of degradation of 4- Chloro Phenol in case of *Bacillus timonensis***



(a) (b)

Figure IVB.11: **3-Dimensional surfaces and 2-Dimensional plots of the interaction effects of: (a) media vol. and inoculums percent (Z4Z5) & (b) media vol. and residence time (Z4Z6) on the percent of degradation of 4- Chloro Phenol in case of *Bacillus cereus***

IVB.2.7 Effects of inoculums size and residence time:

In case of both the strains, these two factors exhibited very significant role individually. In case of *Bacillus timonensis*, p- values of the inoculums size (X5) & residence time (X6) were deducted as 0.0461 and 0.0258 respectively (Table IVB.7) while performing ANOVA.Up to 6% of inoculums of the strain, degradation percent increased gradually. Beyond that, degradation percent of 4- Chloro Phenol stopped almost. Same incident was recorded during the study of residence time. Maximum degradation was obtained after 24 hours. After that, the degradation percent decreased again. So, almost 100percent degradation was achieved after 24 hours of Incubation, when the initial conc. of 4- Chloro Phenol was 800 mg/L. Also, the interaction effect of the inoculums size vs. residence time (X5X6) found to be very much significant on the response i.e. degradation percent of 4- Chloro Phenol. The p- value of this model term found to be < 0.0001(Table IVB.7) which proved its significance. Figure IVB.12 also supported the same.

In case of *Bacillus cereus*, inoculums size and residence time both showed important effects on percent of degradation of 4- Chloro Phenol. Degradation percent increased with increased inoculums percent. Up to 8% inoculums, degradation percent increased simultaneously. After that, degradation percent suspended even after increasing the inoculums size. Similarly in case of residence time, degradation percent increased with increased residence time up to 24 hour. Beyond that, no big changes occurred rather negative effects arose. Complete degradation of 600 mg/L 4- Chloro Phenol was achieved within 24 hours of time. Moreover, the interaction effect in between these two factors (Z5Z6) revealed significant effect. Figure: IVB.13 showed a convex curve which denotes its significance.

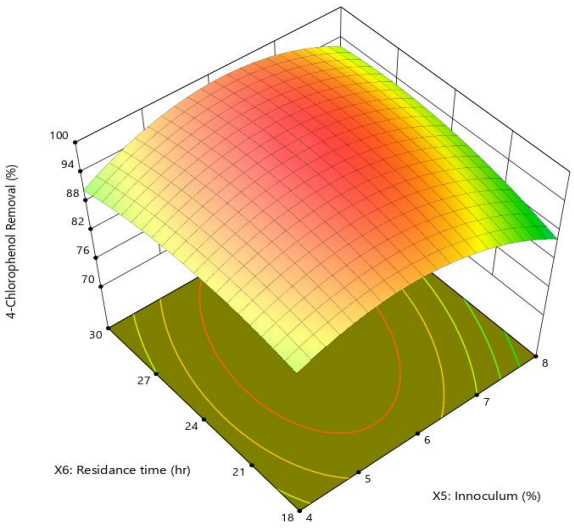


Figure IVB.12: **3-Dimensional surfaces and 2-Dimensional plots of the interaction effects of inoculums percent and residence time (X5X6) on percent of degradation of 4-Chloro Phenol in case of *Bacillus timonensis***

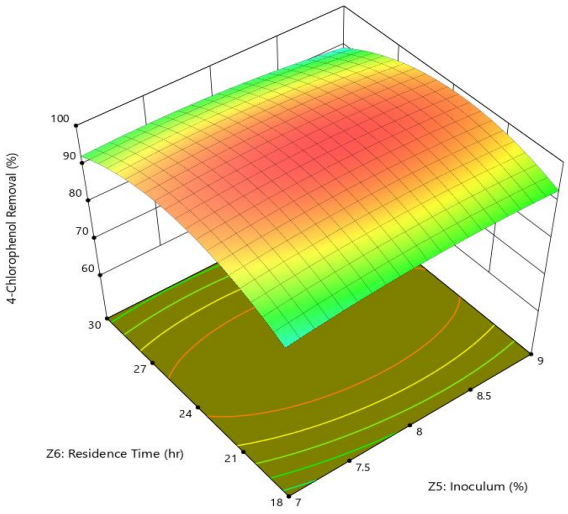


Figure IVB.13: **3-Dimensional surfaces and 2-Dimensional plots of the interaction effects of inoculums percent and residence time (Z5Z6) on percent of degradation of 4-Chloro Phenol in case of *Bacillus cereus***

IVB. 2.8 Optimization of the operating parameters:

The Response Surface Methodology (RSM) was involved in case of the two isolated strains (*Bacillus timonensis* & *Bacillus cereus*) to deduce the optimum conditions for the six independent variables to achieve maximum percent of degradation of 4-Chloro Phenol. Equation 4.7 was defined as objective function for the percent of degradation of 4- Chloro Phenol in case of *Bacillus timonensis* strain 10403023 and the independent factors in their ranges were model constraints. Thus the following optimum conditions, achieved for the maximum percent of degradation of 4- Chloro Phenol were: 800 mg/L initial conc. of 4-Chloro Phenol, 7.5 pH of the bacterial culture, 40^{o}C temperature, 500 mL volume of the culture media, 6percent inoculums size of the strain (i.e. *Bacillus timonenis* strain 10403023) and 24 hour of residence time. 99.99% degradation of the 4- Chloro Phenol was obtained by involving these six favourable conditions. The obtained optimal operating conditions were performed nine more times to validate the predicted values as well to minimise the error and only 3.4% error was obtained which confirmed the acceptance of the model as the values are within 95% CI.

Similarly in case of *Bacillus cereus* strain K1, equation 4.8 was defined as objective function for the percent of degradation of 4- Chloro Phenol and the independent factors in their ranges were model constraints. Thus the following optimum conditions, achieved for the maximum percent of degradation of 4- Chloro Phenol in case of *Bacillus cereus*, were: 600 mg/L initial conc. of 4- Chloro Phenol, 6.5 pH of the bacterial culture, 25^{o}C temperature, 400 mL volume of the culture media, 8percent inoculums size of the strain (i.e. *Bacillus cereus* strain MK789657) and 24 hours of residence time. 99.98% degradation of the 4- Chloro Phenol was obtained by involving these six favourable situations. The obtained optimal operating conditions were performed nine more times to validate the predicted values as well to minimise the error and 99.98% degradation of 4- Chloro Phenol was achieved in case of each experimental results, obtaining only 1.7% of error and thereby, indicating the reliability of this model.

Sub chapter IVC

Optimization of parameters for the Catechol degrading strains via Response Surface Methodology

IVC.1 Materials and Methods:

IVC.1.1 Materials:

Bacillus pseudomycoides strain NBRC 101232 and *Bacillus paramycoides* strain MCCC 1A04098 were isolated from the contaminated soil, collected from South Howrah state General Hospital by enrichment method as described in the chapter II, section IIA.1.5. A large volume of inoculums of the two above mentioned strains were already prepared via acclimatization process in Mineral Salt Medium (MSM) as described in chapter II, section IIA.1.8.

Catechol solution was prepared synthetically in the laboratory. Conc. of the stock solution was maintained as 10 g/L.

IVC.1.2 Experimental set up:

Same as mentioned in the chapter III, section IIIA.1.2.

IVC.1.3 Analytical Method:

Details of this process has been mentioned in chapter II, section IIA.1.7.

IVC.1.4 Experimental design:

In case of *Bacillus pseudomycoides* strain NBRC 101232, six (6) independent variables (Table IVC.1) are X1 (A): 500 – 700 mg/L; X2 (B): 5 – 6; X3 (C): 30^{o} – 40^{o}C; X4 (D): 400 – 800 ml; X5 (E): 5 – 7% and X6 (F): 64 – 80 hours.

In case of *Bacillus paramycoides* strain MCCC 1A04098, six (6) independent variables (Table IVC.2) are Y1 (A): 500 – 700 mg/L; Y2 (B): 5 – 6; Y3 (C): 20^{o} – 30^{o}C; Y4 (D): 400 – 600 ml; Y5 (E): 5 – 7% and Y6 (F): 64 – 80 hours.

Rest of the portion is same as described in sub chapter IVA, section IVA.1.4.

Table IVC.1: Independent variables with coded levels for *Bacillus pseudomycoides*

Independent variable	Symbol	Coded levels		
		-1	0	+1
Initial conc. of Catechol (mg/L)	X1	500	600	700
pH of bacterial media	X2	5	5.5	6
Temperature (^oC)	X3	30	35	40
Volume of bacterial media(mL)	X4	400	600	800
Inoculums percent (percent)	X5	5	6	7
Residence time(hr)	X6	64	72	80

Table IVC.2: Independent variables with coded levels for *Bacillus paramycoides*

Independent variable	Symbol	Coded levels		
		-1	0	+1
Initial conc. of Catechol (mg/L)	Y1	500	600	700
pH of bacterial media	Y2	5	5.5	6
Temperature (^oC)	Y3	20	25	30
Volume of bacterial media(mL)	Y4	400	500	600
Inoculums percent (percent)	Y5	5	6	7
Residence time(hr)	Y6	64	72	80

IVC.2 Results and Discussions:

IVC.2.1 Fitting of the model and Statistical analysis:

For both the strains, total 86 experiments were done as per the design matrix and the only one response was percent of degradation of Catechol. Table IVC.3 & IVC.4 displayed both the predicted and experimental/actual values of percent of degradation of Catechol by *Bacillus pseudomycoides* & *Bacillus paramycoides* respectively. Second order and linear polynomial equations (equation 4.9 & 4.10 for *Bacillus pseudomycoides* & *Bacillus paramycoides* respectively) were fitted to the actual data to obtain the regression equation. To reveal the suitable model, sequential model sum of squares and model summary statistics were deducted (for *Bacillus pseudomycoides*, Table IVC.5 and for *Bacillus paramycoides*, IVC.6 respectively). The Sequential P-value for the quadratic model is less than 0.0001 in both cases; Maximum predicted R^2 and adjusted R^2 values were 0.9614 and 0.9896 respectively for the percent of degradation of the Catechol in case of *Bacillus pseudomycoides*. In case of & *Bacillus paramycoides*, those values were 0.9638 and 0.9734 respectively for the percent of degradation of the Catechol.

Cubic model found to be aliased for both; here the sequential P- values were> 0.05 for both. So, finally the quadratic model was chosen for both strains for further determination of the percent of degradation of Catechol. The following equations clearly displayed that:

percent of Catechol degradation by *Bacillus pseudomycoides* (Y): 95.26– 2.85X1– 0.8623X2+ 2.45X3+2.03X4– 0.0435X5 - 0.3202X6 - 0.4519X1X2+ 0.7262X1X3– 0.5453X1X4+0.6597X1X5 - 1.10X1X6 - 1.41X2X3 - 0.5844X2X4+ 0.2681X2X5– 3.47X2X6+ 0.0956X3X4– 2.52X3X5+ 0.2997X3X6+ 0.6447X4X5 - 0.0256X4X6+2.36X5X6+ 0.8409$X1^2$ + 3.83$X2^2$ – 15.49$X3^2$ + 2.84$X4^2$ - 3.67$X5^2$ - 3.22$X6^2$ (4.9)

Negative coefficients for the model components X1, X2, X5, X6, X1X2, X1X4, X1X6, X2X3, X2X4, X2X6, X3X5, X4X6, $X3^2$, $X5^2$ and $X6^2$ revealed negative impacts on the percent of degradation of Catechol while positive coefficients X3, X4, X1X3, X1X5, X2X5, X3X4, X3X6, X4X5, X5X6, $X1^2$, $X2^2$ and $X4^2$ stands for positive impacts on the percent of degradation of Catechol (Equation 4.9). Moreover, the coefficients, whose values are near 0, revealed lower relative intensity. X5, X3X4 and X4X6 were categorised in this class which could not affect percent of degradation of the Catechol so much.

Percentage of Catechol degradation by *Bacillus paramycoides* (X): 95.02– 1.08Y1– 0.3998Y2+ 1.38Y3 - 1.051Y4– 0.0624Y5 + 0.2423Y6 - 0.2055Y1Y2 - 0.0902Y1Y3+ 0.3717Y1Y4+0.2380Y1Y5 - 1.31Y1Y6 - 0.5911Y2Y3 - 0.8367Y2Y4+ 1.25Y2Y5 – 0.0533Y2Y6 - 1.01Y3Y4+ 0.3711Y3Y5+ 0.8133Y3Y6 - 1.47Y4Y5 + 1.03Y4Y6+0.6564Y5Y6 - 2.17$Y1^2$ + 1.83$Y2^2$ – 4.15$Y3^2$ - 4.12$Y4^2$ - 3.64$Y5^2$ - 1.65$Y6^2$ (4.10)

Negative coefficients for the model components Y1, Y2, Y4, Y5, Y1Y2, Y1Y3, Y1Y6, Y2Y3, Y2Y4, Y2Y6, Y3Y4, Y4Y5, $Y1^2$, $Y3^2$, $Y4^2$, $Y5^2$ and $Y6^2$ revealed negative impacts on the percent of degradation of Catechol while positive coefficients Y3, Y6, Y1Y4, Y1Y5, Y2Y5, Y3Y5, Y3Y6, Y4Y6, Y5Y6 and $Y2^2$ stands for positive impacts on the percent of degradation of Catechol (Equation 4.10). Furthermore, the coefficients, whose values are near 0, revealed lower relative intensity. Y5, Y1Y3 and Y2Y6 were categorised in this class which could not affect percent of degradation of the Catechol so much.

Table IVC.3: Experimental design matrix for the degradation of Catechol by *Bacillus pseudomycoides* strain NBRC 101232

Run order	Space type	X1:Initial Concentration of Catechol (mg/L)	X2: pH	X3: Temperature (°C)	X4: Medium Volume(mL)	X5:Inoculum (percent)	X6: Residence Time(hr)	Y: percent of Catechol degradation		
								Experimental value	Predicted value	Error
1	Factorial	500	6	30	800	7	64	84.35	86.33	-1.98
2	Factorial	500	6	30	400	7	64	78.35	81.19	-2.84
3	Factorial	700	6	40	400	5	80	66.35	70.88	-4.53
4	Factorial	700	5	40	800	7	80	88.36	87.30	1.06
5	Factorial	700	6	40	800	7	80	67.35	74.27	-6.92
6	Factorial	500	5	30	400	5	64	74.35	73.46	0.8898
7	Factorial	700	6	30	400	5	64	78.36	79.85	-1.49
8	Factorial	700	5	30	800	5	64	69.26	74.80	-5.54
9	Factorial	700	5	30	400	7	64	75.35	71.81	3.54
10	Axial	600	5.5	35	600	6	80	87.25	91.72	-4.47
11	Factorial	700	6	40	400	7	64	70.32	75.46	-5.14
12	Factorial	500	5	40	800	5	64	88.35	92.16	-3.81
13	Factorial	700	5	40	800	7	64	82.35	77.93	4.42
14	Factorial	700	6	40	800	7	64	78.36	78.80	-0.4398
15	Factorial	700	6	30	400	5	80	67.52	64.80	2.72
16	Factorial	500	5	40	400	5	80	88.35	91.32	-2.97
17	Axial	600	5.5	35	600	7	72	86.32	91.55	-5.23
18	Centre	600	5.5	35	600	6	72	99.52	95.26	4.26
19	Axial	500	5.5	35	600	6	72	95.35	98.96	-3.61
20	Centre	600	5.5	35	600	6	72	99.52	95.26	4.26
21	Axial	600	5.5	35	600	6	64	88.32	92.36	-4.04
22	Factorial	500	5	40	800	7	64	86.35	81.76	4.59
23	Factorial	500	6	30	800	5	64	87.35	85.59	1.76
24	Centre	600	5.5	35	600	6	72	99.52	95.26	4.26
25	Factorial	500	5	40	800	5	80	95.34	96.50	-1.16
26	Factorial	700	5	40	400	7	64	69.35	72.26	-2.91
27	Axial	600	5.5	35	400	6	72	92.35	96.07	-3.72
28	Factorial	500	6	40	800	7	64	85.32	84.43	0.8874
29	Factorial	500	5	40	400	7	64	70.52	73.90	-3.38
30	Factorial	500	5	30	800	5	80	82.35	81.50	0.8488
31	Centre	600	5.5	35	600	6	72	99.52	95.26	4.26
32	Factorial	500	6	40	400	5	64	87.35	90.82	-3.47
33	Factorial	700	6	30	800	5	64	82.33	80.23	2.10
34	Factorial	500	5	40	400	5	64	88.32	86.88	1.44
35	Factorial	500	5	30	800	7	80	88.12	90.60	-2.48
36	Factorial	700	5	30	400	5	64	71.35	72.08	-0.7324
37	Axial	600	6	35	600	6	72	94.35	98.23	-3.88
38	Factorial	700	5	30	400	5	80	67.32	70.92	-3.60
39	Factorial	700	6	40	800	5	80	73.34	71.54	1.80
40	Factorial	700	5	40	800	5	80	86.35	85.63	0.7203

41	Centre	600	5.5	35	600	6	72	99.52	95.26	4.26
42	Factorial	500	6	30	800	7	80	84.55	85.02	-0.4659
43	Factorial	500	5	30	800	5	64	83.35	78.36	4.99
44	Axial	600	5	35	600	6	72	95.32	99.95	-4.63
45	Factorial	700	6	40	400	5	64	90.11	84.73	5.38
46	Factorial	700	6	30	800	7	64	85.42	83.60	1.82
47	Factorial	500	5	40	800	7	80	92.35	95.53	-3.18
48	Factorial	700	5	30	800	7	80	85.53	85.27	0.2624
49	Factorial	500	6	30	400	7	80	74.23	79.98	-5.75
50	Centre	600	5.5	35	600	6	72	99.52	95.26	4.26
51	Factorial	500	6	30	800	5	80	75.69	74.85	0.8409
52	Factorial	500	5	30	400	5	80	78.35	76.70	1.65
53	Centre	600	5.5	35	600	6	72	99.52	95.26	4.26
54	Factorial	500	5	40	400	7	80	86.35	87.77	-1.42
55	Factorial	700	6	40	400	7	80	71.25	71.04	0.2143
56	Factorial	500	6	40	400	5	80	87.35	81.37	5.98
57	Factorial	500	5	30	400	7	80	88.25	83.22	5.03
58	Axial	600	5.5	35	800	6	72	95.34	100.13	-4.79
59	Factorial	500	6	40	800	7	80	88.32	84.31	4.01
60	Axial	600	5.5	35	600	5	72	88.35	91.63	-3.28
61	Factorial	700	6	30	400	7	80	75.32	75.02	0.2957
62	Centre	600	5.5	35	600	6	72	99.52	95.26	4.26
63	Factorial	700	5	40	800	5	64	90.27	85.70	4.57
64	Factorial	700	6	30	800	7	80	84.61	77.88	6.73
65	Centre	600	5.5	35	600	6	72	99.52	95.26	4.26
66	Factorial	500	6	40	400	7	80	81.35	78.89	2.46
67	Factorial	700	5	40	400	5	64	82.35	82.60	-0.2462
68	Axial	600	5.5	40	600	6	72	77.78	82.22	-4.44
69	Factorial	500	5	30	400	7	64	70.35	70.55	-0.1982
70	Factorial	500	6	40	400	7	64	84.77	78.91	5.86
71	Factorial	500	6	30	400	5	64	84.36	83.03	1.33
72	Factorial	700	6	30	800	5	80	61.21	65.08	-3.87
73	Factorial	500	6	40	800	5	80	85.81	84.22	1.59
74	Factorial	700	6	40	800	5	64	85.36	85.49	-0.1292
75	Factorial	700	5	30	800	5	80	76.24	73.53	2.71
76	Centre	600	5.5	35	600	6	72	99.52	95.26	4.26
77	Factorial	700	6	30	400	7	64	84.35	80.65	3.70
78	Factorial	700	5	30	800	7	64	70.53	77.11	-6.58
79	Factorial	500	6	30	400	5	80	69.33	72.39	-3.06
80	Factorial	500	6	40	800	5	64	88.24	93.76	-5.52
81	Axial	700	5.5	35	600	6	72	88.35	93.25	-4.90
82	Factorial	700	5	30	400	7	80	80.35	80.07	0.2762
83	Factorial	500	5	30	800	7	64	79.18	78.03	1.15
84	Factorial	700	5	40	400	7	80	84.32	81.72	2.60
85	Axial	600	5.5	30	600	6	72	73.25	77.32	-4.07
86	Factorial	700	5	40	400	5	80	84.71	82.63	2.08

Table IVC.4: Experimental design matrix for the degradation of Catechol by *Bacillus paramycoides* strain MCCC 1A04098

Run order	Space type	Y1:Initial Concentration of Catechol (mg/L)	Y2: pH	Y3: Temperature (°C)	Y4: Medium Volume(mL)	Y5:Inoculum (percent)	Y6: Residence Time(hr)	X: percent of Catechol degradation		
								Experimental value	Predicted value	Error
1	Factorial	500	6	30	400	5	64	87.24	83.17	4.07
2	Factorial	700	6	20	600	5	80	76.25	76.60	-0.3462
3	Factorial	700	5	30	600	5	80	84.32	83.54	0.7814
4	Factorial	500	5	20	400	7	80	76.25	80.32	-4.07
5	Factorial	500	5	20	600	7	80	78.36	79.36	-1.00
6	Axial	600	5	25	500	6	72	94.25	97.24	-2.99
7	Factorial	500	5	30	600	7	80	82.35	83.84	-1.49
8	Centre	600	5.5	25	500	6	72	98.56	95.02	3.54
9	Factorial	500	5	20	400	5	80	79.32	79.91	-0.5919
10	Factorial	700	5	20	400	7	64	80.24	80.26	-0.0169
11	Factorial	500	6	20	400	7	64	84.37	84.54	-0.1737
12	Axial	600	5.5	25	500	7	72	88.35	91.32	-2.97
13	Factorial	500	6	30	600	7	80	81.36	82.99	-1.63
14	Axial	600	5.5	25	400	6	72	88.35	92.40	-4.05
15	Centre	600	5.5	25	500	6	72	98.56	95.02	3.54
16	Factorial	500	5	30	400	7	64	85.46	84.73	0.7338
17	Axial	600	6	25	500	6	72	92.35	96.44	-4.09
18	Factorial	700	5	30	400	7	80	85.24	84.01	1.23
19	Factorial	500	5	30	600	7	64	77.25	75.63	1.62
20	Centre	600	5.5	25	500	6	72	98.56	95.02	3.54
21	Factorial	700	5	30	600	7	80	78.25	80.51	-2.26
22	Factorial	700	5	20	600	7	80	74.25	76.39	-2.14
23	Factorial	500	6	30	400	5	80	85.24	84.43	0.8076
24	Factorial	700	5	30	400	5	80	83.24	81.16	2.08
25	Factorial	500	6	30	600	7	64	78.25	75.00	3.25
26	Factorial	500	5	30	600	5	64	79.35	82.24	-2.89
27	Factorial	700	6	20	600	5	64	81.34	79.73	1.61
28	Centre	600	5.5	25	500	6	72	98.56	95.02	3.54
29	Factorial	700	6	20	400	5	64	82.31	80.77	1.54
30	Factorial	700	6	30	400	7	80	90.24	85.68	4.56
31	Factorial	500	5	20	400	5	64	81.26	81.69	-0.4283
32	Factorial	700	6	30	400	5	64	79.35	81.82	-2.47
33	Factorial	700	6	30	600	5	80	77.14	76.87	0.2746
34	Factorial	700	5	20	400	5	80	71.25	74.50	-3.25
35	Axial	600	5.5	20	500	6	72	85.32	89.48	-4.16
36	Axial	700	5.5	25	500	6	72	88.35	91.76	-3.41
37	Factorial	700	5	30	600	7	64	80.32	77.55	2.77
38	Factorial	700	6	20	400	7	64	82.31	84.51	-2.20
39	Axial	600	5.5	25	500	6	80	89.32	93.61	-4.29
40	Factorial	700	5	30	400	7	64	81.24	85.15	-3.91

41	Factorial	500	5	20	600	7	64	77.81	74.41	3.40
42	Axial	600	5.5	25	600	6	72	86.35	89.38	-3.03
43	Axial	600	5.5	25	500	5	72	87.32	91.44	-4.12
44	Factorial	500	6	30	600	5	80	78.25	81.97	-3.72
45	Factorial	500	6	20	600	7	64	71.24	76.14	-4.90
46	Centre	600	5.5	25	500	6	72	98.56	95.02	3.54
47	Factorial	500	6	30	400	7	64	86.35	87.44	-1.09
48	Factorial	700	6	20	400	7	80	82.45	79.90	2.55
49	Factorial	700	5	30	400	5	64	88.25	84.93	3.32
50	Axial	500	5.5	25	500	6	72	90.25	93.92	-3.67
51	Factorial	700	6	30	400	7	64	85.32	87.04	-1.72
52	Factorial	500	6	20	600	7	80	84.32	80.88	3.44
53	Factorial	500	6	20	400	5	64	82.35	81.76	0.5886
54	Factorial	500	5	30	400	7	80	88.26	88.83	-0.5685
55	Factorial	700	6	30	400	5	80	74.25	77.84	-3.59
56	Factorial	700	6	30	600	7	64	76.25	76.09	0.1615
57	Factorial	500	6	30	400	7	80	89.35	91.32	-1.97
58	Factorial	500	6	20	400	5	80	78.32	79.77	-1.45
59	Axial	600	5.5	25	500	6	64	90.32	93.12	-2.80
60	Factorial	700	5	20	600	7	64	78.35	76.68	1.67
61	Factorial	700	5	20	400	7	80	78.25	75.86	2.39
62	Factorial	500	5	30	400	5	64	84.28	85.46	-1.18
63	Factorial	700	5	30	600	5	64	78.32	83.21	-4.89
64	Centre	600	5.5	25	500	6	72	98.56	95.02	3.54
65	Factorial	500	5	20	400	7	64	82.32	79.47	2.85
66	Factorial	500	5	30	400	5	80	88.36	86.94	1.42
67	Factorial	500	6	20	400	7	80	88.32	85.18	3.14
68	Centre	600	5.5	25	500	6	72	98.56	95.02	3.54
69	Factorial	700	5	20	600	5	64	85.32	83.83	1.49
70	Factorial	500	6	30	600	5	64	76.35	76.61	-0.2562
71	Factorial	700	6	30	600	7	80	80.32	78.83	1.49
72	Factorial	700	6	30	600	5	64	80.32	76.75	3.57
73	Centre	600	5.5	25	500	6	72	98.56	95.02	3.54
74	Factorial	700	6	20	600	7	64	76.25	77.59	-1.34
75	Factorial	700	6	20	600	7	80	75.26	77.08	-1.82
76	Axial	600	5.5	30	500	6	72	89.32	92.24	-2.92
77	Centre	600	5.5	25	500	6	72	98.56	95.02	3.54
78	Factorial	500	5	20	600	5	80	89.32	84.83	4.49
79	Factorial	700	5	20	600	5	80	81.32	80.90	0.4151
80	Factorial	500	5	30	600	5	80	92.25	87.82	4.43
81	Factorial	500	6	20	600	5	80	82.32	81.34	0.9770
82	Factorial	500	6	20	600	5	64	80.25	79.23	1.02
83	Factorial	500	5	20	600	5	64	77.35	82.50	-5.15
84	Factorial	700	6	20	400	5	80	73.25	73.54	-0.2881
85	Centre	600	5.5	25	500	6	72	98.56	95.02	3.54
86	Factorial	700	5	20	400	5	64	83.27	81.52	1.75

Table IVC.5: Adequacy of the models tested for Catechol degradation (for *Bacillus pseudomycoides* strain NBRC 101232)

Sequential Model Sum of Squares						
Source	**Sum of Squares**	**df**	**Mean Square**	**F-value**	**p-value**	**Remarks**
Mean vs. Total	6.045E+05	1	6.045E+05			
Linear vs. Mean	1264.11	6	210.69	2.54	0.0265	
2FI vs. Linear	1890.04	15	126.00	1.73	0.0666	
Quadratic vs. 2FI	**3534.19**	**6**	**589.03**	**30.47**	**< 0.0001**	**Suggested**
Cubic vs. Quadratic	357.43	26	13.75	0.5758	0.9237	Aliased
Residual	763.95	32	23.87			
Total	6.123E+05	86	7119.55			

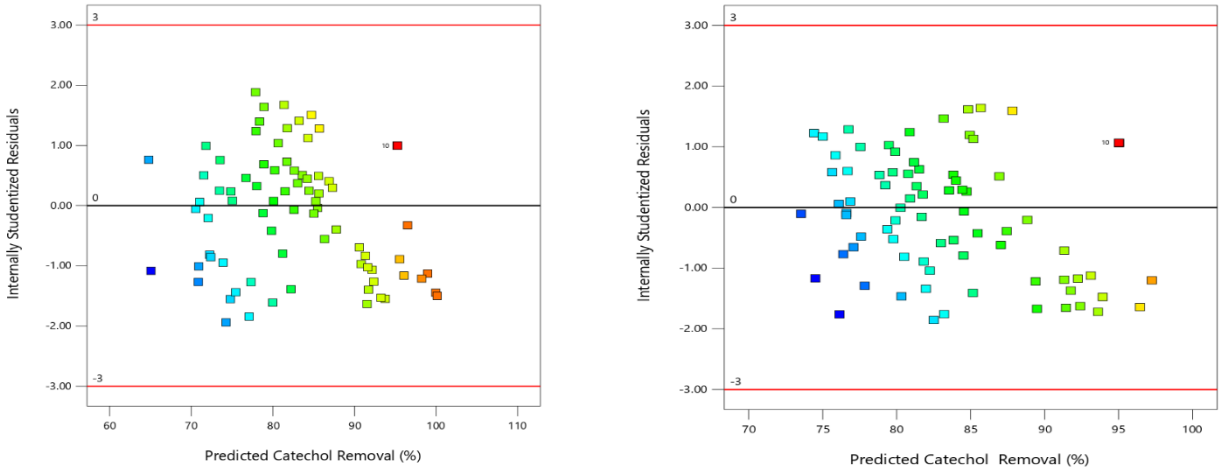
Model Summary Statistics						
Source	**Std. Dev.**	**R^2**	**Adjusted R^2**	**Predicted R^2**	**PRESS**	**Remarks**
Linear	9.10	0.1619	0.0982	0.0463	7448.12	
2FI	8.53	0.4039	0.2083	0.1580	6576.03	
Quadratic	**4.40**	**0.9564**	**0.9896**	**0.9614**	**2644.56**	**Suggested**
Cubic	4.89	0.9022	0.7402	-12.9659	1.091E+05	Aliased

Table IVC.6: Adequacy of the models tested for Catechol degradation (for *Bacillus paramycoides* strain MCCC 1A04098)

Sequential Model Sum of Squares						
Source	**Sum of Squares**	**df**	**Mean Square**	**F-value**	**p-value**	**Remarks**
Mean vs. Total	6.117E+05	1	6.117E+05			
Linear vs. Mean	367.78	6	61.30	1.20	0.3133	
2FI vs. Linear	642.32	15	42.82	0.8106	0.6621	
Quadratic vs. 2FI	**2702.06**	**6**	**450.34**	**38.47**	**< 0.0001**	**Suggested**
Cubic vs. Quadratic	267.28	26	10.28	0.7990	0.7190	Aliased
Residual	411.70	32	12.87			
Total	6.161E+05	86	7163.67			

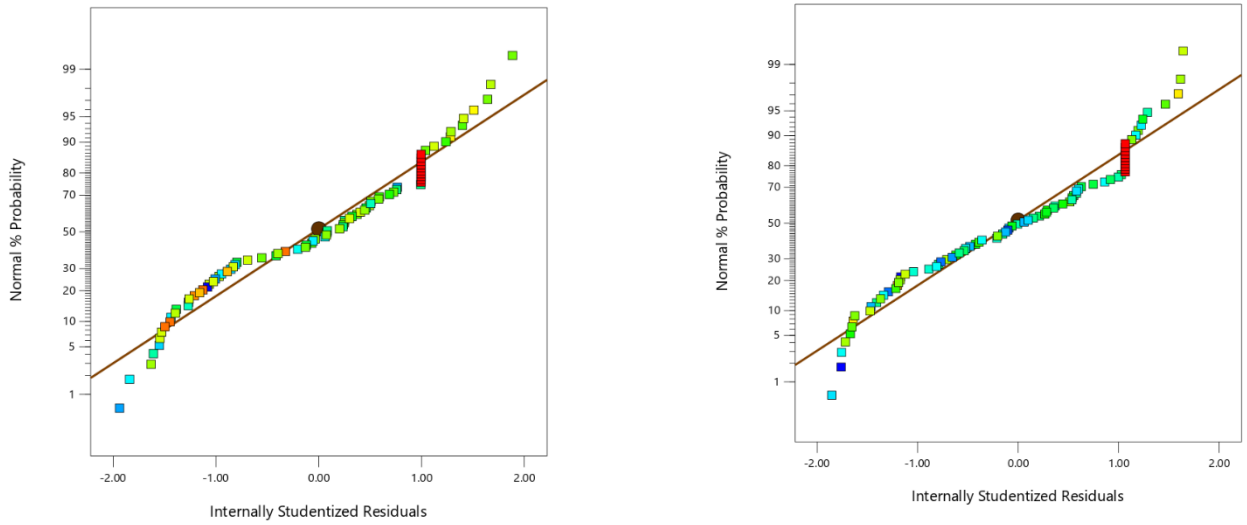
Model Summary Statistics						
Source	**Std. Dev.**	**R^2**	**Adjusted R^2**	**Predicted R^2**	**PRESS**	**Remarks**
Linear	7.14	0.0838	0.0142	-0.0230	4492.31	
2FI	7.27	0.2300	-0.0226	-0.0822	4752.04	
Quadratic	**3.42**	**0.9854**	**0.9734**	**0.9638**	**1608.16**	**Suggested**
Cubic	3.59	0.9062	0.7510	-15.9923	74615.33	Aliased

In case of both strains, the constant variance assumptions were investigated by plotting internally studentized residual vs. predicted values of percent degradation of Catechol (Figure IVC.1 (a) & IVC.1 (b)). Studentized residuals were deducted by dividing the residuals by their standard deviations displaying a randomly scattered pattern within the detection limits -3 to +3 and so, prediction of model described in the equation no (4.9 & 4.10 respectively) for the percent degradation of Catechol is satisfactory. The normal probability plot of residuals (Figure IVC.2 (a) & IVC.2 (b)) for the percent degradation of Catechol showed a straight line pattern for the both strains, rather than S shaped followed by the points on the plot. As the residuals are distributed normally, transformation of response is not required. Relation between the predicted and experimental values of responses have been displayed in Figure IVC.3 (a) & IVC.3 (b) for *Bacillus pesudomycoides* strain NBRC 101232& *Bacillus paramycoides* strain MCCC 1A04098 respectively. In both the figures, very little discrepancies were found by the straight trend line pointing a good relationship in between the predicted and experimental values.



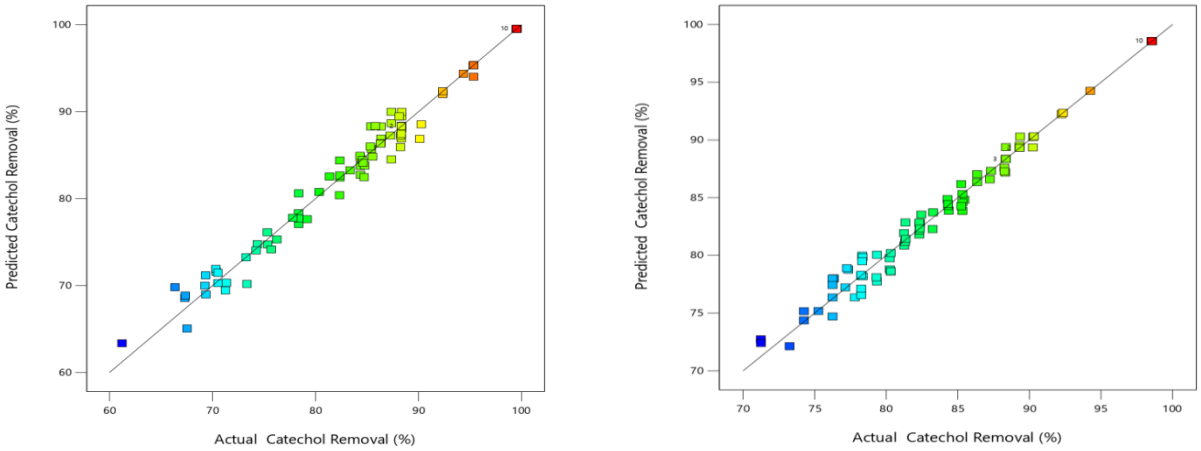
(a) (b)

Figure IVC.1: Internally studentized residuals vs. predicted values (a) *Bacillus pseudomycoides* (b) *Bacillus paramycoides*



(a) (b)

Figure IVC.2: Internally studentized residuals vs. Normal probability (a) *Bacillus pseudomycoides* (b) *Bacillus paramycoides*



(a) (b)

Figure IVC.3: Actual experimental degradation data vs. predicted data (a) *Bacillus pseudomycoides* (b) *Bacillus paramycoides*

IVC.2.2 ANOVA Test:

Outputs of ANOVA for percent of degradation of Catechol by the two strains have been given in Table IVC.7 & IVC.8 respectively. In Table IVC.7 ((ANOVA for percent degradation of Catechol by *Bacillus pseudomycoides*), the Model F-value of 12.81 implies the model is significant. There is only a 0.01% chance that an F-value this large could occur due to noise. X1, X2, X3, X4, X5, X6, X1X3, X1X4, X1X5, X1X6, X2X3, X2X5, X2X6, X3X4, X3X5, X3X6, X4X5, X4X6, X5X6 and $X3^2$ are significant model terms. X1X2, X2X4, $X1^2$, $X2^2$, $X4^2$, $X5^2$ and $X6^2$ are not significant model terms.

Table IVC.7: ANOVA of the second order polynomial equation for the degradation of Catechol by *Bacillus pseudomycoides*

Source	Sum of Squares	df	Mean Square	F-value	p-value	Remarks
Model	6688.34	27	247.72	12.81	< 0.0001	significant
X1-Initial Concentration	537.80	1	537.80	27.82	< 0.0001	significant
X2-pH	49.07	1	49.07	2.54	0.0066	significant
X3-Temperature	397.19	1	397.19	20.54	< 0.0001	significant
X4-Medium Volume	273.16	1	273.16	14.13	0.0004	significant
X5-Inoculum	0.1248	1	0.1248	0.0065	0.0062	significant
X6-Residence Time	6.76	1	6.76	0.3499	0.0065	significant
X1X2	13.07	1	13.07	0.6759	0.4144	Not significant
X1X3	33.76	1	33.76	1.75	0.0116	significant
X1X4	19.03	1	19.03	0.9843	0.0153	significant
X1X5	27.85	1	27.85	1.44	0.0249	significant
X1X6	77.70	1	77.70	4.02	0.0097	significant
X2X3	126.96	1	126.96	6.57	0.0130	significant
X2X4	21.86	1	21.86	1.13	0.2921	Not significant
X2X5	4.60	1	4.60	0.2380	0.0175	significant
X2X6	771.31	1	771.31	39.89	< 0.0001	significant
X3X4	0.5852	1	0.5852	0.0303	0.0225	significant
X3X5	405.42	1	405.42	20.97	< 0.0001	significant
X3X6	5.75	1	5.75	0.2973	0.0377	significant
X4X5	26.60	1	26.60	1.38	0.0256	significant
X4X6	0.0420	1	0.0420	0.0022	0.0130	significant
X5X6	355.51	1	355.51	18.39	< 0.0001	significant
$X1^2$	1.69	1	1.69	0.0873	0.7686	Not significant
$X2^2$	34.96	1	34.96	1.81	0.1840	Not significant
$X3^2$	573.37	1	573.37	29.66	< 0.0001	significant
$X4^2$	19.21	1	19.21	0.9935	0.3230	Not significant
$X5^2$	32.24	1	32.24	1.67	0.2017	Not significant
$X6^2$	24.83	1	24.83	1.28	0.2618	Not significant
Residual	1121.38	58	19.33			
Lack of Fit	1121.38	49	22.89			
Pure Error	0.0000	9	0.0000			
Cor Total	7809.72	85				

Outputs of ANOVA for percent of degradation of Catechol (by *Bacillus paramycoides*) have been given in Table IVC.8. In that table, the Model F-value of 11.74 implies the model is

significant. There is only a 0.01% chance that an F-value this large could occur due to noise. Y1, Y2, Y3, Y4, Y5, Y6, Y1Y3, Y1Y4, Y1Y5, Y1Y6, Y2Y3, Y2Y4, Y2Y5, Y2Y6, Y3Y4, Y3Y5, Y3Y6, Y4Y5, Y4Y6, Y5Y6 and $Y5^2$ are significant model terms. Values greater than 0.1000 indicate the model terms are not significant. So, Y1Y2, $Y1^2$, $Y2^2$, $Y3^2$, $Y4^2$, and $Y6^2$ are not significant model terms.

Table IVC.8: ANOVA of the second order polynomial equation for the degradation of Catechol by *Bacillus paramycoides*

Source	Sum of Squares	df	Mean Square	F-value	p-value	Remarks
Model	3712.16	27	137.49	11.74	< 0.0001	significant
Y1-Initial Concentration	77.00	1	77.00	6.58	0.0129	significant
Y2-pH	10.55	1	10.55	0.9014	0.0063	significant
Y3-Temperature	125.94	1	125.94	10.76	0.0018	significant
Y4-Medium Volume	150.15	1	150.15	12.83	0.0007	significant
Y5-Inoculum	0.2572	1	0.2572	0.0220	0.0027	significant
Y6-Residence Time	3.87	1	3.87	0.3309	0.0073	significant
Y1Y2	2.70	1	2.70	0.2308	0.6327	Not significant
Y1Y3	0.5202	1	0.5202	0.0444	0.0138	significant
Y1Y4	8.84	1	8.84	0.7554	0.0284	significant
Y1Y5	3.62	1	3.62	0.3096	0.0201	significant
Y1Y6	110.12	1	110.12	9.41	0.0033	significant
Y2Y3	22.36	1	22.36	1.91	0.0122	significant
Y2Y4	44.81	1	44.81	3.83	0.0352	significant
Y2Y5	100.03	1	100.03	8.54	0.0049	significant
Y2Y6	0.1817	1	0.1817	0.0155	0.0013	significant
Y3Y4	64.98	1	64.98	5.55	0.0219	significant
Y3Y5	8.81	1	8.81	0.7529	0.0091	significant
Y3Y6	42.33	1	42.33	3.62	0.0022	significant
Y4Y5	138.09	1	138.09	11.80	0.0011	significant
Y4Y6	67.34	1	67.34	5.75	0.0197	significant
Y5Y6	27.58	1	27.58	2.36	0.0203	significant
$Y1^2$	11.28	1	11.28	0.9640	0.3303	Not significant
$Y2^2$	7.97	1	7.97	0.6805	0.4128	Not significant
$Y3^2$	41.21	1	41.21	3.52	0.0657	Not significant
$Y4^2$	40.61	1	40.61	3.47	0.0676	Not significant
$Y5^2$	31.62	1	31.62	2.70	0.0057	significant
$Y6^2$	6.53	1	6.53	0.5579	0.4581	Not significant
Residual	678.97	58	11.71			
Lack of Fit	678.97	49	13.86			
Pure Error	0.0000	9	0.0000			
Cor Total	4391.14	85				

IVC.2.3 Effect of initial conc. of Catechol:

While studying *Bacillus pseudomycoides*, initial conc. of Catechol found to be an important factor as beyond a certain conc. an inhibitory effect arose. To understand the effect of initial conc. of Catechol, 3D and contour plots were showed in Figure IVC.4. It was observed that increase in initial conc. of Catechol up to 600 mg/L; almost 100% degradation was obtained. Beyond this concentration, the degradation was decreased. The p- value of X1 (<0.0001) also indicated its significance on the response. From the 3D and contour plots, it can be showed that the interaction effects between initial conc. of Catechol vs. temperature (X1X3), initial conc. vs. media volume (X1X4), initial conc. vs. inoculums size (X1X5) and initial conc. vs. residence time (X1X6) possesses very significant effects on percent of degradation of Catechol (Figure: IVC.4 (a), (b), (c) & (d)).

Also in case of *Bacillus paramycoides*, initial conc. of Catechol was an important factor as beyond a certain conc. an inhibitory effect arose. To make out the effect of initial conc. of Catechol, 3D and contour plots were displayed in Figure IVC.5. It was observed that increase in initial conc. of Catechol up to 600 mg/L; almost 99% degradation was obtained. Beyond this concentration, the degradation was decreased. The p- value of Y1 (0.0129) also indicated its significance. From the 3D surfaces and contour plots, it can be showed that the interaction effects between initial conc. of Catechol vs. temperature (Y1Y3), initial conc. vs. media volume (Y1Y4), initial conc. vs. inoculums size (Y1Y5) and initial conc. vs. residence time (Y1Y6) possesses significant effects on percent of degradation of Catechol (Figure: IVC.5 (a), (b), (c) & (d)).

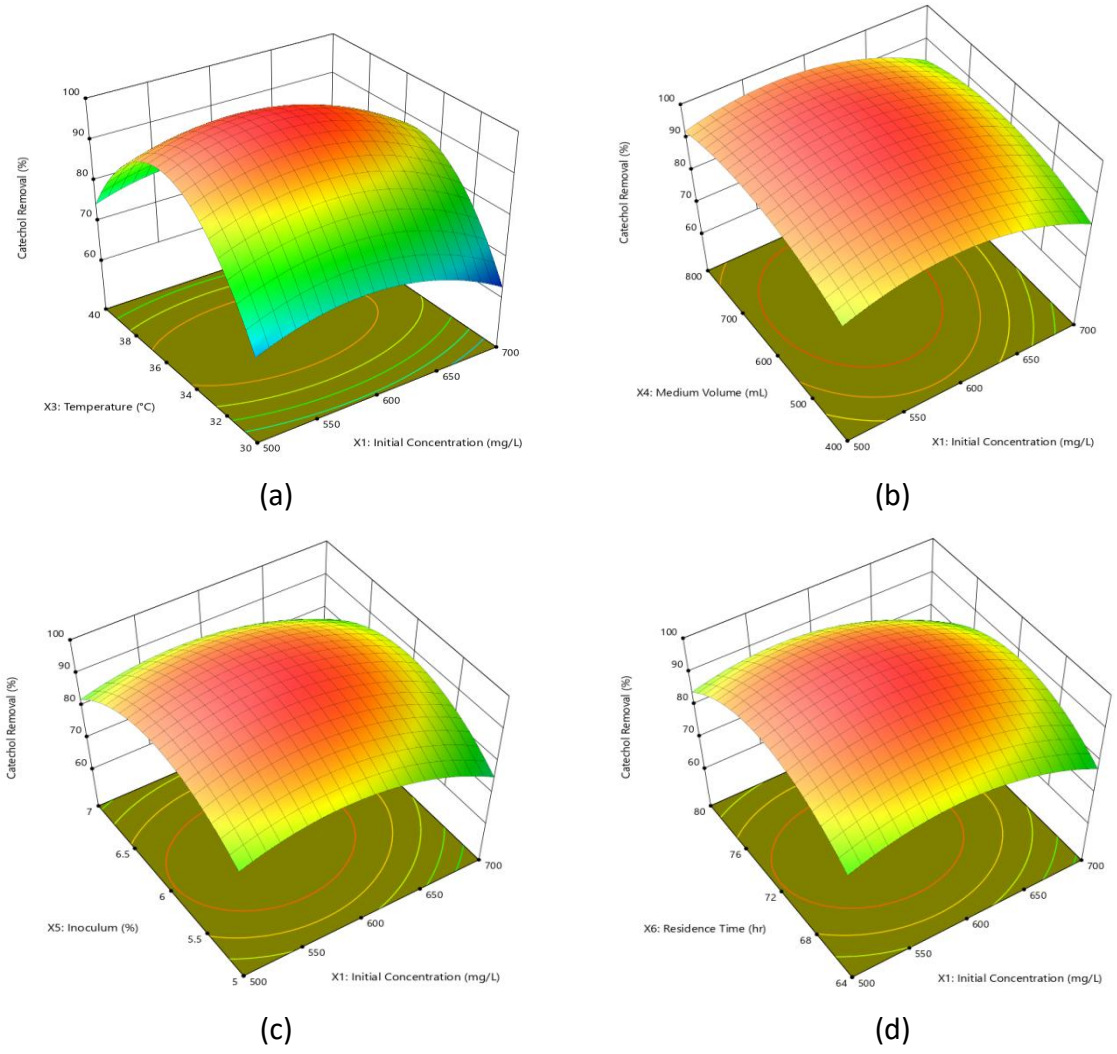


Figure IVC.4: 3- Dimensional surfaces and 2-Dimensional plots of the interaction effects of: (a) initial conc. of Catechol and temperature (X1X3), (b) initial conc. of Catechol and media vol. (X1X4), (c) initial conc. of Catechol and inoculums percent (X1X5), (d) initial conc. of Catechol and residence time (X1X6) on the percent of degradation of Catechol in case of *Bacillus pseudomycoides*

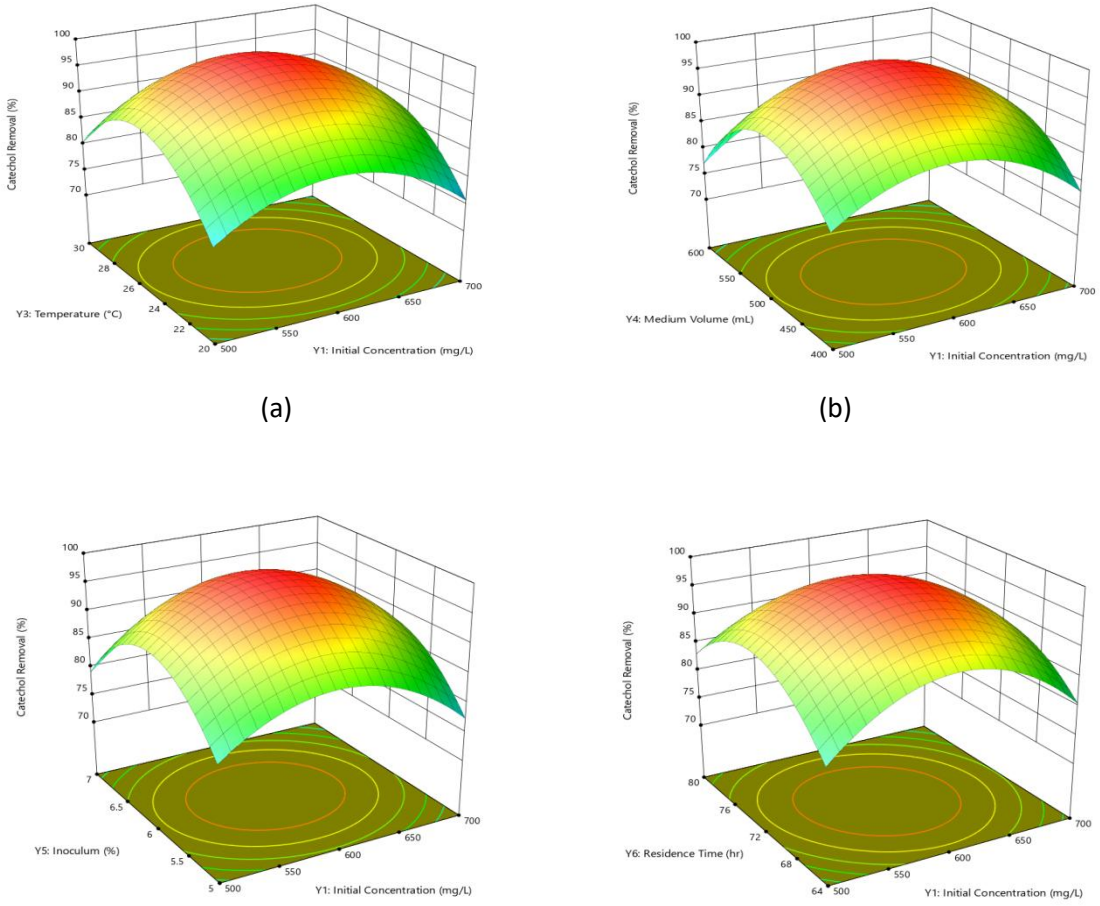


Figure IVC.5: 3- Dimensional surfaces and 2-Dimensional plots of the interaction effects of: (a) initial conc. of Catechol and temperature (Y1Y3), (b) initial conc. of Catechol and media vol. (Y1Y4), (c) initial conc. of Catechol and inoculums percent (Y1Y5), (d) initial conc. of Catechol and residence time (Y1Y6) on the percent of degradation of Catechol in case of *Bacillus paramycoides*

IVC.2.4 Effect of pH of media:

In case of *Bacillus pseudomycoides*, effect of pH i.e. concentration of H^+ ions revealed a significant role in percent degradation of Catechol. P- Value of the X2 (0.0066) revealed the same. Percent of degradation of Catechol was maximum (more than 90percent) when the pH value of the culture media was 5.5. Percent degradation was decreased in both the situations while the pH value of the media increased and decreased indicating that maximum degradation was occurred particularly at pH 5.5. pH values below and above that condition is not favourable for the strain to remove Catechol. From the 3D and contour plots, it can be showed that the interaction effects of: pH of the media vs. temperature (X2X3) , pH of the media vs. inoculums size (X2X5) and pH of the media vs. residence time (X2X6) had significant effects on percent degradation of Catechol as the plots are convex shaped (Figure: IVC.6 (a), (c) & (d)). But interaction effects between pH of the media vs. volume of media (X2X4) did not exhibit significant effects on the degradation percent of Catechol. Plot is almost flat here (Figure: IVC.6 (b)).

Almost similar results were obtained while studying *Bacillus paramycoides*. Here also, the effect of pH exhibited a significant role in percent degradation of Catechol. P- Value of the Y2 (0.0063) supported the same. Here also, percentage of degradation of Catechol was maximum (more than 92%) when the pH value of the culture media was 5.5. Like that of previous strain, percent of degradation was decreased in both the situations while the pH value of the media increased or decreased indicating that maximum degradation would be occurred at pH 5.5. pH values below and above that condition is not favourable for the strain to remove Catechol. From the 3D and contour plots, it can be displayed clearly that the interaction effects of: pH of the media vs. temperature (Y2Y3), pH of the media vs. volume of media (Y2Y4), pH of the media vs. inoculums size (Y2Y5) and pH of the media vs. residence time (Y2Y6) all had significant effects on percent degradation of Catechol as all the plots are umbrella shaped (Figure: IVC.7 (a), (b), (c) & (d)).

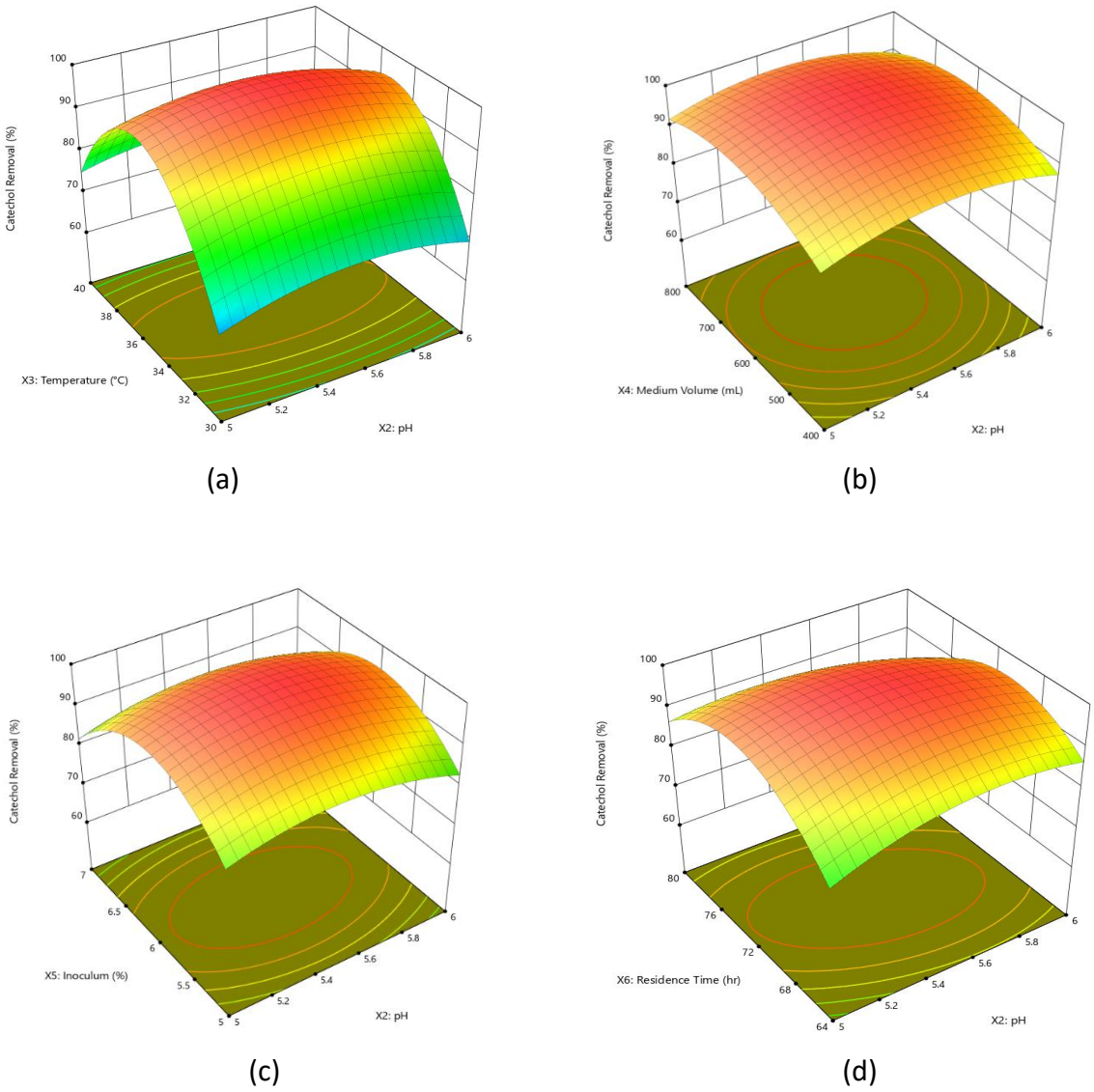


Figure IVC.6: 3-Dimensional surfaces and 2-Dimensional plots of the interaction effects of: (a) pH of the media and temperature (X2X3), (b) pH of the media and media vol. (X2X4), (c) pH of media and inoculums percent (X2X5), (d) pH of media and residence time (X2X6) on the percent of degradation of Catechol in case of *Bacillus pseudomycoides*

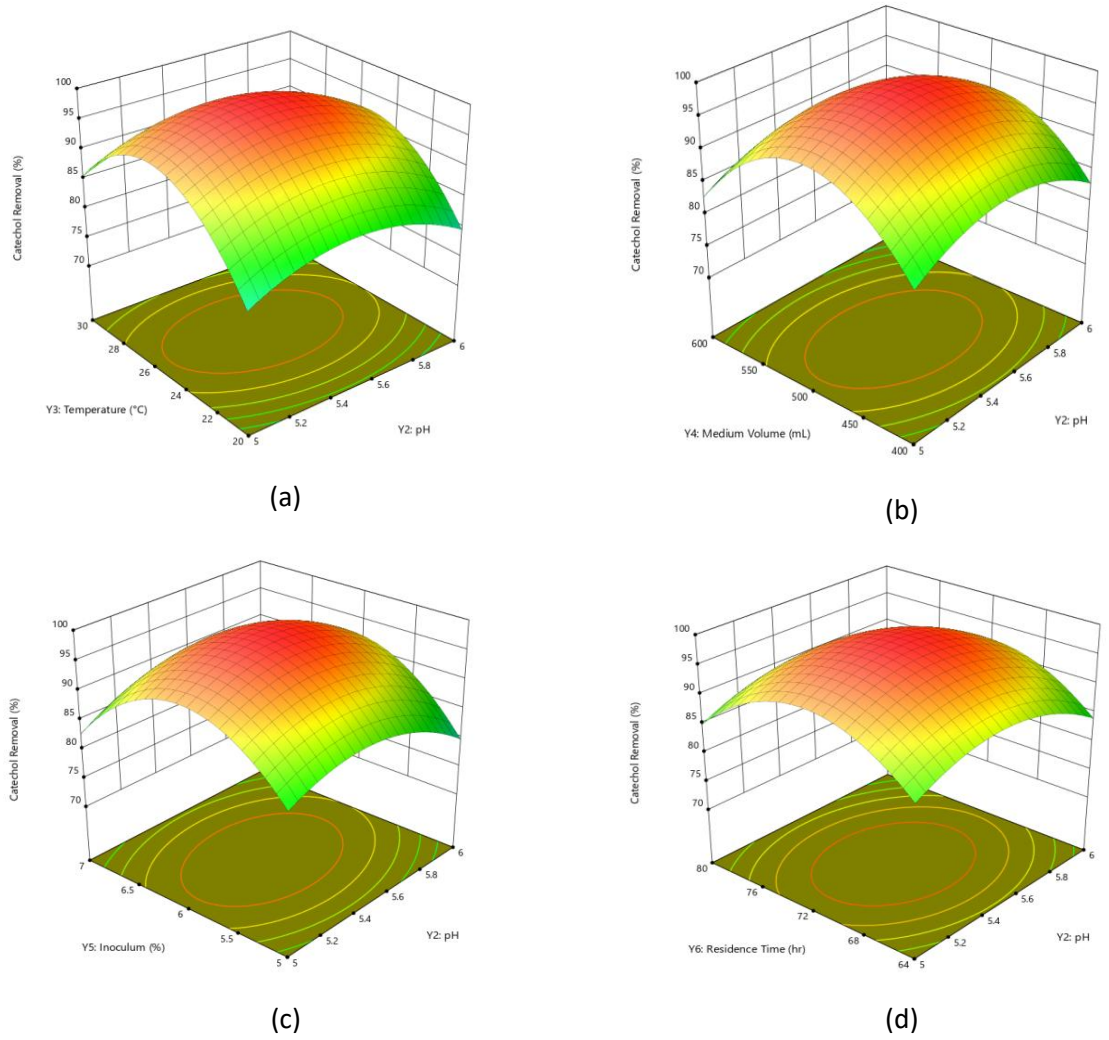


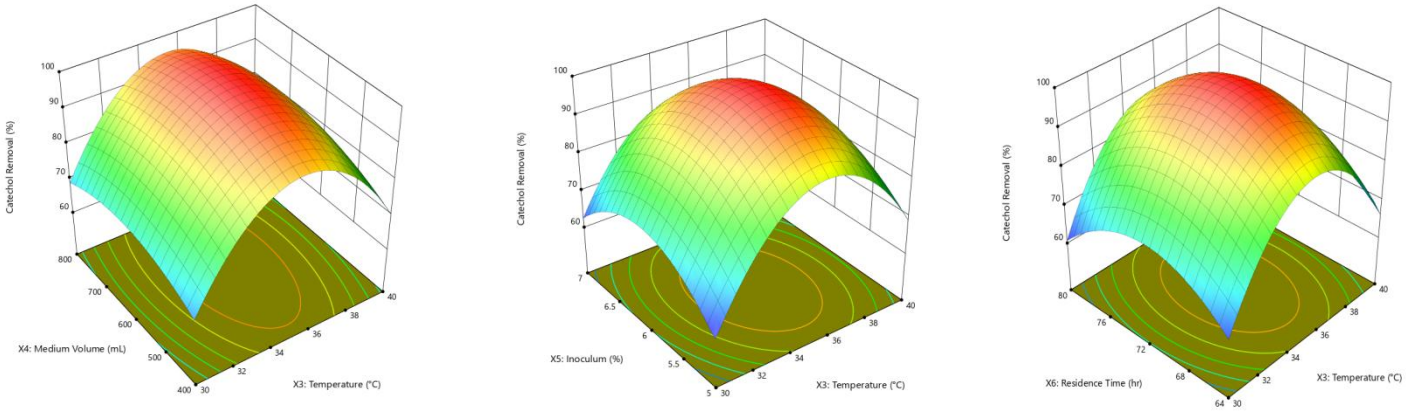
Figure IVC.7: 3-Dimensional surfaces and 2-Dimensional plots of the interaction effects of: (a) pH of the media and temperature (Y2Y3), (b) pH of the media and media vol. (Y2Y4), (c) pH of media and inoculums percent (Y2Y5), (d) pH of media and residence time (Y2Y6) on the percent of degradation of Catechol in case of *Bacillus paramycoides*

IVC.2.5 Effect of temperature:

While studying *Bacillus pseudomycoides*, temperature was found to be very important factor (p- value < 0.0001 in the Table IVC.7). Degradation percent of Catechol was increased rapidly while temperature increased to 35^{o}C. Maximum degradation was also obtained at 35^{o}C temperature. But below and above this temperature, degradation percent decreased. The interaction effects of temperature vs. media volume (X3X4), temperature vs. inoculums percent (X3X5) and temperature vs. residence time (X3X6) all exhibited significant effects

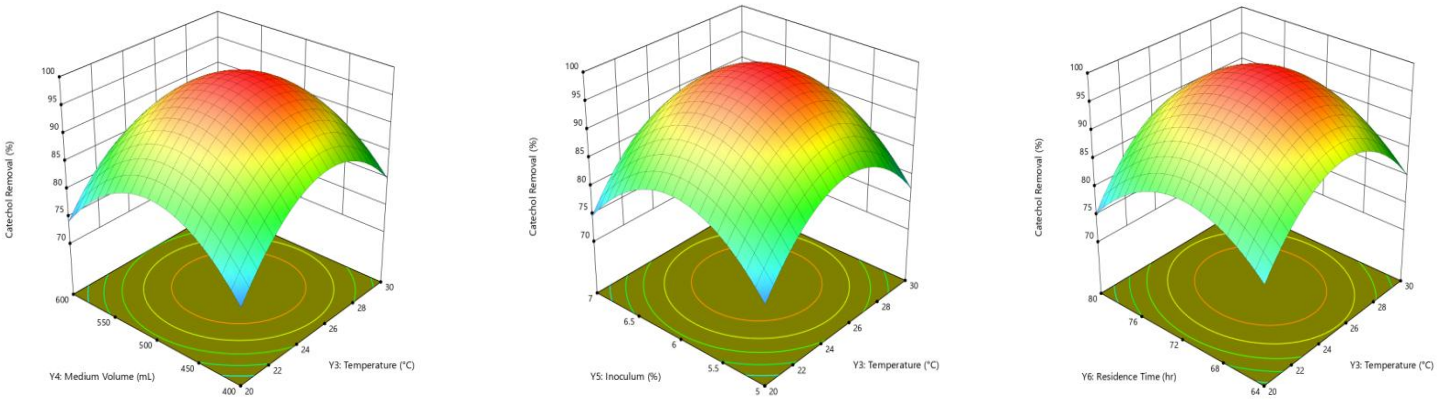
on the percent of degradation of Catechol. The convex appearances of the 3D surfaces and 2D plots (Figure IVC.8 (a), (b) & (c)) revealed significant effects on the response i.e. percent degradation of Catechol. Besides, being the p- Values of these model terms less than 0.05 (Table IVC.7), these are definitely very significant.

On the other hand, in case of *Bacillus paramycoides*, temperature was found to be very important factor also, during the experiments (p- value < 0.0018 in the Table IVC.8). Degradation percent of Catechol was increased rapidly while temperature increased to 25^{o}C. Maximum degradation was also obtained at 25^{o}C temperature. But below and above this temperature (at 20^{o}C & 30^{o}C respectively), degradation percent decreased. The interaction effects of temperature vs. media volume (Y3Y4), temperature vs. inoculums percent (Y3Y5) and temperature vs. residence time (Y3Y6) all exhibited significant effects on the percent of degradation of Catechol. The umbrella shaped appearances of the 3D surfaces and 2D plots (Figure IVC.9 (a), (b) & (c)) revealed significant effects on the response i.e. percent degradation of Catechol. Also, P –Values of Y3Y4, Y3Y5 and Y3Y6 (0.0219, 0.0091 & 0.0022) indicated the same.



(a) (b) (c)

Figure IVC.8: 3-Dimensional surfaces and 2-Dimensional plots of the interaction effects of: (a) temperature and media vol. (X3X4), (b) temperature and inoculums percent (X3X5), (c) temperature and residence time (X3X6) on the percent of degradation of Catechol in case of *Bacillus pseudomycoides*



(a) (b) (c)

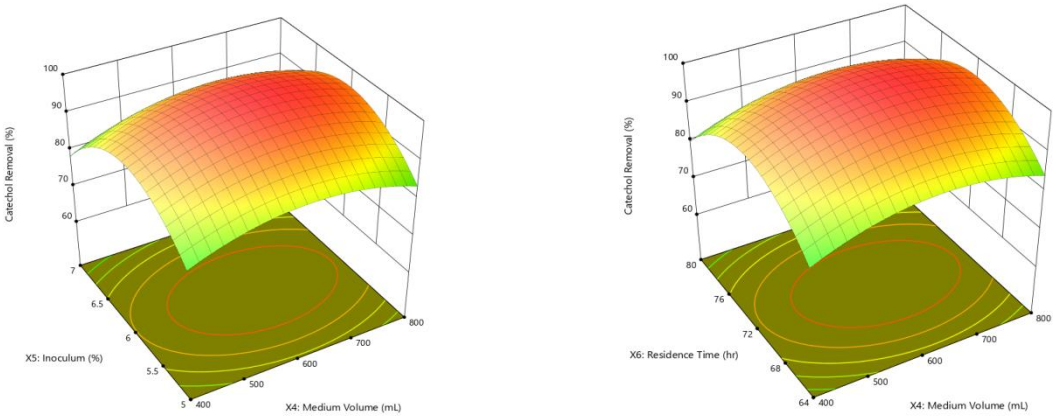
Figure IVC.9: 3-Dimensional surfaces and 2-Dimensional plots of the interaction effects of: (a) temperature and media vol. (Y3Y4), (b) temperature and inoculums percent (Y3Y5), (c) temperature and residence time (Y3Y6) on the percent of degradation of Catechol in case of *Bacillus paramycoides*

IVC.2.6 Effect of media volume:

Volume of the culture media played a vital role on degradation percent of Catechol in case of both the strains. In case of *Bacillus pseudomycoides*, P- value of the media volume (X4) found to be 0.0004 (Table IVC.7) which stood for an evidence of its significance. Percent of degradation of Catechol increased with the increasing volume of the culture media. Up to 600 ml volume, degradation percent increased steadily. But beyond that, when the volume further increased up to 800, the degradation percent remained almost remained constant. In case of interaction effects of media volume vs. inoculums percent (X4X5) and media volume vs. residence time (X4X6), both left very significant roles on degradation percent of Catechol. P-Values of the model terms were 0.0256 and 0.0130 respectively (Table IVC.7) indicating their significances. Moreover, convex shaped curves (Figure: IVC.10 (a) & (b)) supported the same.

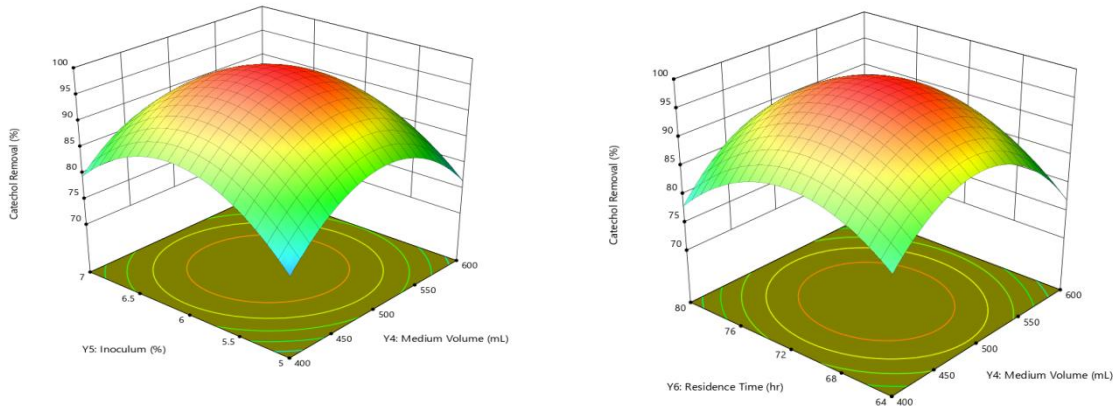
Also in case of *Bacillus paramycoides*, volume of the culture media played a vital role on degradation percentage of Catechol. P- Value of the Y4 is 0.0007 (Table IVC.8) which stands for an evidence of its significant effect. Percent of degradation of Catechol increased with the increasing volume of the culture media. Up to 500 ml volume, degradation percent increased steadily. But beyond that, when the volume increased up to 600 mL, the degradation percent remained almost constant. In case of interaction effects between media

volume vs. inoculums percent (Y4Y5) and media volume vs. residence time (Y4Y6), both left very significant roles on degradation percent of Catechol. P- Values of the model terms were 0.0011 and 0.0197 respectively (Table IVC.8) indicating their significance. Moreover, convex shaped curves (Figure: IVC.11 (a) & (b)) supported the same.



(a) (b)

Figure IVC.10: 3-Dimensional surfaces and 2-Dimensional plots of the interaction effects of: (a) media volume vs. inoculums size (X4X5) and (b) media volume vs. residence time (X4X6) on the percentage of degradation of Catechol in case of *Bacillus pseudomycoides*



(a) (b)

Figure IVC.11: 3-Dimensional surfaces and 2-Dimensional plots of the interaction effects of: (a) media volume vs. inoculums size (Y4Y5) and (b) media volume vs. residence time (Y4Y6) on the percentage of degradation of Catechol in case of *Bacillus paramycoides*

IVC.2.7 Effects of inoculums size and residence time:

In case of *Bacillus pseudomycoides*, inoculums size and residence time both showed important effects on percent of degradation of Catechol. Degradation percent increased with increasing inoculums percent. Up to 6% size of inoculums, degradation percent increased simultaneously. After that, degradation percent suspended even after increasing the inoculums size. Similarly in case of residence time, degradation percent increased with increased residence time up to 72 hour. Beyond that, no big changes appeared rather negative effects arose. Almost complete degradation of 600 mg/L Catechol was achieved within 72 hours of time involving 6percent of inoculums. P- Values of these model terms (X5 & X6) were 0.0062 and 0.0065 respectively (Table IVC.7) which implied their significant roles on the response individually. Also, the interaction effect of these two factors (X5X6) revealed significant effect on the degradation percent of Catechol. Figure: IVC.12 showed an umbrella shaped curve which denoted its significance. Also the p- value of this model term was obtained to be < 0.0001 (Table IVC.7) indicating the significant effect of the interaction.

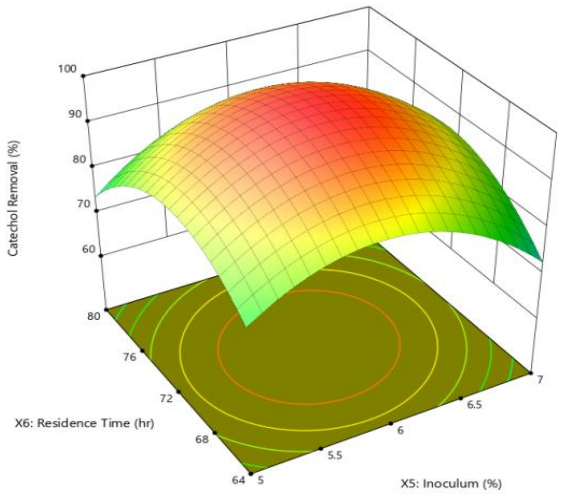


Figure IVC.12: 3-Dimensional surface and 2-Dimensional plot of the interaction effects of inoculums percent and residence time (X5X6) on percent of degradation of Catechol in case of *Bacillus pseudomycoides*

Similarly in case of *Bacillus paramycoides*, inoculums size and residence time both found to show important effects on percent of degradation of Catechol. Degradation percent increased when inoculums size was increased. Here also, up to 6percent inoculums, degradation percent increased steadily. After that, degradation percent stopped almost even after increasing the inoculums size further. Also in case of residence time, degradation percent increased with increased residence time up to 72 hours. Beyond that, no big changes appeared; rather negative effects appeared i.e. degradation percentage decreased. Almost 99% degradation of 600 mg/L Catechol was obtained within 72 hours of incubation time and 6percent inoculums. P- Values of these model terms (Y5 & Y6) were also found to be 0.0027 and 0.0073 respectively while performing ANOVA (Table IVC.8) which implied their significant roles on the response individually. Also, the interaction effect in between these two factors (Y5Y6) revealed significant effect on the degradation percent of Catechol. Figure: IVC.13 showed an umbrella shaped curve which denoted its significance. Also the p- value of this model term was found to be 0.0203 (Table IVC.8) indicating the significant effect of this interaction.

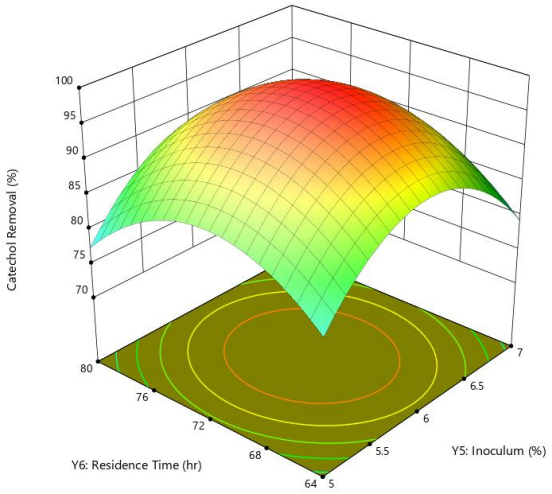


Figure IVC.13: 3-Dimensional surface and 2-Dimensional plot of the interaction effects of inoculums percent and residence time (Y5Y6) on percent of degradation of Catechol in case of *Bacillus paramycoides*

IVC.2.8 Optimization of the operating parameters:

The Response Surface Methodology (RSM) was involved in case of the two isolated strains (*Bacillus pseudomycoides* & *Bacillus paramycoides*) to deduce the optimum conditions for the six independent variables to achieve maximum percent of degradation of Catechol. Equation 4.9 was defined as objective function for the percent of degradation of Catechol in case of *Bacillus pseudomycoides* strain NBRC 101232 and the independent factors in their ranges were model constraints. Thus the following optimum conditions, achieved for the maximum percent of degradation of Catechol were: 600 mg/L initial conc. of Catechol, 5.5 pH of the bacterial culture, 35^{o}C temperature, 600 mL volume of the culture media, 6percent inoculums size of the strain (i.e. *Bacillus pseudomycoides* strain NBRC 101232) and 72 hours of residence time. 99.52% degradation of the Catechol was obtained by involving these six favourable situations. The obtained optimal operating conditions were performed nine more times to validate the predicted values as well to minimise the error and only 4.3% error was obtained indicating the reliability of this model.

Similarly in case of *Bacillus paramycoides*, equation 4.10 was defined as objective function for the percent of degradation of Catechol and the independent factors in their ranges were model constraints. Thus the following optimum conditions, achieved for the maximum percent of degradation of Catechol in case of *Bacillus paramycoides* strain MCCC 1A04098,

were: 600 mg/L initial conc. of Catechol, 5.5 pH of the bacterial culture, 25^{o}C temperature, 500 mL volume of the culture media, 6percent inoculums size of the strain (i.e. *Bacillus paramycoides* strain MCCC 1A04098) and 72 hours of residence time. 98.56% degradation of the Catechol was obtained by involving these six favourable situations. The obtained optimal operating conditions were performed nine more times to validate the predicted values as well to minimise the error and only 3.5% error was achieved in each of experiments indicating the reliability of this model.

Conclusions:

The Response Surface Methodology (RSM) was applied to optimize the process parameters in case of all the six isolated strains individually. The developed statistical as well as mathematical model is able to provide a comprehensive exploration of the cross- factor interactive effects of the independent variables (six parameters) on the response. Maximum cross-factors were found to be significant and some of them were very much significant on the response i.e. degradation percentage of Phenol (in case of *Brevibacillus formosus* & *Pseudomonas otitidis*) or 4- Chloro Phenol (in case of *Bacillus timonensis* & *Bacillus cereus*) or Catechol (in case of *Bacillus pseudomycoides* & *Bacillus paramycoides*) whichever was applicable. The proposed model, explaining the treatment of pharmaceutical waste water by involving six strains individually, was found to be suitable for the future studies to design a bioreactor, modelling. Experiment in pilot- scale is also recommended for the future study. Furthermore, here only single strains were utilized against single Phenolic substances. In future, two or three of these substances can be treated together by involving a consortium of the above mentioned strains.

Chapter V

Determination of growth kinetics of the isolated species while degrading the toxic Phenolic substances

Introduction:

From the previous chapter, the optimization of the operating parameters were deducted by Response Surface Methodology (RSM) in case of the all the six isolated bacterial strains. In some favourable parameters, up to a certain concentration of toxic Phenolic compounds (Phenol or 4- Chloro Phenol or Catechol) can be degraded by those microbial strains. But at high concentrations, Phenolic substances exhibit inhibition effects on the cultures (Hill and Robinson; 1975 & Yang and Humphrey; 1975). Numerous processes that impact the entire microbial growth process have been linked to the suppression of microbes by substrates (Edward, 1970). Thus, determination of the substrate inhibition has become an important factor in case of biodegradation of the toxic Phenolic compounds in culture media. Different types of mathematical models are there to determine the inhibitory effects of the toxic substrates (such as Phenol, 4- Chloro Phenol, Catechol etc) on the growth kinetics of microbes (Nweke and Okpokwasili, 2014). Some of them are: Haldane model, Monode kinetic model, Han- Levenspiel model, Luong model, Edward model, Yano- Koga model, Sum kinetic model etc. According to Goudar et al., 2000, Haldane model is the most common equation to describe the growth inhibition kinetics of microbes.

Kumar et al., 2005 conducted the studies of biodegradation kinetics of Phenol and Catechol by deploying *Pseudomonas putida* strain MTCC 1194. Haldane's growth kinetics model validated the data of growth kinetics there. Decay coefficients were found to be 0.0056 h^{-1} & 0.0067 h^{-1} respectively in case of Phenol and Catechol. Yield coefficients for the growth of Phenol Catechol were found to be 0.65 and 0.50 respectively.

Several studies have been conducted to deduce the growth inhibition kinetics of a varieties of microbes while degrading Phenolic substances. Nweke and Okpokwasili, 2014 conducted a study to investigate the kinetics of growth on two microbial strains *Pseudomonas* sp. DAF_1 and *Pseudomonas* sp. RWW_2 in batch culture at 1000 mg/L initial conc. of phenol. The growth was narrated by Haldane model. In case of *Pseudomonas* sp. DAF_1, kinetics constants were: $\mu_m = 0.025\ h^{-1}$, $K_s = 12.718$ mg/L & $K_i = 1632.086$ mg/L. On the other hand, in case of *Pseudomonas* sp. RWW_2, those constants were found to be: $\mu_m = 0.024\ h^{-1}$, $K_s = 14.628$ mg/L & $K_i = 2986.159$ mg/L. the average yield of biomass ($Y_{x/s}$) in the culture medias was 0.001 λ_{600} units.L/mg. Both the cultures followed the substrate inhibition kinetics there.

Pseudomonas putida 548's biodegradation of batch phenol was examined by Monterio et al., 2000. They found that the length of the lag phase increases linearly with initial phenol

concentration, and they varied the initial phenol concentration from 1 to 100 mg/L. It was discovered that *Pseudomonas putida* has a maximum specific growth rate of 0.436 h^{-1}.

In the current study, *Brevibacillus formosus* strain NRRL NRS-863 and *Pseudomonas otitidis* strain MCC10330 were utilized to determine the inhibition effects of Phenol on these two strains and growth kinetics of these two strains against Phenol. Similarly, *Bacillus timonensis* strain 10403023 and *Bacillus cereus* strain K1 were deployed in case of 4- Chloro Phenol and *Bacillus pseudomycoides* strain NBRC 101232 & *Bacillus paramycoides* strain MCCC 1A04098 were deployed in case of Catechol to determine their inhibitory effects.

Sub chapter V A

Determination of growth kinetics of the Phenol degrading strains and inhibitory effect of Phenol

VA.1 Materials and Methods:

VA.1.1 Materials:

Same as mentioned in chapter III, section IIIA.1.1.

VA.1.2 Experimental set up:

All the experiments were performed at 1L conical flasks with cotton plugged. Readymade Mineral Salt Medium (MSM) was utilized as the culture media. Composition and preparatory method of this medim has been described in chapter II, section IIA.4.3. pH of the media was adjusted by using 1N HCl and 1N NaOH as per requirement. To maintain the temperature and dynamic condition, BOD incubator shaker was used for the experiments with an average speed of 140- 150 RPM. Out of the six parameters, five parameters (Temperature, pH, Time, Media volume & Inoculums size) were maintained as the centre points of the RSMs (Chapter IV) of the respective microbial strains because at the centre points, maximum degradations were obtained. Only the initial conc. of Phenol was varied to determine the inhibition effect. The five selected parameters in case of the two microbial strains have been described in Table VA.1.

Table VA.1: Five optimized parameters in case of two microbial strains

Name of the strain	Temperature (^{o}C)	pH	Incubation time (hour)	Media volume (mL)	Inoculums size (percent)
Brevibacillus formosus strain NRRL NRS-863	30	7	40	500	6
Pseudomonas otitidis strain MCC10330	40	7	40	500	6

In case of *Brevibacillus formosus*, the strain was cultured in eight different concentrations. In one medium, Phenol was not added (i.e. control). Initial Phenol conc. in rest of the seven mediums were maintained as 50 mg/L, 100 mg/L, 200 mg/L, 400 mg/L, 500 mg/L, 600 mg/L & 800 mg/L respectively. In case of *Pseudomonas otitidis*, 50 mg/L, 100 mg/L, 200 mg/L,

400 mg/L, 600 mg/L, 800 mg/L & 1000 mg/L initial conc. of Phenol was maintained in seven different mediums respectively along with a control. The highest initial conc. of Phenol was maintained as 800 mg/L and 1000 mg/L in case of *Brevibacillus formosus* and *Pseudomonas otitidis* respectively because, these two initial concentrations were obtained at the centre points of RSM respectively. Periodically after 2 hours, all the samples were analyzed for biomass (for all the samples including the controls) and degradation percentage of Phenol (excluding the controls).

VA.1.3 Analytical Method:

The method of determination of residual Phenol in the media was same as mentioned in chapter II, section IIA.1.7. Cell mass was analyzed at 600 nm wavelengths in spectrophotometer.

VA.1.4 Calculations:

The rates of the Phenol degradation (Q_s) were calculated from the plots of S_0 - S_t (amount of degraded Phenol) vs. $t - t_0$ (incubation time). Specific growth rates were analyzed from the plots of $\ln(X/X_0)$ vs. $t - t_0$ for each initial concentrations of Phenol. The yield coefficients (Y) were calculated by plotting $X - X_0$ vs. $S_0 - S$.

$$Q_s = \frac{S0 - S}{t - t0} \qquad V.1$$

$$\ln \frac{X}{X0} = \mu\,(t - t_0) \qquad V.2$$

$$X - X_0 = Y\,(S_0 - S) \qquad V.3$$

Here, S_0 = initial conc. of Phenol at t_0 time (mg/L)

S = Conc. of Phenol at time t (mg/L)

X= Conc. Of biomass at λ_{600} at time t

X_0 = Conc. Of biomass at λ_{600} at time t_0

Microbial growth representation by Haldane equation:

$$\mu = \frac{\mu_m S}{K_s + S + \frac{S^2}{K_i}} \qquad \text{V.4}$$

Here, μ = specific growth rate in h^{-1}

S= Substrate conc. in mg/L

μ_m = maximum specific growth rate in h^{-1}

K_s = half saturation coefficient in mg/L

K_i = inhibition coefficient in mg/L

Now, the production of biomass can be represented by:

$$\frac{dx}{dt} = \mu X \qquad \text{V.5}$$

Substituting the μ in the equation no V.5 from the equation no V.4,

$$\frac{dx}{dt} = \frac{\mu_m SX}{K_s + S + \frac{S^2}{K_i}} \qquad \text{V.6}$$

From the equation no V.3, it can be represented that,

$$S = S_0 - \frac{X - X_0}{Y} \qquad \text{V.7}$$

Here, X = conc. of biomass

Y = yield coefficient (x/s)

S = substrate conc.

t = time

S_0 = initial substrate conc.

X_0 = initial biomass conc.

Now, in the equation no V.6, S can be substituted by from the equation no V.7

$$\frac{dx}{dt} = \frac{\mu_m \left(S_0 - \frac{X - X0}{Y}\right)X}{K_s + \left(S_0 - \frac{X - X0}{Y}\right) + \frac{\left(S_0 - \frac{X - X0}{Y}\right)^2}{K_i}} \qquad \text{V.8}$$

Now integrating the both side of the equation no V.8, equation V.9 obtained,

$$\mu_m t = \left[\frac{KsY}{YS_0 + X_0} + 1 + \frac{YS_0 + X_0}{K_i Y}\right] \ln \frac{X}{X_0} - \frac{KsY}{YS_0 + X_0} \ln \frac{YS_0 + X_0 - X}{YS_0} \qquad \text{V.9}$$

The rate of biodegradation can be represented as

$$\frac{ds}{dt} = -\mu \frac{X}{Y} \qquad \text{V.10}$$

Now, substituting the μ from the equation no V.4,

$$\frac{ds}{dt} = - \frac{\mu_m SX}{\left(K_S + S + \frac{S^2}{K_i}\right)Y} \quad \text{V.11}$$

Applying mass balance,

$$X = Y\,(S_0 - S) + X_0 \quad \text{V.12}$$

Now, in the equation no V.11, X can be substituted from the equation no V.13

$$\frac{ds}{dt} = - \frac{\mu_m S\,[Y(S_0 - S) + X_0}{\left(K_S + S + \frac{S^2}{K_i}\right)Y} \quad \text{V.13}$$

Now, integrating the equation no V.13, equation no V.14 can be achieved,

$$\mu_m t = \left[\frac{KsY}{YS_0 + X_0} + 1 + \frac{YS_0 + X_0}{K_i Y}\right] \ln \frac{YS_0 + X_0 - YS}{X_0} - \frac{KsY}{YS_0 + X_0}\, ln\, \frac{S}{S_0} \quad \text{V.14}$$

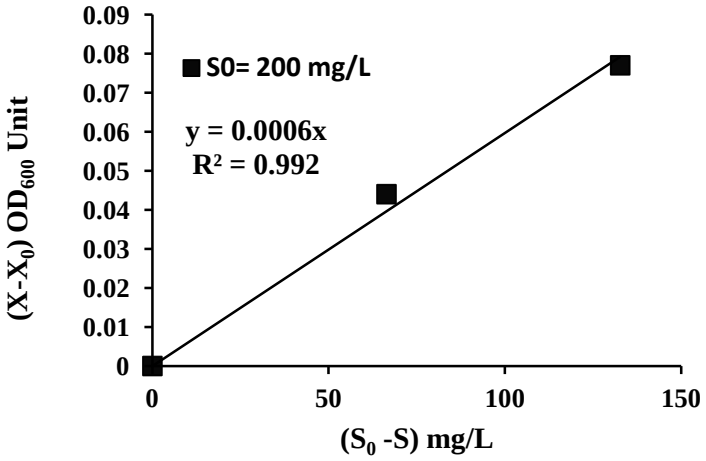
Equation V.9 was fitted with the biomass production data, and Table Curve 2D v5.01 was used in an iterative approach to estimate the kinetic parameters. The progress curve analysis started with basic estimations derived from the yield coefficients found in the X-X_0 versus S_0-S plots. Equation V.14 describes the kinetic parameters that were derived from the biomass production data in order to model substrate depletion.

VA.2 Results and Discussions:

VA.2.1 Degradation of Phenol & biomass production:

Two bacterial strains were utilized in this study. For *Brevibacillus formosus*, the highest initial conc. of Phenol was 800mg/L and in case of *Pseudomonas otitidis*, it was 1000 mg/L. Incubation time varied with respect to the initial conc. of Phenol. *Brevibacillus formosus* and *Pseudomonas otitidis*, both strains spent 40 hours to remove 800 mg/L and 1000 mg/L Phenol respectively. When the initial conc. of Phenol was low, it was removed within very

short time span. *Brevibacillus formosus* completely removed 50 mg/L and 100 mg/L Phenol within just 2 hours. And thereby, 200 mg/L, 400 mg/L, 500 mg/L and 600 mg/L Phenol was degraded by this strain within 6hours, 18 hours, 24 hours and 30 hours respectively. On the other hand, *Pseudomonas otitidis* degraded 50 mg/L and 100 mg/L Phenol within 2 hours. 200 mg/L, 400 mg/L, 600 mg/L and 800 mg/L Phenol was degraded by this strain completely within 6 hours, 16 hours, 28 hours and 32 hours respectively. Usually, degradation of Phenol was faster in case of *Pseudomonas otitidis*, as it degraded 1000 mg/L Phenol within 40 hours while same time span was utilized by *Brevibacillus formosus* to degrade 800 mg/L Phenol. The effects of initial conc. of the Phenol on the growth of the two strains with respect to that of the biomass yield have been shown in Figure: VA.1 & VA.2 for *Brevibacillus formosus* & *Pseudomonas otitidis* respectively.The yield of biomass per unit mass of consumed Phenol ($Y_{x/s}$) have been displayed in the Table VA.2. Maximum yield of 0.0014 OD units. L/mg was achieved by *Brevibacillus formosus* when initial conc. was 800 mg/L.



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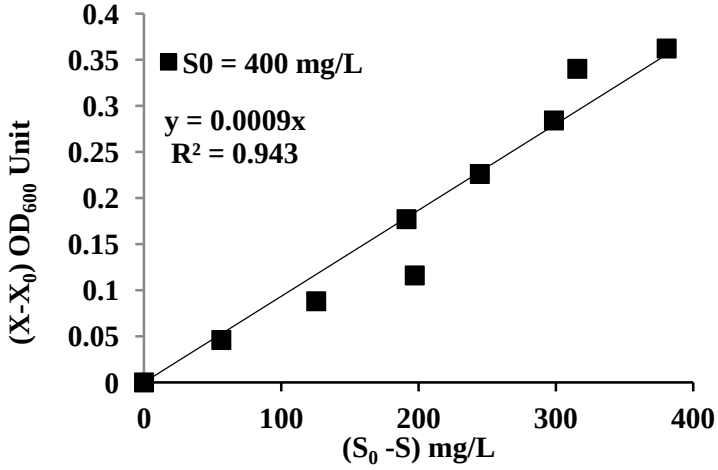

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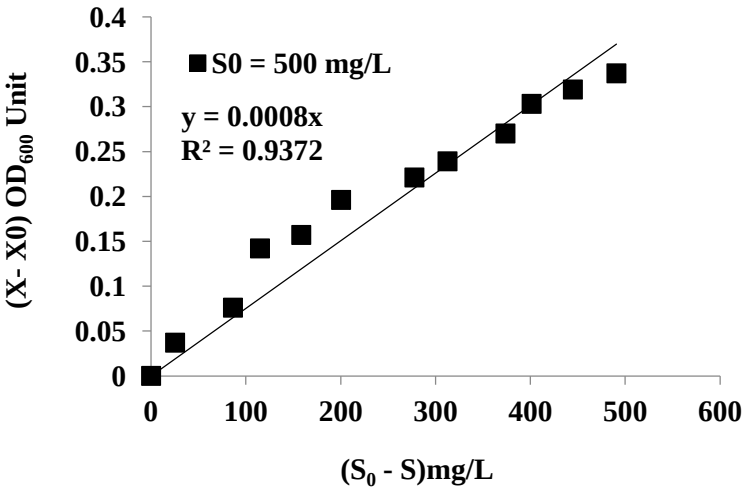

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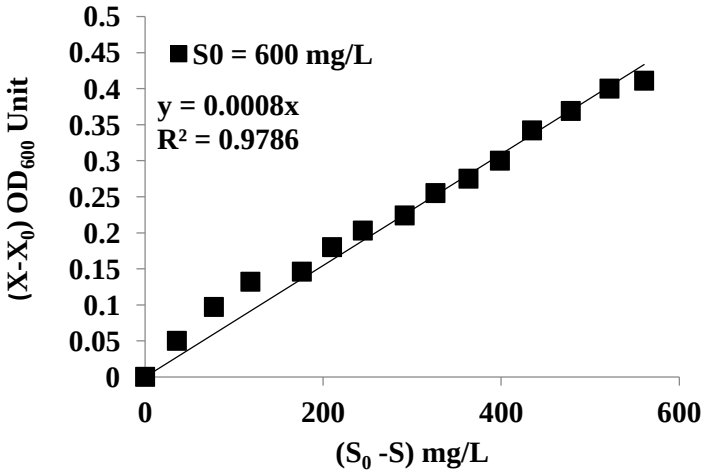

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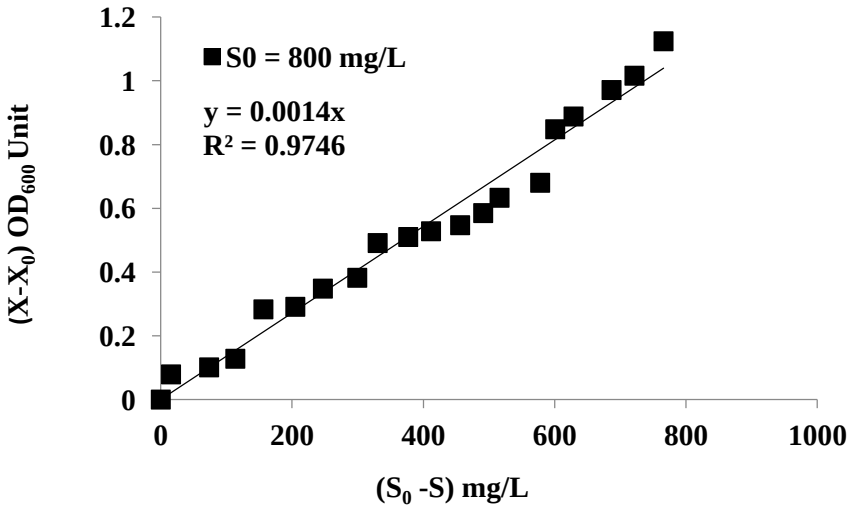

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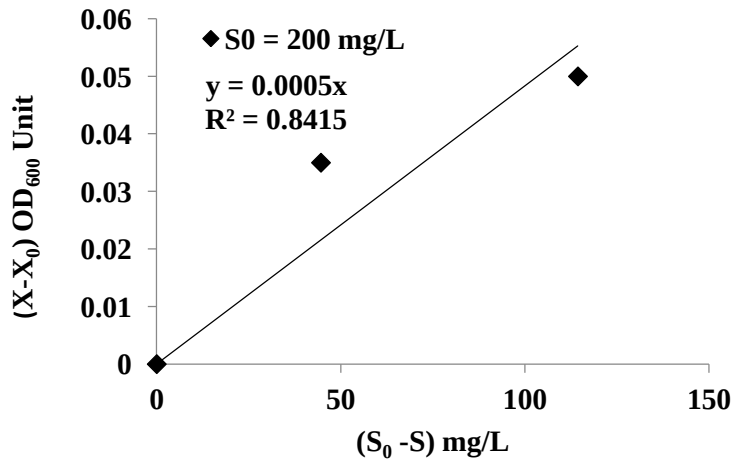
Figure VA.1: Yield coefficients for *Brevibacillus formosus*; growth on Phenol



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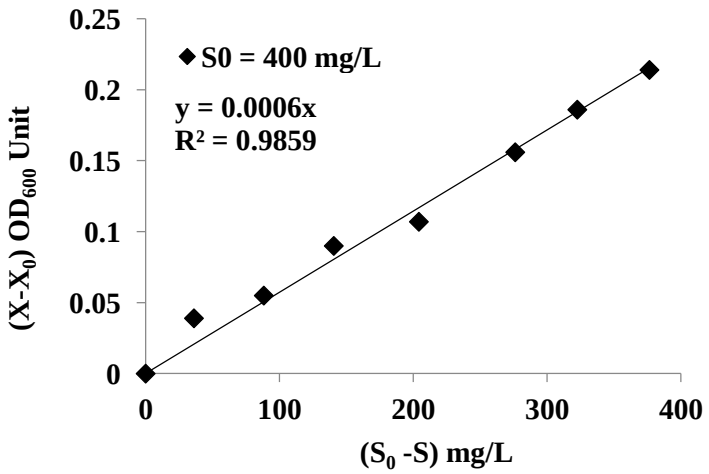

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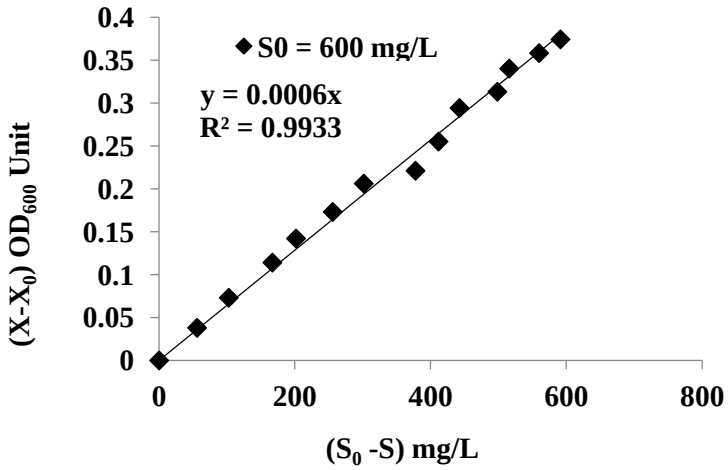

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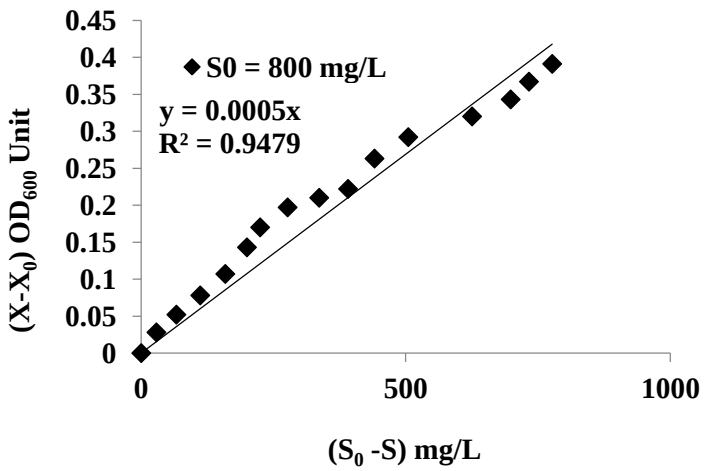

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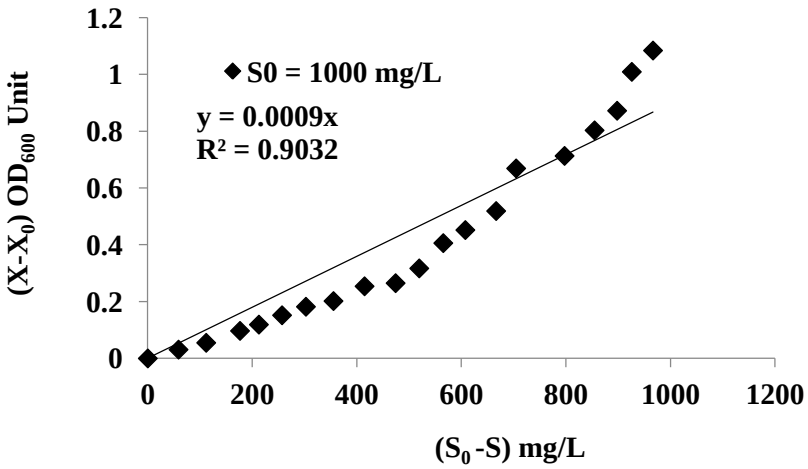

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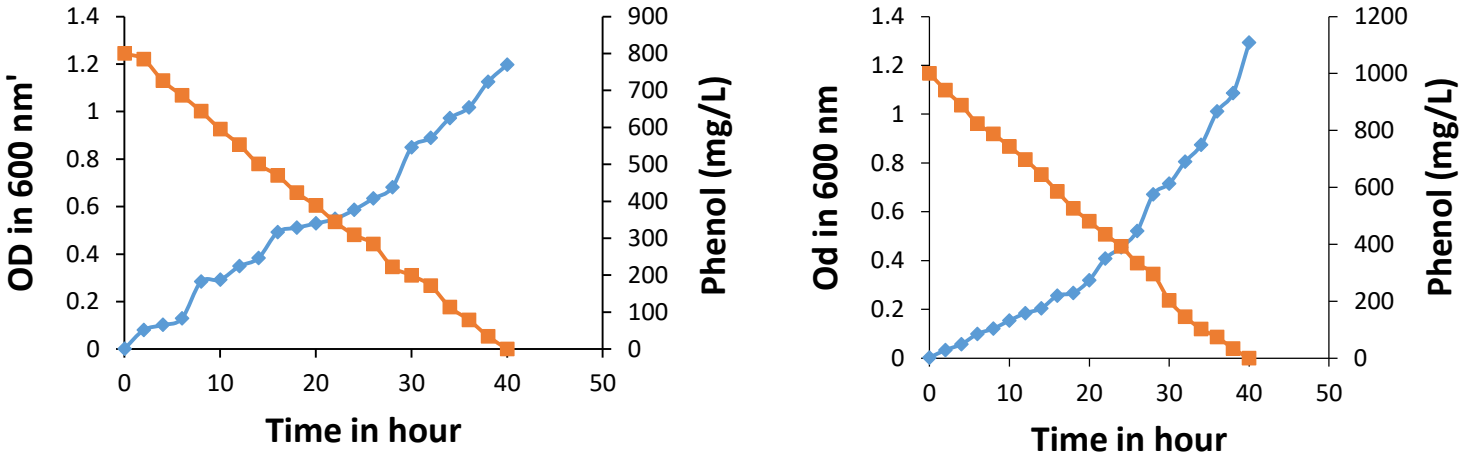
Figure VA.2: Yield coefficients for *Pseudomonas otitidis*; growth on Phenol

Table VA.2: Yield coefficients for the bacterial growth on the Phenol determined from the plots

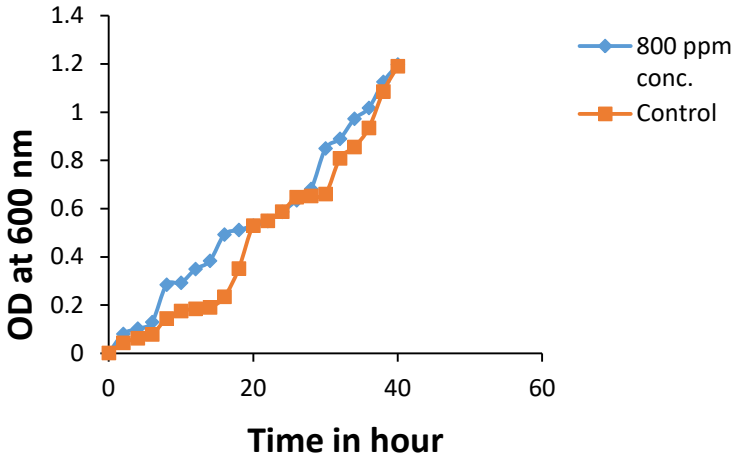
S_0 (mg/L)	Yield coefficients ($Y_{x/s}$) [l_{600} units. L/mg]	
	Brevibacillus formosus	***Pseudomonas otitidis***
200	0.0006	0.0005
400	0.0009	0.0006
500	0.0008	Nil
600	0.0008	0.0006
800	0.0014	0.0005
1000	Nil	0.0009

In case of both the strains, yield coefficients were not deducted for 50 mg/L and 100 mg/L initial conc. of Phenol. The time duration of the microbial growth and Phenol degradations for 800 mg/L (in case of *Brevibacillus formosus***)** and 1000 mg/L (in case of *Pseudomonas otitidis***)** have been shown in Figure VA.3. The growth profile of *Brevibacillus formosus* and *Pseudomonas otitidis* both in the highest Phenol conc. and control medium has been displayed in Figure VA.4. OD value and the incubation time to reach the stationary phase depend upon the initial conc. of Phenol. The highest optical density (λ_{600}) of 1.197 and 1.293 were attained by *Brevibacillus formosus* and *Pseudomonas otitidis* respectively at 800 mg/L and 1000 mg/L Phenol respectively. The corresponding highest OD values of 1.125 & 1.086 for the two respective strains were obtained at the same initial concentrations of Phenol i.e. 800 mg/L and 1000 mg/L respectively. The similar values of yield coefficients achieved for the two respective microbial strains at different initial conc. of Phenol indicate that the highest OD value depends upon the initial conc. of Phenol. The growths had exponential and stationary phase with increasing lag phase. Duration of the lag phase increased with the increasing initial conc. of Phenol and in turn, increasing the incubation time to attain the complete degradation of Phenol.



(a) (b)

Figure VA.3: Time duration of the microbial growth and Phenol degradation in case of (a) *Brevibacillus formosus* and (b) *Pseudomonas otitidis*



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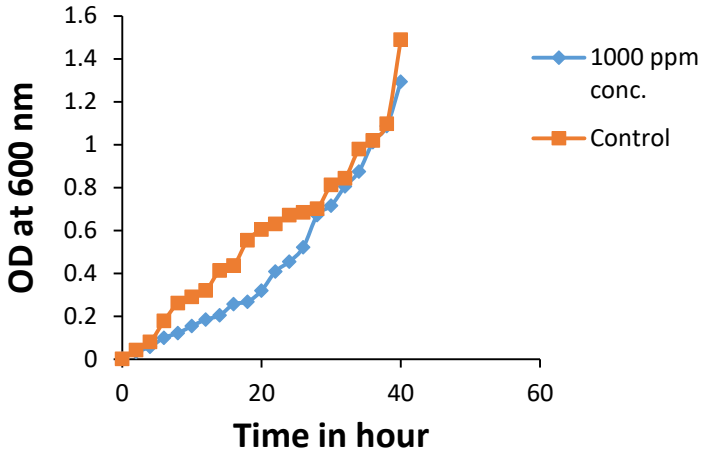
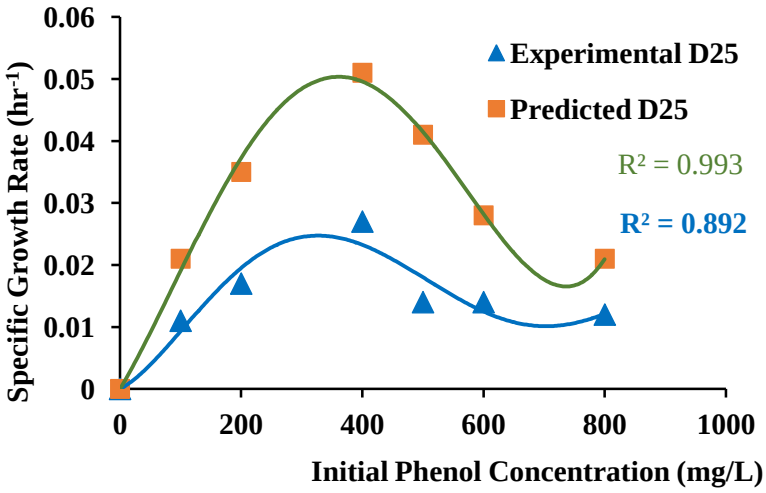


(b)

Figure VA.4: The growth profile of (a) *Brevibacillus formosus* and (b) *Pseudomonas otitidis* in the highest Phenol conc. and control medium

VA.2.2 Kinetics of degradation of Phenol and production of biomass:

The growth and degradation data might be explained by an integrated Haldane's substrate inhibition model with R^2 values more than 0.9, according to the progress curve analysis. The pattern of substrate inhibition was followed by the growth of the bacterial strain and the biodegradation of phenol. The growth rate and Phenol degradation was initially increased when the concentration of Phenol increased. After that, both the rates decreased subsequently with the increasing conc. of Phenol. The Haldane model described overall growth of D108 i.e. *Pseudomonas otitidis* (R^2 = 0.9551) better than that of the D25 i.e. *Brevibacillus formosus* where R^2 = 0.892 at the S_0 range of 0 – 1000 mg/L and 0 – 800 mg/L respectively. The kinetic parameters of the Haldane model have been displayed in Table V.1. Haldane model with R^2 values of 0.9959 and 1 for *Brevibacillus formosus* & *Pseudomonas otitidis* respectively described the overall degradation rates very well.



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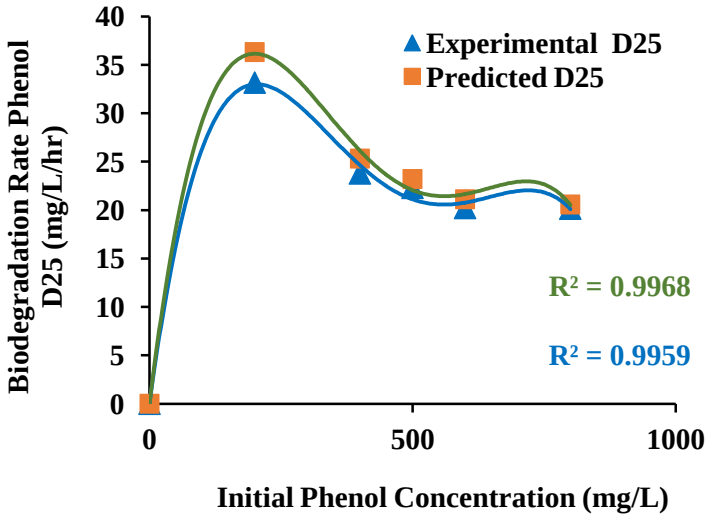

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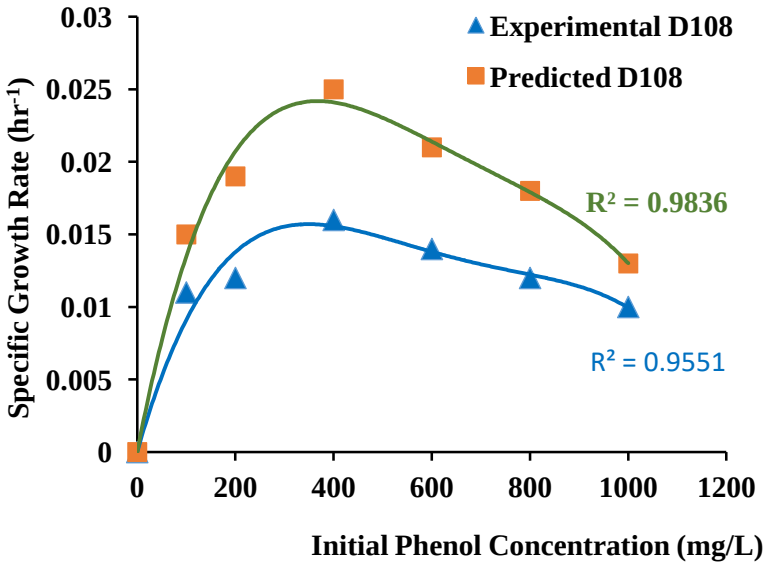

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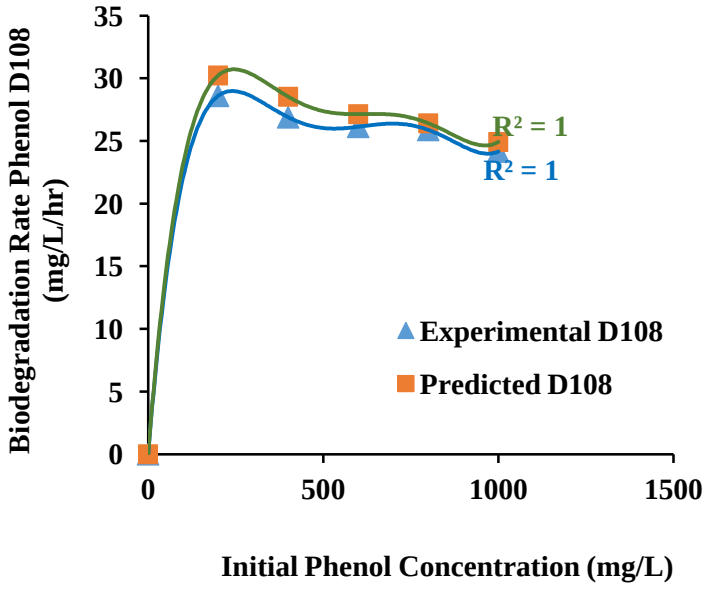


(d)

Fig VA.5: Experimental and predicted specific growth and degradation rate of *Brevibacillus formosus* strain NRRL NRS-863 and *Pseudomonas otitidis* strain MCC10330 during the biodegradation of Phenol

Sub chapter VB

Determination of growth kinetics of the 4- Chloro Phenol degrading strains and inhibitory effect of 4- Chloro Phenol

VB.1 Materials and Methods:

VB.1.1 Materials:

Same as mentioned in chapter III, section IIIB.1.1.

VB.1.2 Experimental set up:

The five selected parameters in case of the two microbial strains have been described in Table VB.1.

Table VB.1: Five optimized parameters in case of two microbial strains

Name of the strain	Temperature (^{o}C)	pH	Incubation time (hour)	Media volume (mL)	Inoculums size (percent)
Bacillus timonensis strain 10403023	40	7.5	24	500	6
Bacillus cereus strain K1	25	6.5	24	400	8

In case of *Bacillus timonensis*, the strain was cultured in seven different concentrations. Initial 4-Chloro Phenol conc. in the seven mediums were maintained as 50 mg/L, 100 mg/L, 200 mg/L, 400 mg/L, 500 mg/L, 600 mg/L & 800 mg/L respectively. In case of *Bacillus cereus*, 50 mg/L, 100 mg/L, 200 mg/L, 400 mg/L, 500 mg/L & 600 mg/L initial conc. of 4-Chloro Phenol was maintained in six different mediums respectively. A control medium was kept in each of the two strains. The highest initial conc. of 4- Chloro Phenol was maintained as 800 mg/L and 600 mg/L in case of *Bacillus timonensis* and *Bacillus cereus* respectively because, these two initial concentrations were obtained at the centre points of RSM respectively. Periodically after 2 hours, all the samples were analyzed for biomass and degradation percentage of 4- Chloro Phenol.

Rest of the portion is same as described in the sub chapter VA, section VA.1.2.

VB.1.3 Analytical Method:

The method of determination of residual 4- Chloro Phenol in the media was same as mentioned in chapter II, section IIA.1.7. Cell mass was analyzed at 600 nm wavelengths in spectrophotometer.

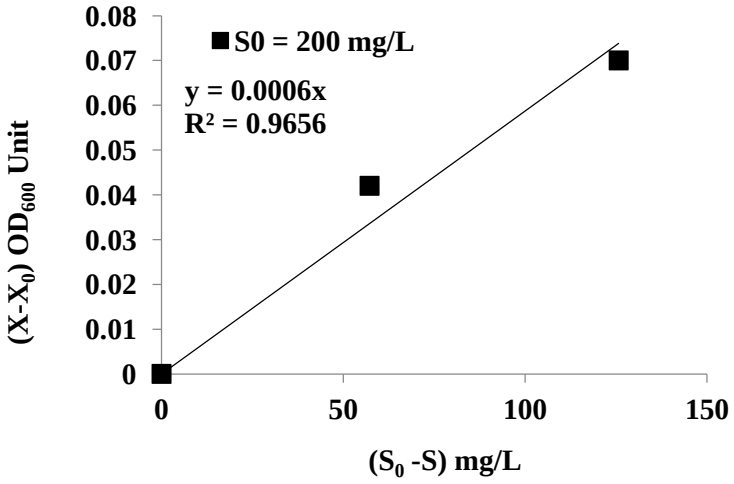
VB.1.4 Calculations:

Same as described the sub chapter VA, section VA.1.4.

VB.2 Results and Discussions:

VB.2.1 Degradation of 4- Chloro Phenol & biomass production:

Two bacterial strains were utilized in this study. For *Bacillus timonensis*, the highest initial conc. of 4- Chloro Phenol was 800mg/L and in case of *Bacillus cereus,* it was 600 mg/L. Incubation time varied with respect to the initial conc. of 4- Chloro Phenol. *Bacillus timonensis* and *Bacillus cereus,* both strains spent 24 hours to remove 800 mg/L and 600 mg/L 4- Chloro Phenol respectively. When the initial conc. of 4- Chloro Phenol was low, it was removed within very short time span. *Bacillus timonensis* completely removed 50 mg/L and 100 mg/L 4- Chloro Phenol within just 2 hours. And thereby, 200 mg/L, 400 mg/L, 500 mg/L and 600 mg/L 4- Chloro Phenol was degraded by this strain within 6 hours, 10 hours, 12 hours and 20 hours respectively. On the other hand, *Bacillus cereus* degraded 50 mg/L and 100 mg/L 4- Chloro Phenol within 2 hours. 200 mg/L, 400 mg/L and 500 mg/L of 4- Chloro Phenol was degraded by this strain completely within 6 hours, 10 hours and 16 hours respectively. Usually, degradation of 4- Chloro Phenol was faster in case of *Bacillus timonensis*, as it degraded 800 mg/L 4- Chloro Phenol within 24 hours while same time span was utilized by *Bacillus cereus* to degrade 600 mg/L 4- Chloro Phenol. The effects of initial conc. of the 4- Chloro Phenol on the growth of the two strains with respect to that of the biomass yield have been shown in Figure: VB.1 & VB.2 for *Bacillus timonensis* & *Bacillus cereus* respectively.The yield of biomass per unit mass of consumed 4- Chloro Phenol ($Y_{x/s}$) have been displayed in the Table VB.2. Maximum yield of 0.0013 OD units. L/mg was achieved by *Bacillus cereus* when initial conc. was 600 mg/L.



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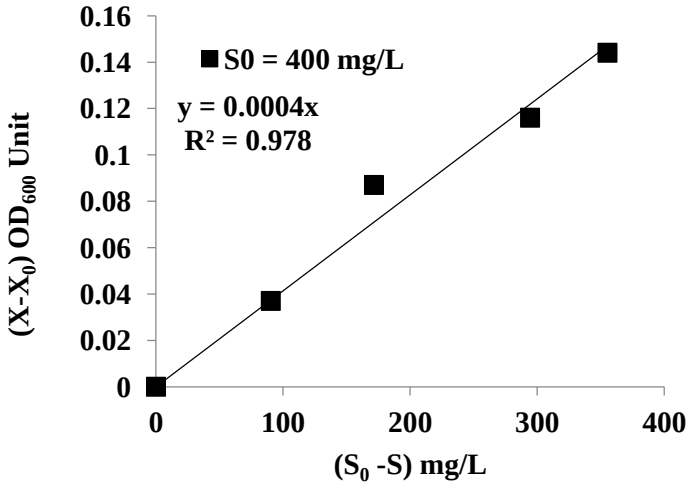

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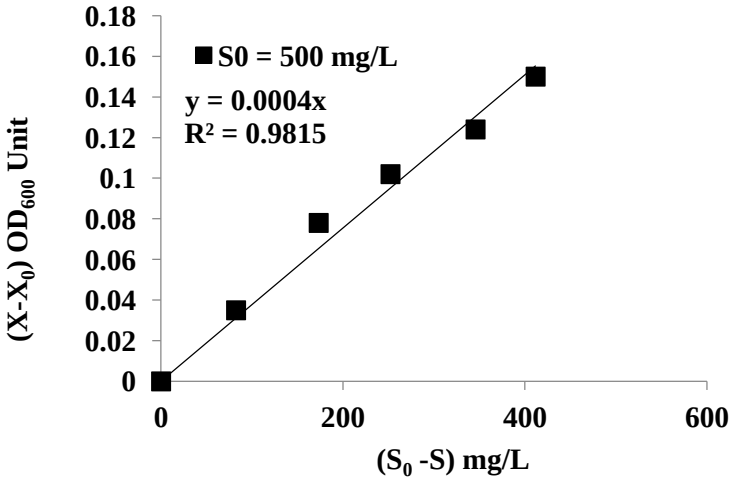

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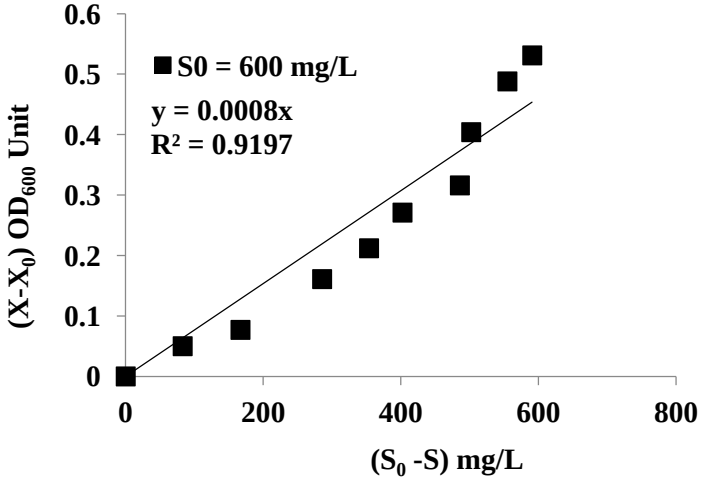

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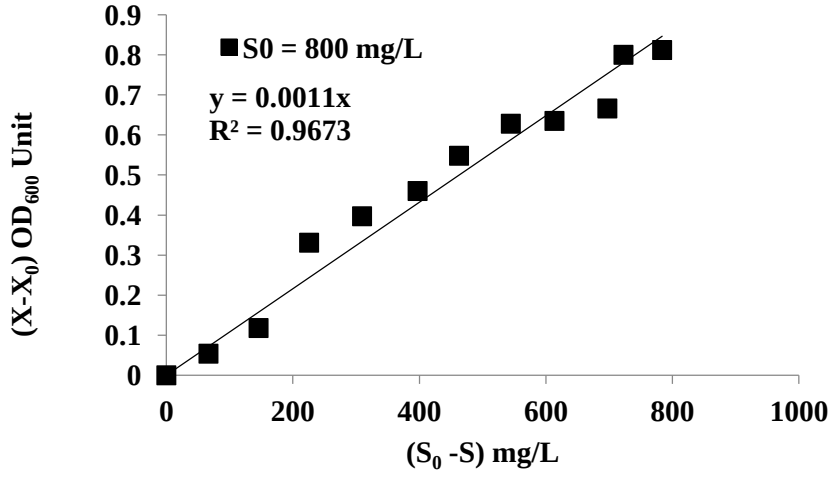
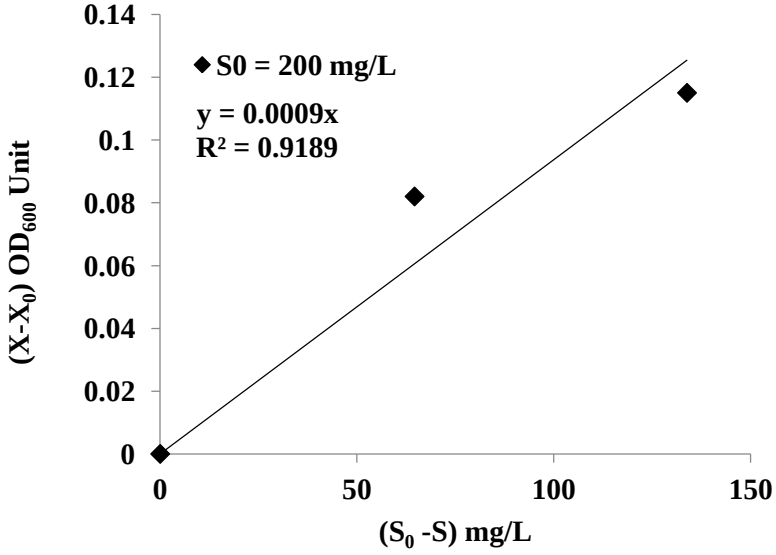


(e)

Figure VB.1: Yield coefficients for *Bacillus timonensis*; growth on 4- Chloro Phenol



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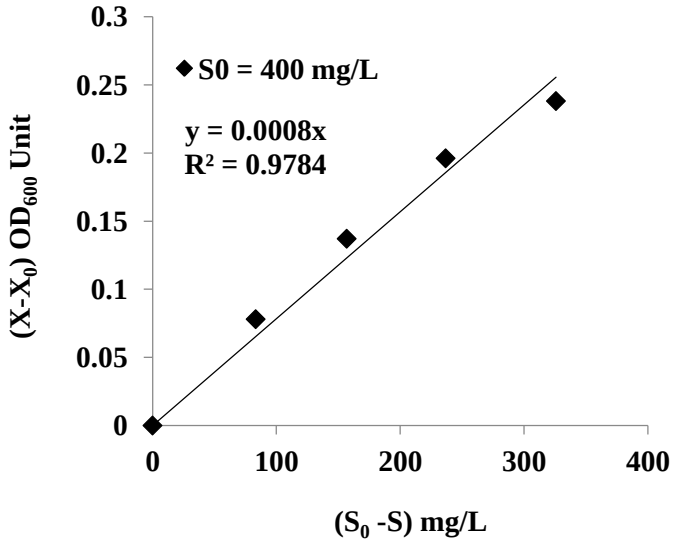

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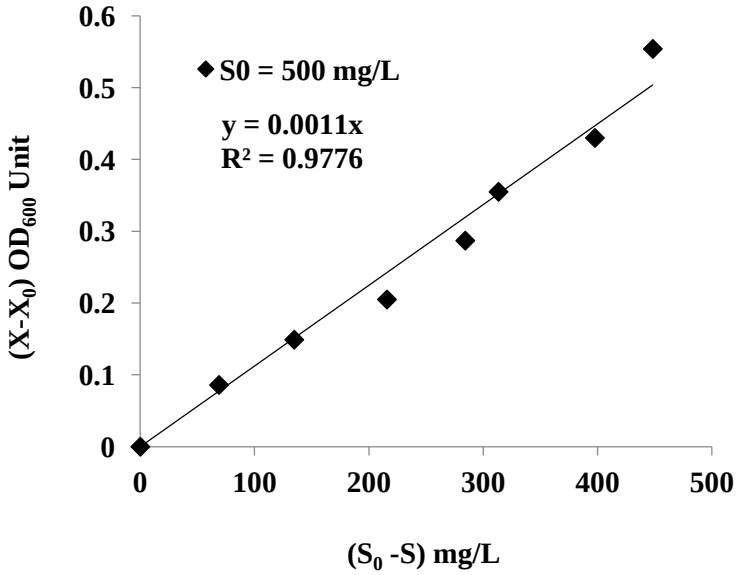

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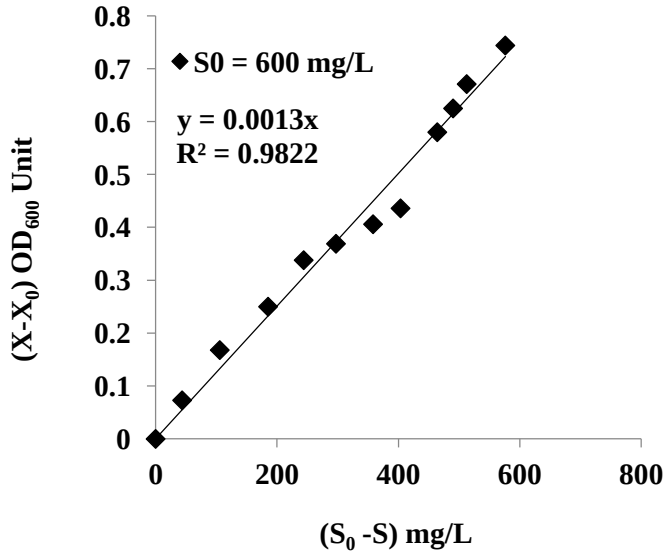


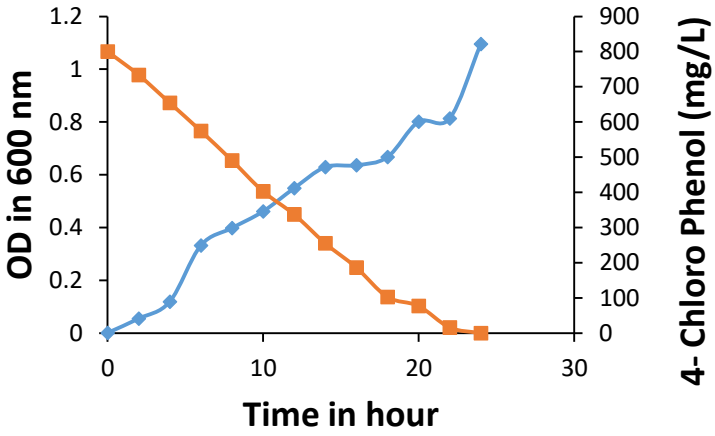
(d)

Figure VB.2: Yield coefficients for *Bacillus cereus*; growth on 4- Chloro Phenol

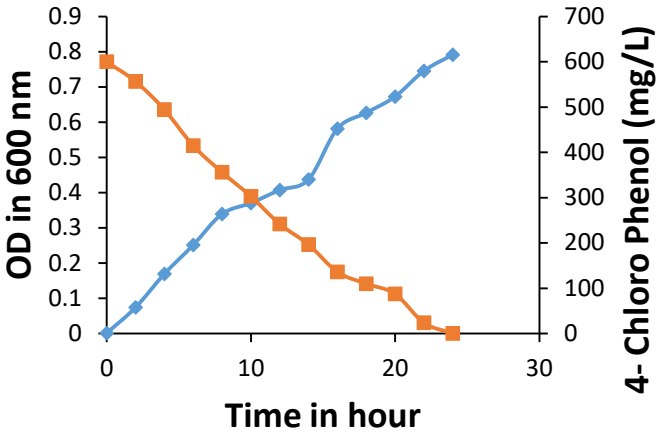
Table VB.2: Yield coefficients for the bacterial growth on the 4- Chloro Phenol determined from the plots

S_0 (mg/L)	Yield coefficients ($Y_{x/s}$) [l_{600} units. L/mg]	
	Bacillus timonensis	***Bacillus cereus***
200	0.0006	0.0009
400	0.0004	0.0008
500	0.0004	0.0011
600	0.0008	0.0013
800	0.0011	Nil

In case of both the strains, yield coefficients were not deducted for 50 mg/L and 100 mg/L initial conc. of 4- Chloro Phenol. The time duration of the microbial growth and 4- Chloro Phenol degradations for 800 mg/L (for *Bacillus timonensis*) and 600 mg/L (*Bacillus cereus*) have been shown in Figure VB.3. The growth profile of *Bacillus timonensis* and *Bacillus cereus* both in the highest Phenol conc. and control medium has been displayed in Figure VB.4. The highest OD value and the incubation time to reach the stationary phase depend upon the initial conc. of 4- Chloro Phenol. The highest optical density (λ_{600}) of 1.095 and 0.79 were attained by *Bacillus timonensis* and *Bacillus cereus* respectively at 800 mg/L and 600 mg/L 4-Chloro Phenol respectively. The corresponding highest OD values of 0.81 & 0.74 for the two respective strains were obtained at the same initial concentrations of 4-Chloro Phenol i.e. 800 mg/L and 600 mg/L respectively. The similar values of yield coefficients achieved for the two respective microbial strains at different initial conc. of 4-Chloro Phenol indicate that the highest OD value depends upon the initial conc. of 4- Chloro Phenol. The growths had exponential and stationary phase with increasing lag phase. Duration of the lag phase increased with the increasing initial conc. of 4- Chloro Phenol and in turn, increasing the incubation times to attain the complete degradation of 4- Chloro Phenol.

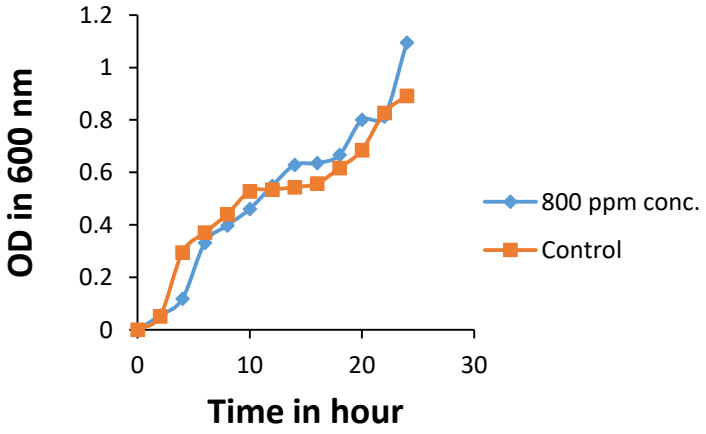


(a)



(b)

Figure VB.3: Time duration of the microbial growth and Phenol degradation in case of (a) *Bacillus timonensis* and (b) *Bacillus cereus*



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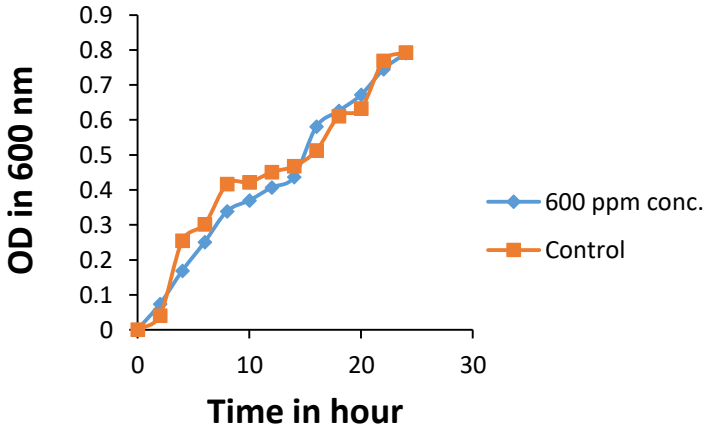


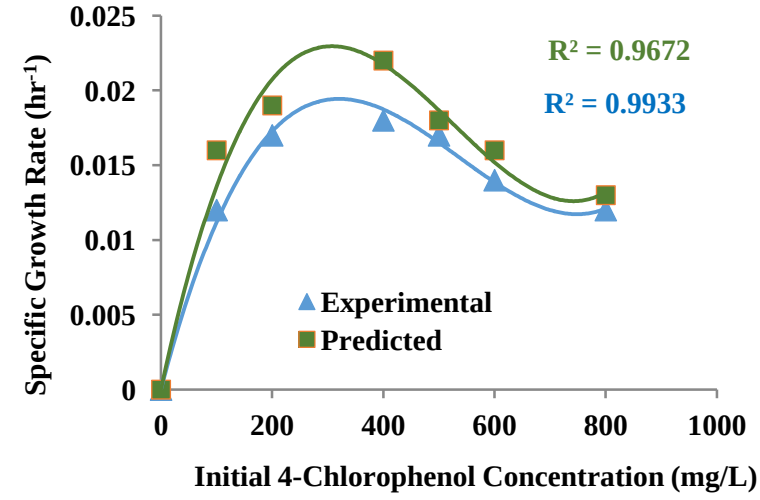
(b)

Figure VB.4: The growth profile of (a) *Bacillus timonensis* and (b) *Bacillus cereus* in the highest Phenol conc. and control medium

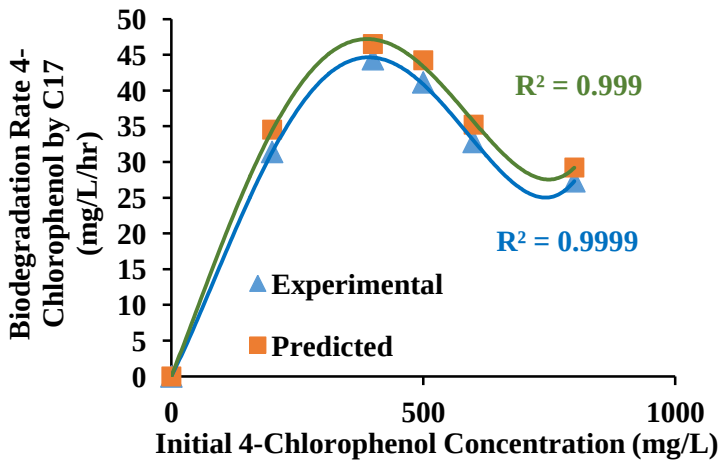
VB.2.2 Kinetics of degradation of 4- Chloro Phenol and production of biomass:

The growth and degradation data might be explained by an integrated Haldane's substrate inhibition model with R^2 values more than 0.9, according to the progress curve analysis. The pattern of substrate inhibition was followed by the growth of the bacterial strain and the

biodegradation of 4- Chloro phenol. The growth rate and 4- Chloro Phenol degradation was initially increased when the concentration of 4- Chloro Phenol increased. After that, both the rates decreased subsequently with the increasing conc. of 4- Chloro Phenol. The Haldane model described overall growth of C17 i.e. *Bacillus timonensis* (R^2 = 0.9933) better than that of the C19 i.e. *Bacillus cereus* where R^2 = 0.9858 at the S_0 range of 0 – 800 mg/L and 0 – 600 mg/L respectively. The kinetic parameters of the Haldane model have been displayed in Table V.1. Haldane model with R^2 values of 0.9999 and 1 for *Bacillus timonensis* & *Bacillus cereus* respectively described the overall degradation rates very well.



(a)



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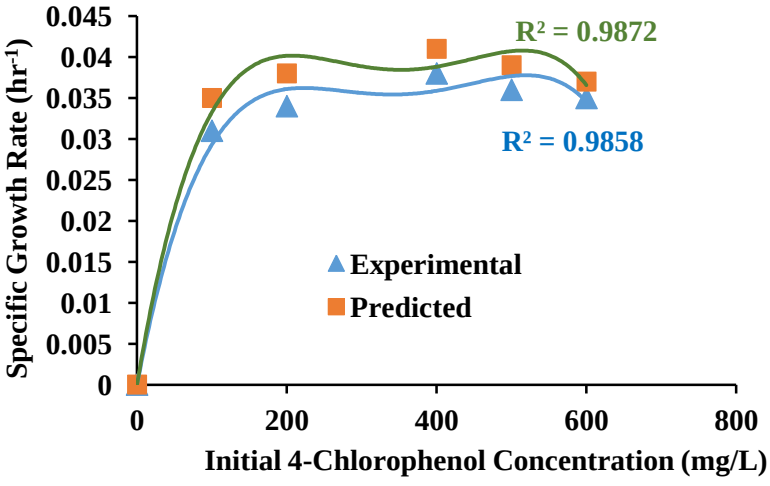

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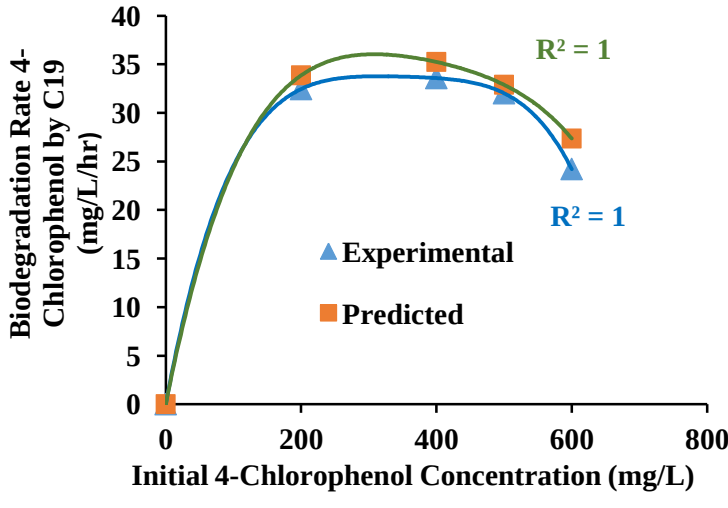


(d)

Fig VB.5: Experimental and predicted specific growth and degradation rate of *Bacillus timonensis* strain 10403023 and *Bacillus cereus* strain K1during the biodegradation of 4-Chloro Phenol

Sub chapter VC

Determination of growth kinetics of the Catechol degrading strains and inhibitory effect of Catechol

VC.1 Materials and Methods:

VC.1.1 Materials:

Same as mentioned in chapter III, section IIIC.1.1.

VC.1.2 Experimental set up:

The five selected parameters in case of the two Catechol degrading microbial strains have been described in Table VC.1.

Table VC.1: Five optimized parameters in case of two microbial strains

Name of the strain	Temperature (^{o}C)	pH	Incubation time (hour)	Media volume (mL)	Inoculums size (percent)
Bacillus Pseudomycoides strain NBRC 101232	35	5.5	72	600	6
Bacillus paramycoides strain MCCC 1A04098	25	5.5	72	500	6

In case of *Bacillus Pseudomycoides*, the strain was cultured in six different concentrations along with a control. Initial Catechol conc. in the six mediums were maintained as 50 mg/L, 100 mg/L, 200 mg/L, 400 mg/L, 500 mg/L & 600 mg/L respectively. In case of *Bacillus paramycoides* also, 50 mg/L, 100 mg/L, 200 mg/L, 400 mg/L, 500 mg/L & 600 mg/L initial conc. of Catechol was maintained in six different mediums respectively along with a control. The highest initial conc. of Catechol was maintained as 600 mg/L in case of both the strains because, these two initial concentrations were obtained at the centre points of RSM respectively. Periodically after 4 hours, all the samples were analyzed for biomass and degradation percentage of Catechol.

Rest of the portion is same as described in the sub chapter VA, section VA.1.2.

VC.1.3 Analytical Method:

The method of determination of residual Catechol in the media was same as mentioned in chapter II, section IIA.1.7. Cell mass was analyzed at 600 nm wavelengths in spectrophotometer.

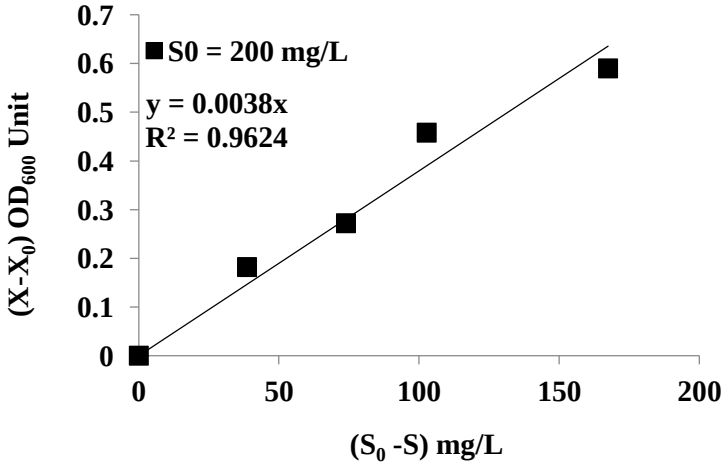
VC.1.4 Calculations:

Same as described the sub chapter VA, section VA.1.4.

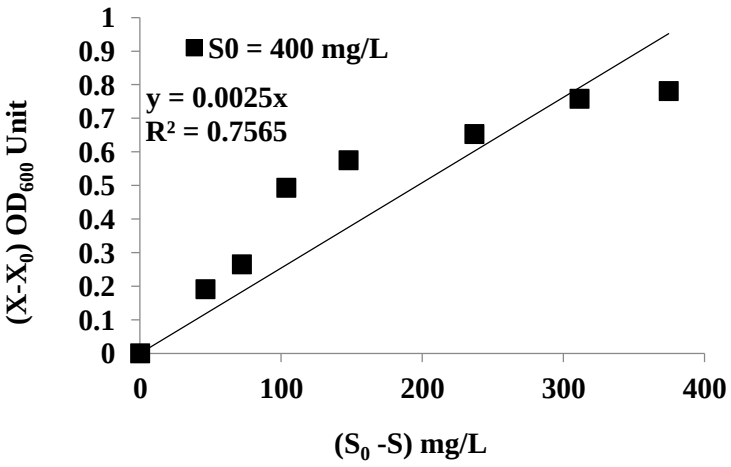
VC.2 Results and Discussions:

VC.2.1 Degradation of Catechol & biomass production:

Two bacterial strains were utilized in this study. For *Bacillus Pseudomycoides,* the highest initial conc. of Catechol was 600mg/L and in case of *Bacillus paramycoides,* it was also 600 mg/L. Incubation time varied with respect to the initial conc. of Catechol. *Bacillus Pseudomycoides* and *Bacillus paramycoides,* both strains spent 72 hours to remove 600 mg/L of Catechol respectively. When the initial conc. of Catechol was low, it was removed within very short time span. *Bacillus Pseudomycoides* completely removed 50 mg/L and 100 mg/L Catechol within just 4 hours. And thereby, 200 mg/L, 400 mg/L & 500 mg/L of Catechol was degraded by this strain within 20 hours, 32 hours and 60 hours respectively. On the other hand, *Bacillus paramycoides* degraded 50 mg/L and 100 mg/L Catechol within 4 hours as usual. 200 mg/L, 400 mg/L and 500 mg/L of Catechol was degraded by this strain completely within 20 hours, 32 hours and 52 hours respectively. Usually, degradation of Catechol was faster in case of *Bacillus paramycoides,* (up to 500mg/L of Catechol) as it degraded 500 mg/L Catechol within 52 hours while 60 hours time period was utilized by *Bacillus Pseudomycoides* to degrade 500 mg/L Catechol. The effects of initial conc. of the Catechol on the growth of the two strains with respect to that of the biomass yield have been shown in Figure: VC.1 & VC.2 for *Bacillus Pseudomycoides* & *Bacillus paramycoides* respectively.The yield of biomass per unit mass of consumed Catechol ($Y_{x/s}$) have been displayed in the Table VC.2. Maximum yield of 0.0042 OD units. L/mg was achieved by *Bacillus paramycoides* when initial conc. was 200 mg/L.



(a)



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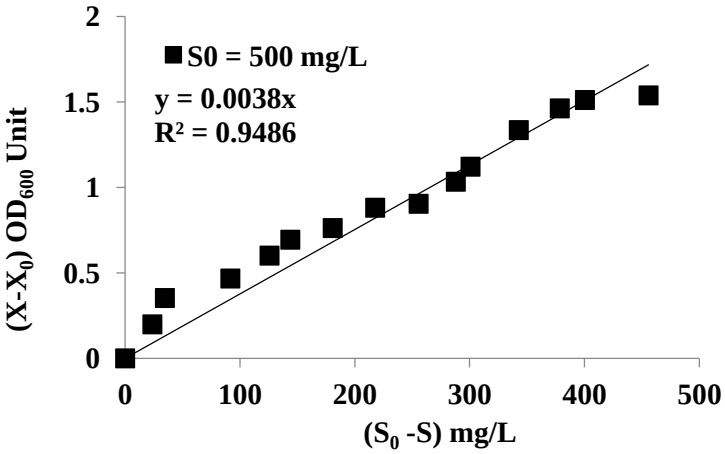

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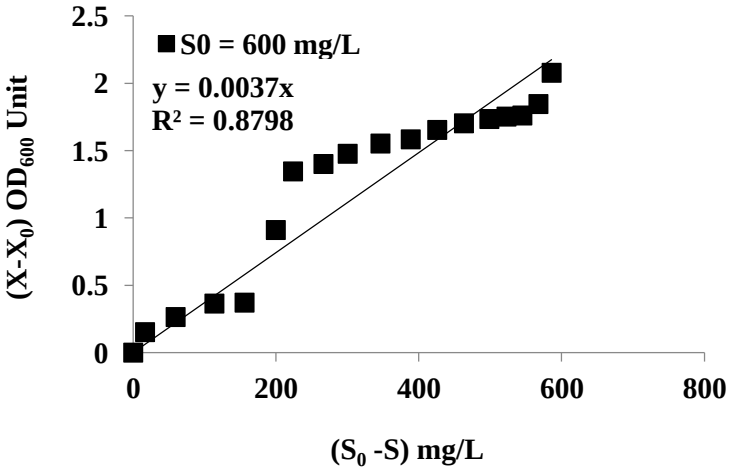
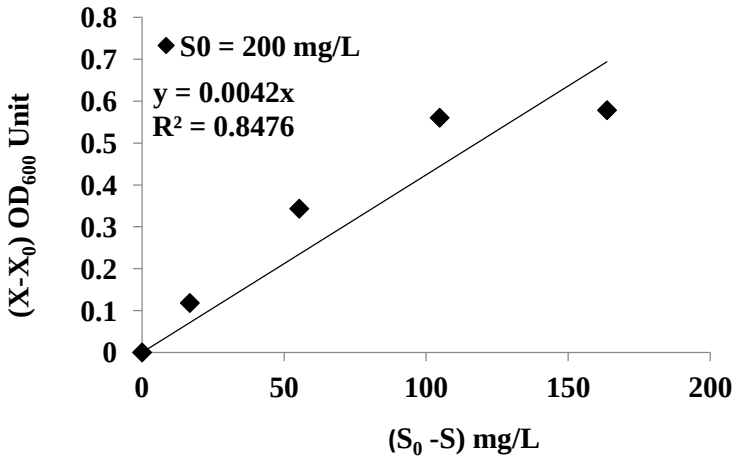


(d)

Figure VC.1: Yield coefficients for *Bacillus Pseudomycoides*; growth on Catechol



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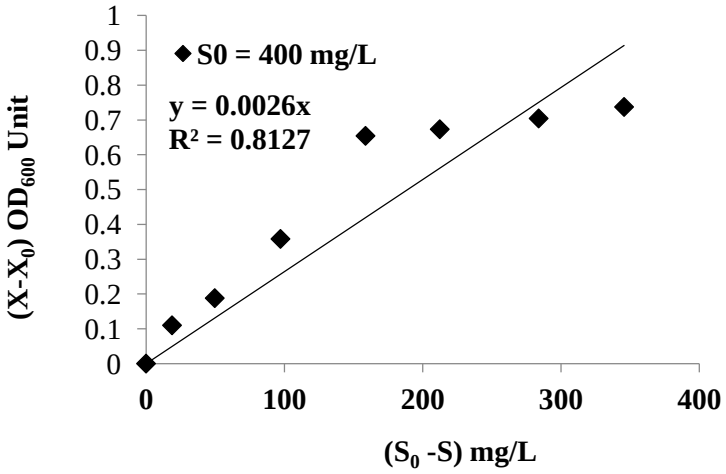

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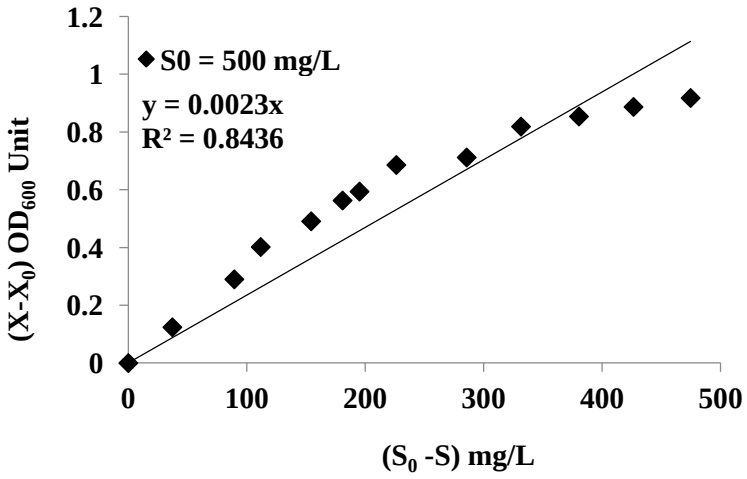

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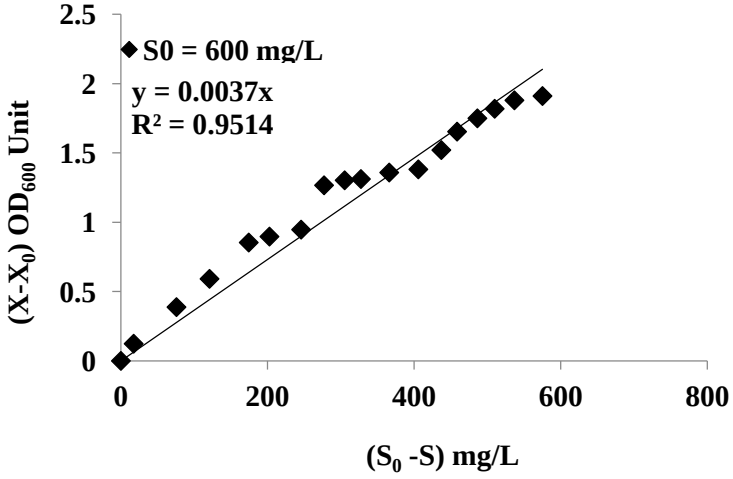


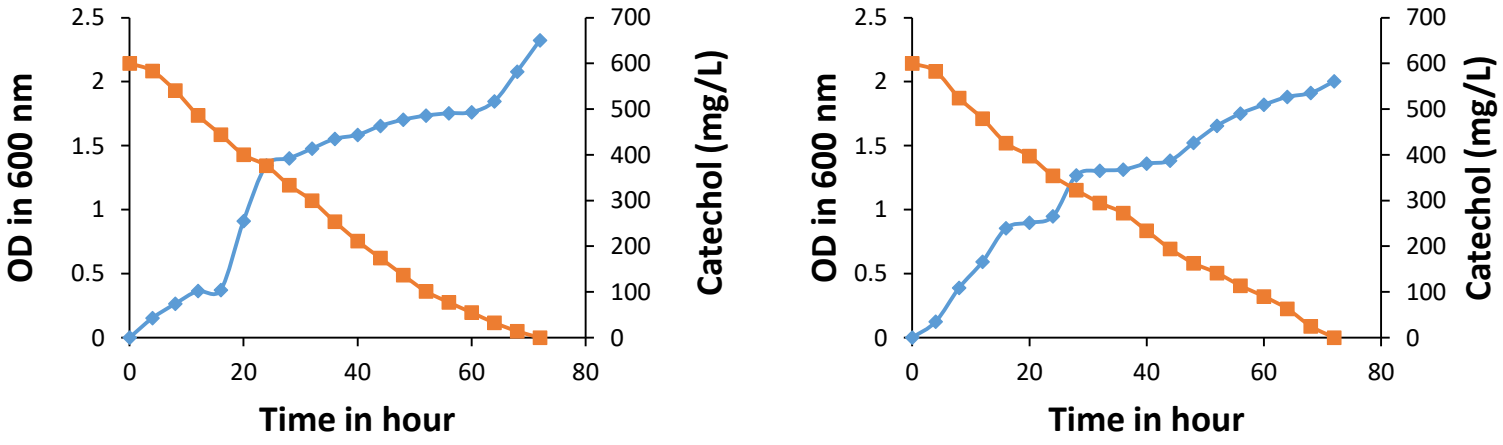
(d)

Figure VC.2: Yield coefficients for *Bacillus paramycoides*; growth on Catechol

Table VC.2: Yield coefficients for the bacterial growth on the Catechol determined from the plots

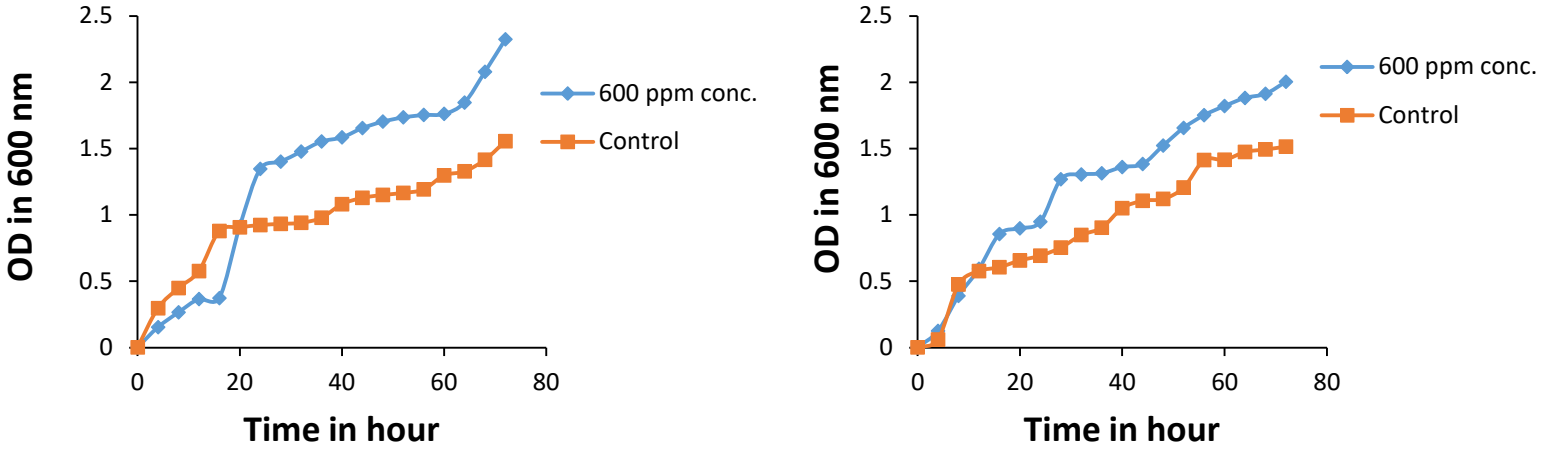
S_0 (mg/L)	Yield coefficients ($Y_{x/s}$) [l_{600} units. L/mg]	
	Bacillus Pseudomycoides	***Bacillus paramycoides***
200	0.0038	0.0042
400	0.0025	0.0026
500	0.0038	0.0023
600	0.0037	0.0037

In case of both the strains, yield coefficients were not deducted for 50 mg/L and 100 mg/L initial conc. of Catechol. The time duration of the microbial growth and Catechol degradations for 600 mg/L conc. By the two strains, have been shown in Figure VC.3. The growth profile of *Bacillus Pseudomycoides* and *Bacillus paramycoides* both in the highest Catechol conc. and control medium has been displayed in Figure VC.4. The highest OD value and the incubation time to reach the stationary phase depend upon the initial conc. of Catechol. The highest optical density (λ_{600}) of 2.323 and 2.003 were attained by *Bacillus Pseudomycoides* and *Bacillus paramycoides* respectively at 600 mg/L of Catechol. The corresponding highest OD values of 2.078 & 1.912 for the two respective strains were obtained at the same initial concentrations of Catechol i.e. 600 mg/L. The similar values of yield coefficients achieved for the two respective microbial strains at different initial conc. of Catechol indicate that the highest OD value depends upon the initial conc. of Catechol. The growths had exponential and stationary phase with increasing lag phase. Duration of the lag phase increased with the increasing initial conc. of Catechol and in turn, increasing the incubation time to attain the complete degradation of Catechol.



(a) (b)

Figure VC.3: Time duration of the microbial growth and Phenol degradation in case of (a) *Bacillus Pseudomycoides* and (b) *Bacillus paramycoides*



(a) (b)

Figure VC.4: The growth profile of (a) *Bacillus Pseudomycoides* and (b) *Bacillus paramycoides* in the highest Phenol conc. and control medium

VC.2.2 Kinetics of degradation of Catechol and production of biomass:

The growth and degradation data might be explained by an integrated Haldane's substrate inhibition model with R^2 values more than 0.9, according to the progress curve analysis. The pattern of substrate inhibition was followed by the growth of the bacterial strain and the biodegradation of Catechol. The growth rate and Catechol degradation was initially increased when the concentration of Catechol increased. After that, both the rates decreased subsequently with the increasing conc. of Catechol. The Haldane model described overall growth of S12 i.e. *Bacillus Pseudomycoides* (R^2 = 0.9991) better than that of the S37 i.e. *Bacillus paramycoides* where R^2 = 0.9875 at the S_0 range of 0 – 600 mg/L and 0 – 600 mg/L respectively. The kinetic parameters of the Haldane model have been displayed in Table V.1. Haldane model with R^2 values of 0.9356 and 0.9933 for *Bacillus Pseudomycoides* & *Bacillus paramycoides* respectively described the overall degradation rates very well.

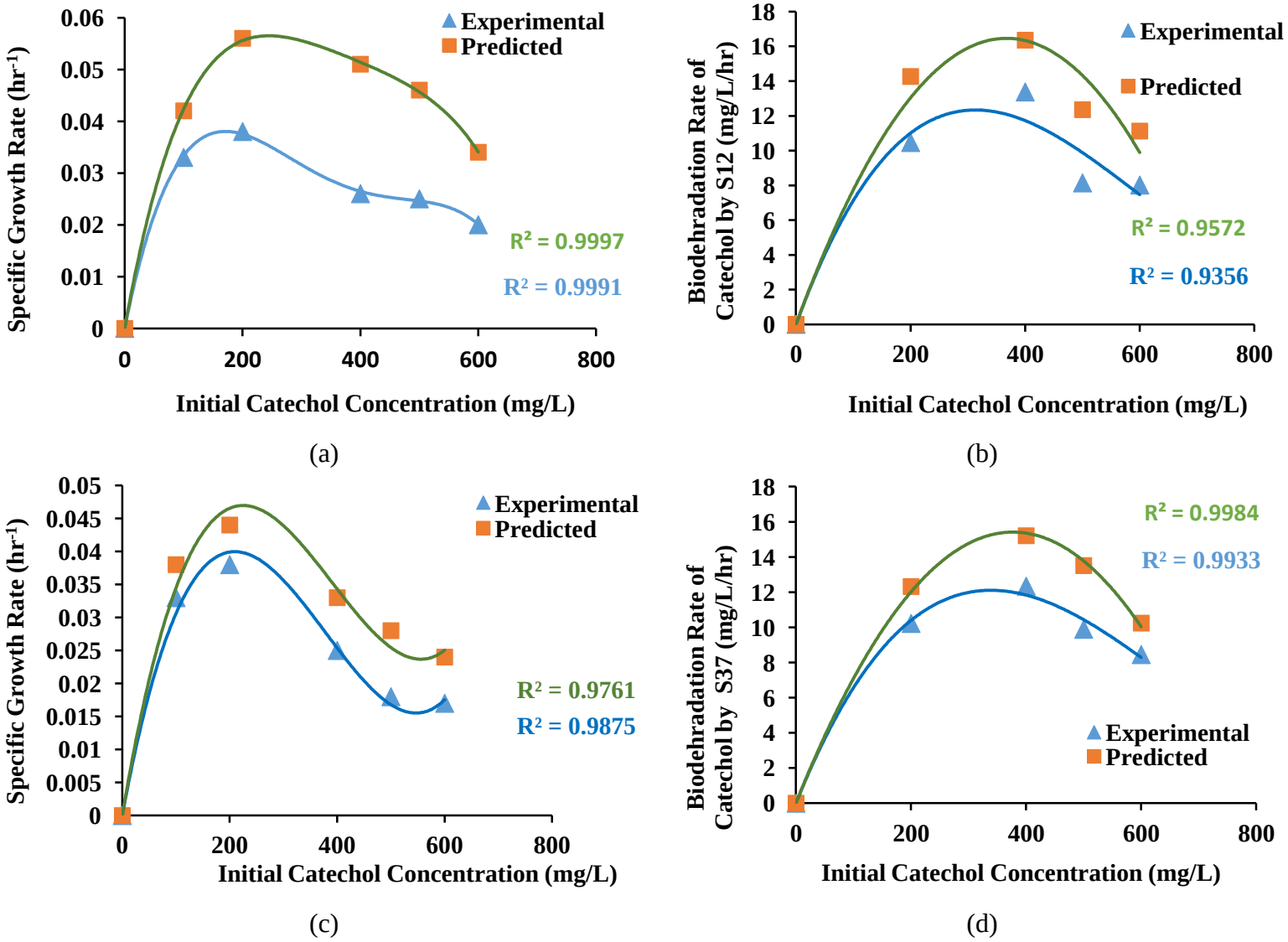


Fig VC.5: Experimental and predicted specific growth and degradation rate of *Bacillus Pseudomycoides* strain 10403023 and *Bacillus paramycoides* strain K1during the biodegradation of Catechol

Conclusions:

In this review, coordinated Haldane model with factors of most extreme specific growth rate, half immersion, biomass yield and inhibition constants were used to assess the progress of cell development during the degradation of phenol, 4- Chloro Phenol and Catechol respectively at various initial concentrations. In such manner, non-linear least squares mechanized curve fitting system of Table Curve 2D was utilized to gauge the kinetic parameters. The values of μ_m, K_s, K_i & $Y_{x/s}$ are displayed in Table V.1. For the most part, the Haldane model gave great description of the experimental information where $R^2 > 0.9$. From

the Table no V.1, it can be displayed that the values of the kinetic parameters, obtained in the current study, are almost similar to that of the previous studies. So, it can be concluded that the biomass production data were successfully fitted into the integrated Haldane model. Moreover, the obtained data implied that inhibitory effects of Phenol, 4- Chloro Phenol or Catechol arose at higher initial concentrations.

Table V.1: Haldane Kinetic parameters for the biodegradation of Phenol, 4- Chloro Phenol and Catechol in batch cultures of respective strains (Previous and current study)

Name of the bacterial strain	Initial conc. of Phenol (mg/L)	Initial conc. of 4-Chloro Phenol (mg/L)	Initial conc. of Catechol (mg/L)	μ_m (h^{-1})	K_s (mg/L)	K_i (mg/L)	Q_s (mg/l/h)	Reference
Pseudomonas putida Q5	0 - 200	Nil	Nil	0.119	5.27	377.0	-	Kotturi et al., 1991
Pseudomonas putida LY1	0 - 800	Nil	Nil	0.217	24.4	121.7	-	Li et al., 2010
Brevibacillus formosus strain NRRL NRS-863	0 - 800	Nil	Nil	0.052	22.25	116.35	36.51	Current study
Pseudomonas otitidis strain MCC10330	0 - 1000	Nil	Nil	0.024	20.58	118.65	30.03	Current study
Bacillus timonensis strain 10403023	Nil	0 – 800	Nil	0.024	26.54	124.65	48.75	Current study
Bacillus cereus strain K1	Nil	0 - 600	Nil	0.042	29.35	126.58	34.81	Current study
Bacillus Pseudomycoides strain NBRC 101232	Nil	Nil	0 - 600	0.056	28.52	126.5	17.01	Current study
Bacillus paramycoides strain MCCC 1A04098	Nil	Nil	0 - 600	0.046	24.24	136.4	15.91	Current study

Chapter VI

Optimization of parameters in case of Bi-solute & Tri-solute mixtures of the Phenolic compounds using consortium of microorganisms

Introduction:

In the previous chapters, it was described in brief how the six microbes were isolated and identified, and how some selected parameters were optimised in case of those single strains against single Phenolic compounds. As the current study is dealing with the bioremediation of pharmaceutical waste water, so definitely there will be a mixture of Phenolic compounds while the waste water is discharged into the nature. So, it should be kept in mind that a mixture of Phenolic compounds is to be treated (whether two or three or four compounds together) while designing a bioreactor. In case of single compounds as well as single strains, the parameters have already been optimized. Now, the same experiment of parameter optimization is to be performed in case of consortium of the earlier isolated bacterial strains against particular mixtures of previously selected Phenolic compounds.

Like that of the previous studies, here also the parameters were optimized in batch culture at first. In this chapter, bi- solute and tri- solute mixtures of the Phenolic compounds have been considered. As, six different bacterial strains were isolated against three different Phenolic substances respectively, in the current study, four different consortiums as well as three different bi- solute mixtures and a tri- solute mixture have been prepared. Those mixtures are as follows: **Phenol & 4- Chloro Phenol**, **4- Chloro Phenol & Catechol**, **Phenol & Catechol and Phenol & 4- Chloro Phenol & Catechol**. Four different types of consortiums were prepared in the following way: *Brevibacillus formosus* strain NRRL NRS- 863, *Pseudomonas otitidis* strain MCC10330, *Bacillus timonensis* strain 10403023 and *Bacillus cereus* strain K1 were utilized together to treat the bi- solute mixture of Phenol & 4- Chloro Phenol (as the first two microbes were isolated to treat Phenol as a single compound and the second two microbes were isolated to treat 4- Chloro Phenol as a single compound). Similarly, *Bacillus timonensis* strain 10403023, *Bacillus cereus* strain K1, *Bacillus pseudomycoides* strain NBRC 101232 and *Bacillus paramycoides* strain MCCC 1A04098 were utilized against the bi- solute mixture of 4- Chloro Phenol & Catechol. The consortium of *Brevibacillus formosus* strain NRRL NRS- 863, *Pseudomonas otitidis* strain MCC10330, *Bacillus pseudomycoides* strain NBRC 101232 and *Bacillus paramycoides* strain MCCC 1A04098 was deployed to treat the bi- solute mixture of Phenol & Catechol. And finally, the mixture of all the six bacterial strains was utilized to deal with the tri- solute mixture of Phenol & 4- Chloro Phenol & Catechol.

Sub chapter VI.A

Bioremediation of Phenol & 4- Chloro Phenol as a bi- solute mixture by involving a microbial consortium: optimisation of process parameters in batch reactor

VIA.1 Materials and Methods:

VIA.1.1 Materials:

As the mixture of Phenol & 4- Chloro Phenol was to be degraded, a bacterial consortium of *Brevibacillus formosus* strain NRRL NRS- 863, *Pseudomonas otitidis* strain MCC10330, *Bacillus timonensis* strain 10403023 and *Bacillus cereus* strain K1 were utilized in the study. All of these strains were isolated from the contaminated soil through enrichment method as described in the chapter II, section IIA.1.5. A large volume of inoculums of the four above mentioned strains were already prepared via acclimatization procedure in Mineral Salt Medium (MSM) as described in chapter II, section IIA.1.8.

Phenol and 4- Chloro Phenol solutions were prepared synthetically in the laboratory. Conc. of the stock solutions was maintained as 10 g/L in case of both.

VIA.1.2 Experimental set up:

Same as mentioned in the chapter III, section IIIA.1.2.

VIA.1.3 Analytical Method:

Percentage of degradation of the bi- solute mixture (Phenol & 4- Chloro Phenol) from the waste water was measured in the UV-VIS spectrophotometer at 510 nm wavelength followed by 4- Amino Antipyrene method as suggested by Yang, R.D., et al. 1975. For that purpose a standard curve as well as a standard equation was prepared with the known concentrations of the bi-solute mixture (i.e. Phenol & 4- Chloro Phenol) and the residual content of the bi-solute mixture in an unknown sample was calculated from that equation. Details of this process has been mentioned in chapter II, section IIA.1.7.

VIA.2 Results and Discussions:

Six parameters were considered for this study. Those were: temperature, pH, incubation time, media volume, inoculums size and initial conc. of the bi- solute mixture (i.e. Phenol & 4- Chloro Phenol).

In case of percent of inoculums, total percent of inoculums of the all four bacterial strains was taken into account where percent of each of the four strains was maintained equally (1:1:1:1 ratio). Similarly in case of initial conc. of the mixture to be removed i.e. Phenol and

4- Chloro Phenol, total initial conc. (mg/L) of the two compounds was used where individual initial conc. of the compounds was kept equal (1:1 ratio).

VIA.2.1 Effect of temperature:

The consortium of the four bacterial strains (*Brevibacillus formosus*, *Pseudomonas otitidis*, *Bacillus timonensis* and *Bacillus cereus*) was cultured in six different temperatures ranging between 25^{o}C to 50^{o}C with an interval of 5^{o}C to find out the most suitable one. 45^{o}C temperature was found to be the most favourable temperature for this consortium while removing the bi- solute mixture of Phenol & Catechol. Almost complete degradation was achieved in this temperature when the initial conc. of the mixture was 500 mg/L (250 mg/L Phenol & 250 mg/L 4- Chloro Phenol). Below and above that temperature, the degradation efficacy of the consortium decreased. In 25^{o}C, 90.25% degradation was obtained and in 30^{o}C, 94.25% degradation was achieved. Similarly, when temperature increased, gradual increase in the degradation percentage was observed. 96.25%, 98.57% degradations were achieved in 35^{o}C and 40^{o}C temperature respectively. And above the optimum temperature, the degradation efficacy again decreased. ~95% degradation was observed when the temperature was 50^{o}C (Fig: VIA.1 (a)).

VIA.2.2 Effect of pH:

Almost complete degradation was achieved at pH 8.5 which was found to be the optimal pH for this consortium. Three tests were performed in acidic pH where pH was maintained at 6.5 & 6.0 & 5.5. 94.37%, 94% & 91.68% degradations were observed in those acidic pH mediums respectively. In neutral pH (i.e. pH 7), around 94.6% degradation was observed. Finally, four experiments were carried out in basic pH mediums where pH values were 7.5, 8.0, 8.5 & 9.0 and 94.62%, 95.63%, 99.99% & 89.64% of degradations were found from those pH mediums respectively (Fig: VIA.1 (b)) indicating the optimal pH at 8.5.

VIA.2.3 Effect of inoculums size:

Size of inoculums played a vital role in degrading the mixture. Here in the study, a very small range of variation was obtained while changing the percentage of inoculums. 89.26% and 90.22% degradations were observed when the inoculums size was 2% (0.5% inoculums of each of the four strains) and 4% (1% inoculums of each of the four strains) respectively. 92.37% and 95.56% degradations were achieved when the inoculums size was increased up to 6% & 8% respectively. When 10% inoculums added to the media, 95.74% degradation was observed which was almost unchanged with respect to that of the previous inoculums size. It was increased up to 99.99%, when 12% inoculums were added. But, in case of 14% inoculums, degradation percentage again decreased to around 95% indicating the 12% inoculums size (3% inoculums of each of the strains) to be the optimum inoculums size for this consortium while degrading the bi- solute mixture of Phenol & 4- Chloro Phenol (Fig: VIA.1 (c)). As mentioned above, in each case of inoculums size, 1:1:1:1 ratio was maintained properly.

VIA.2.4 Effect of incubation time:

One of the most vital parameter was the incubation time. Five (5) different times were selected for the study viz. 12 hr, 24 hr, 48 hr, 72 hr and 96 hr. A wide variation was found during this study. Only 63.41% degradation of the mixture was observed after 12 hour and after 24 hour, it increased up to 88.39%. After 48 hours of incubation, ~100% of degradation was achieved but after 72 and 96 hours of incubation, 98.03% & 94.56% degradations were obtained which implied that degradation percent decreased. So, the optimal time for this consortium was found to be 48 hours (Fig: VIA.1 (d)).

VIA.2.5 Effect of media volume:

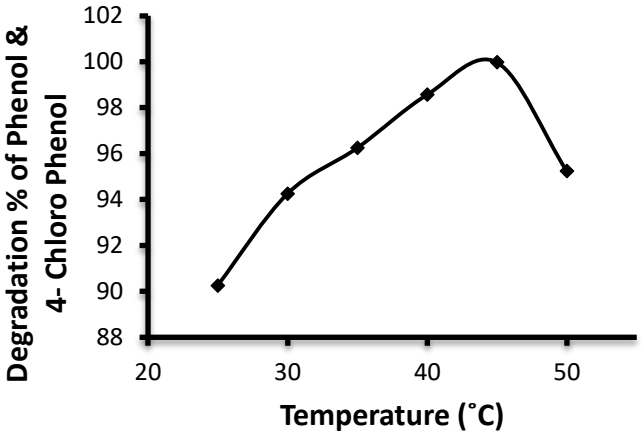
Five different volumes of media were utilized to check the effect of the media volume on the degradation efficacy of the bacterial consortium. 200 mL, 400 mL, 500 mL, 600 mL & 800 mL media volumes were chosen to conduct the study.

During this study, a very small scale of variation was noticed in case of the outcome i.e. percent degradation of the bi- solute mixture. Around 97% degradations were achieved in both case when the volume of the culture media was 200 mL and 400 mL respectively which

implied no changes in the outcome in between these media volumes. Slight increase was observed when the media volume was increased up to 500 mL. The peak was found at 600 mL of media volume where almost 100% degradation was achieved. Beyond that volume, the degradation efficacy of the consortium was decreased again. Around 95% degradation was obtained when the media volume was 800 mL. Though there was not so much variation, 600 mL media volume was optimized for further studies (Fig: VIA.1 (e)).

VIA.2.6 Effect of initial concentration of bi- solute mixture (Phenol & 4- Chloro Phenol):

It was a vital parameter for the microbial consortium. Degradation percentage furiously declined while increasing the initial conc. of the bi- solute mixture when the earlier parameters were not optimized. But when all the earlier optimized parameters were applied together to treat higher conc. of the bi- solute mixture, the scenario totally changed. Up to a certain concentration as well as in all the optimized parameters, the degradation percentage increased gradually. Beyond a certain concentration, degradation percentage decreased again. 96.24% & 97.29% degradations were achieved when the initial conc. of the mixture was 600 mg/L (300 mg/L Phenol & 300 mg/L 4- Chloro Phenol) and 700 mg/L (350mg/L Phenol & 350 mg/L 4- Chloro Phenol) respectively. Similarly, around 98% & 98.25% degradations were obtained when the initial conc. was 800 mg/L & 900 mg/L respectively. Maximum degradation was observed at 1000mg/L initial conc. where almost complete degradation was observed. Approximately 96percent degradation was gained at 1200 mg/L initial conc. where again a gradual fall in the outcome was recorded. So, 1000 mg/L initial conc. of the bi- solute mixture was optimized for this particular consortium (Fig: VIA.1 (f)).



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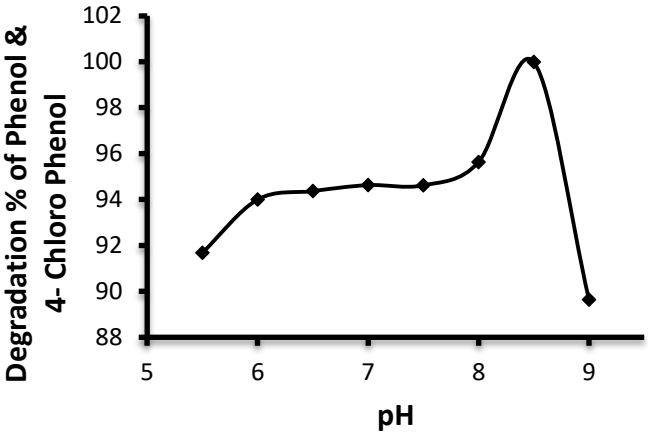

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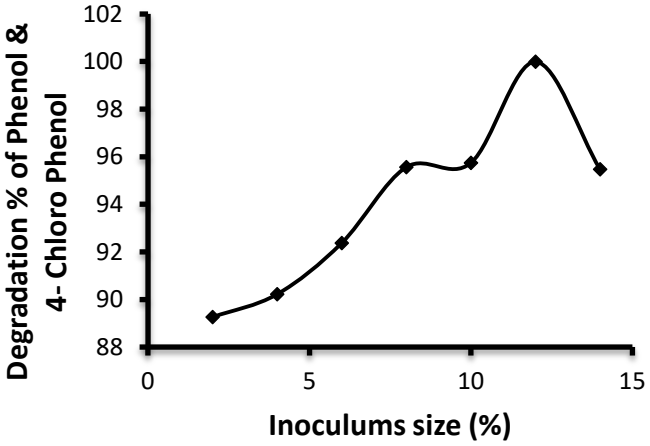

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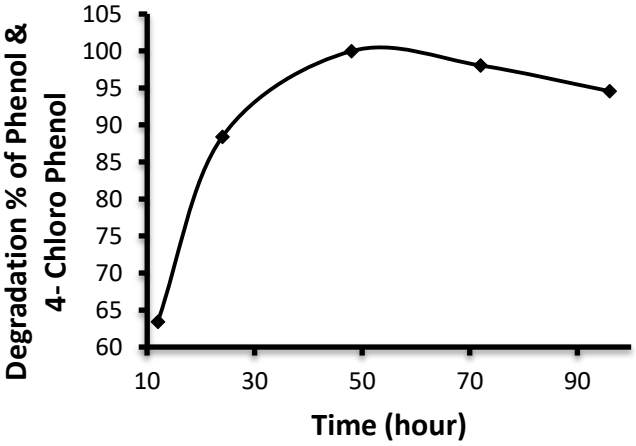

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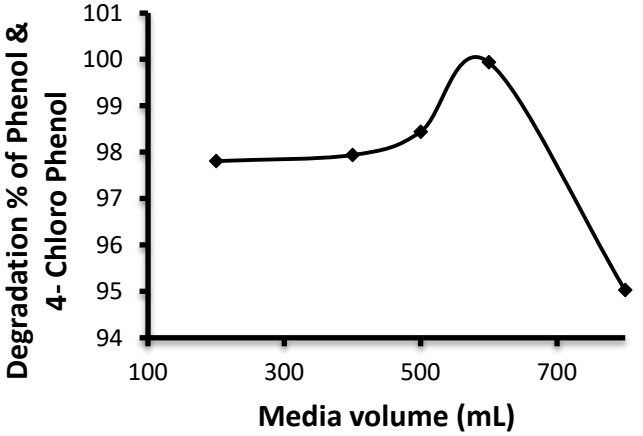

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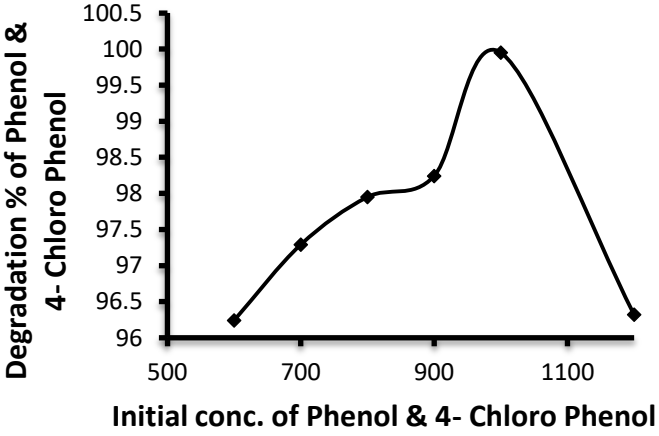


(f)

Fig VIA.1: Effect in the degradation efficacy of the consortium in six different parameters; (a) Temperature (b) pH (c) Inoculums size (d) Time (e) Media volume & (f) Initial conc. of bi-solute mixture (Phenol & 4- Chloro Phenol)

Sub chapter VIB

Bioremediation of 4- Chloro Phenol & Catechol as a bi- solute mixture by involving a microbial consortium: optimization of process parameters in batch reactor

VIB.1 Materials and Methods:

VIB.1.1 Materials:

As the mixture of 4- Chloro Phenol & Catechol was to be degraded, a bacterial consortium of *Bacillus timonensis* strain 10403023, *Bacillus cereus* strain K1, *Bacillus Psudomucoides* strain NBRC 101232 and *Bacillus paramycoides* strain MCCC 1A04098 were utilized in the study. All of these strains were isolated from the contaminated soil through enrichment method as described in the chapter II, section IIA.1.5. A large volume of inoculums of the four above mentioned strains were already prepared via acclimatization procedure in Mineral Salt Medium (MSM) as described in chapter II, section IIA.1.8.

4- Chloro Phenol and Catechol solutions were prepared synthetically in the laboratory. Conc. of the stock solutions was maintained as 10 g/L in case of both.

VIB.1.2 Experimental set up:

Same as mentioned in the chapter III, section IIIA.1.2.

VIB.1.3 Analytical Method:

Percentage of degradation of the bi- solute mixture (4- Chloro Phenol & Catechol) from the waste water was measured in the UV-VIS spectrophotometer at 510 nm wavelength followed by 4- Amino Antipyrene method as suggested by Yang, R.D., et al. 1975. For that purpose a standard curve as well as a standard equation was prepared with the known concentrations of the bi-solute mixture (i.e. 4- Chloro Phenol & Catechol) and the residual content of the bi-solute mixture in an unknown sample was calculated from that equation. Details of this process has been mentioned in chapter II, section IIA.1.7.

VIB.2 Results and Discussions:

In case of percent of inoculums and of initial conc. of the mixture to be removed i.e. 4-Chloro Phenol & Catechol, 1:1:1:1 ratio and 1:1 ratio was maintained respectively as mentioned in the sub chapter VIA, section VIA.2.

VIB.2.1 Effect of temperature:

The bacterial consortium was cultured in six different temperatures ranging between 25^{o}C to 50^{o}C with an interval of 5^{o}C to find out the most favourable one. 45^{o}C temperature was found to be the most favourable temperature for this particular consortium while removing the mixture of 4- Chloro Phenol & Catechol. 99.55% degradation was achieved in this temperature when the initial conc. was 500 mg/L (250 mg/L 4- Chloro Phenol & 250 mg/L Catechol). Below that temperature, the degradation efficacy of the consortium decreased. At 25^{o}C, lowest degradation was obtained i.e. 72.54% and at 30^{o}C, 78.25% degradation was achieved. At 35^{o}C, almost 86% degradation was found and in turn, 94.2% degradation was recorded at 40^{o}C. Similarly, when the temperature increased from the optimum temperature, decrease in the degradation percentage was occurred. 88.24% degradation was achieved at 50^{o}C temperature (Fig: VIB.1 (a)).

VIB.2.2 Effect of pH:

99.68percent degradation was found at the optimal pH value i.e. 8.5 which implied that basic pH could have been favourable for the consortium. A moderate range of variation was found in case of the degradation percent of the mixture. Degradation efficacy of the consortium was only 70.25% when the pH was maintained as 5.5. Degradation efficacy increased while increasing the pH. 76.24% degradation was obtained at pH 6.0 whereas, 79.25% degradation was found at pH 6.5. At the neutral pH, around 84% degradation was gained and in turn, approximately 89% degradation was obtained at the pH of 7.5. Sudden decrease was noticed when the pH value further increased above the optimal value. 86.24% degradation was obtained at pH 9.0 (Fig: VIB.1 (b)).

VIB.2.3 Effect of inoculums size:

Seven experiments were carried out with seven different ranges of the inoculums, ranging between 2% to 14% with an interval of 2%. A moderate range of variation was obtained while changing the percentage of inoculums. 78.22% and 83.52% degradations were observed when the inoculums size of the consortium was 2% and 4% respectively. 87.32% and 90.25% degradations were achieved when the inoculums size was increased up to 6% & 8% respectively. When 10% inoculums added to the media, 94.25% degradation was observed and it was increased up to 99.6% almost, when 12% inoculums were added. But beyond that, in case of 14% inoculums, degradation percentage again decreased to around 92%. Hence 12% of the inoculums (3% inoculums of each of the four strains) was found to be optimum for this particular consortium while degrading the mixture of 4- Chloro Phenol &Catechol (Fig: VIB.1 (c)).

VIB.2.4 Effect of incubation time:

While culturing this consortium, five (5) different times were selected for the study viz. 12 hr, 24 hr, 48 hr, 72 hr & 96 hr. A wide range of variation was found during this study. Only around 68% degradation of the mixture was observed after 12 hours and after 24 hours, it increased only up to ~ 88%. After 48 hours of incubation, complete degradation was achieved. Beyond this time span, after 72 and 96 hours of incubation, 94.27% & 90.44% degradations were obtained, indicating the decrease in the degradation efficacy of the consortium again. So, the optimal residence time for this consortium was found to be 48 hours (Fig: VIB.1 (d)).

VIB.2.5 Effect of media volume:

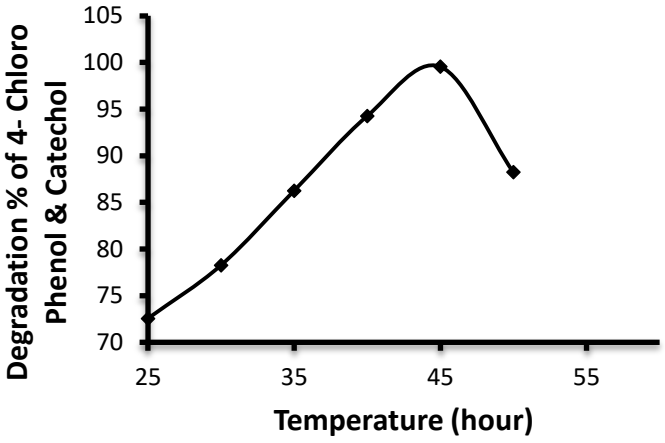
Like that of the previous study, here also five different volumes of the media were deployed to check the effect of the media volume on the degradation efficacy of the consortium (as mentioned in the sub chapter VIA).

While studying, a very small range of variation was noticed in case of the outcomes. Around 97% & 98% degradations were achieved when the volume of the culture media was 200 mL and 400 mL respectively. At 500 mL of the media volume, 99.24% degradation was achieved. Beyond that volume, the degradation efficacy of the consortium increased almost

up to 100% when the media volume further increased to 600 mL. Finally at the volume of 800 mL, again the output slightly decreased up to 97.28%. 600 mL media volume was optimized for the further studies as complete degradation of the mixture occurred in that volume (Fig: VIB.1 (e)).

VIB.2.6 Effect of initial concentration of the mixture (4- Chloro Phenol & Catechol):

It was one of the most important parameters for the consortium. Same phenomena (as described in VIA.2.6) were noticed here also. Around 97% & 98% degradations were achieved when the initial conc. of the mixture was maintained as 600 mg/L and 700 mg/L respectively. Then the degradation continued to increase. At 800mg/L initial conc. 98.77% degradation was observed. When the initial conc. of the bi- solute mixture further increased up to 900 mg/L & 1000 mg/L, around 99.2% & 99.63% degradations were found respectively which were excellent. Beyond that concentration, the degradation efficacy of the consortium started to decrease again as approximately 96% and 88% degradations were gained when the initial conc. of the mixture was maintained at 1200 mg/L & 1400 mg/L respectively. Hence, the consortium was found to remove maximum amount of the bi- solute mixture when the initial conc. was 1000 mg/L (500 mg/L 4- Chloro Phenol & 500 mg/L Catechol) (Fig: VIB.1 (f)).



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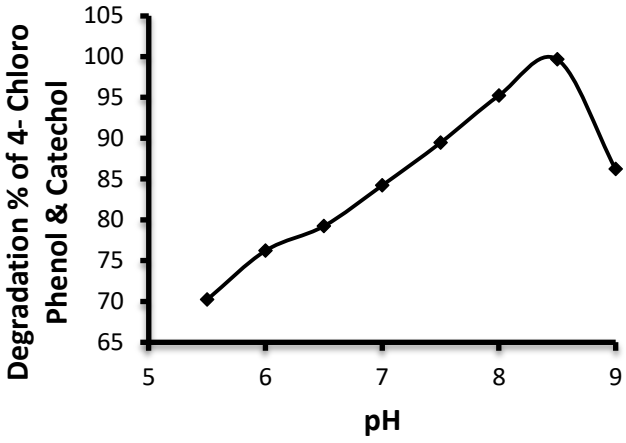

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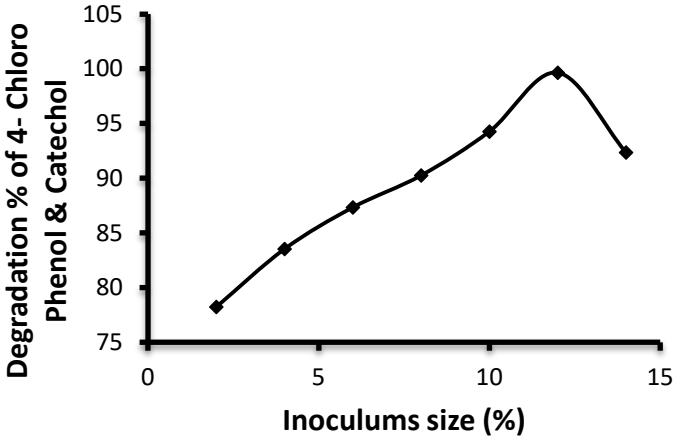

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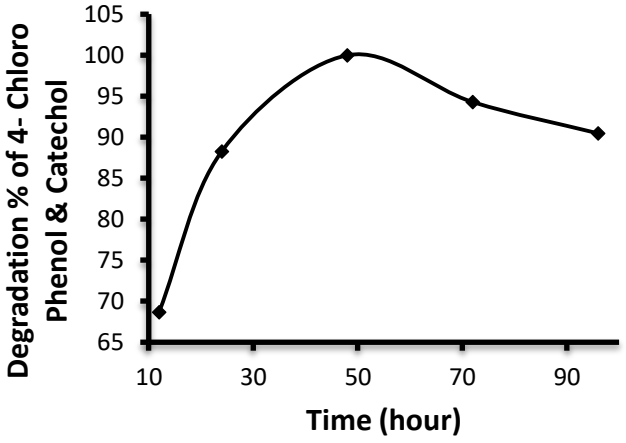

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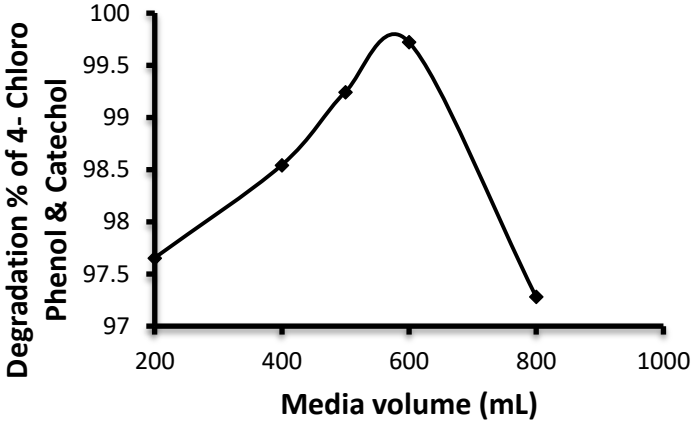

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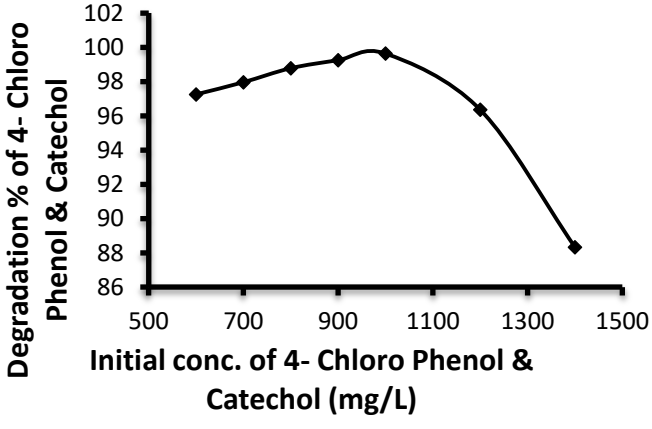


(f)

Fig VIB.1: Effect in the degradation efficacy of the consortium in six different parameters; (a) Temperature (b) pH (c) Inoculums size (d) Time (e) Media volume & (f) Initial conc. of bi-solute mixture (4- Chloro Phenol & Catechol)

Sub chapter VIC

Bioremediation of Phenol & Catechol as a bi- solute mixture by involving a microbial consortium: optimisation of process parameters in batch reactor

VIC.1 Materials and Methods:

VIC.1.1 Materials:

As the mixture of Phenol & Catechol was to be degraded, a bacterial consortium of *Brevibacillus formosus* strain NRRL NRS- 863, *Pseudomonas otitidis* strain MCC10330, *Bacillus Psudomucoides* strain NBRC 101232 and *Bacillus paramycoides* strain MCCC 1A04098 were utilized in this study. All of these strains were isolated from the contaminated soil through enrichment technique as described in the chapter II, section IIA.1.5. A large volume of inoculums of the four above mentioned strains were prepared via acclimatization procedure in Mineral Salt Medium (MSM) as described in chapter II, section IIA.1.8.

Phenol and Catechol solutions were prepared synthetically in the laboratory. Conc. of the stock solutions was maintained as 10 g/L in case of both.

VIC.1.2 Experimental set up:

Same as mentioned in the chapter III, section IIIA.1.2.

VIC.1.3 Analytical Method:

Percentage of degradation of the bi- solute mixture (Phenol & Catechol) from the waste water was measured in the UV-VIS spectrophotometer at 510 nm wavelength followed by 4-Amino Antipyrene method as suggested by Yang, R.D., et al. 1975. For that purpose a standard curve as well as a standard equation was prepared with the known concentrations of the bi-solute mixture (i.e. Phenol & Catechol) and the residual content of the bi- solute mixture in an unknown sample was calculated from that equation. Details of this process has been mentioned in chapter II, section IIA.1.7.

VIC.2 Results and Discussions:

In case of percent of inoculums and of initial conc. of the mixture to be removed i.e. Phenol & Catechol, 1:1:1:1 ratio and 1:1 ratio was maintained respectively as mentioned in the sub chapter VIA, section VIA.2.

VIC.2.1 Effect of temperature:

In this study also, 45^{o}C temperature was found to be most suitable for this consortium during degradation of the bi- solute mixture of Phenol & Catechol. A wide variation in the degradation percentage was observed during this study. 62.19% degradation was achieved in 25^{o}C temperature. Here, the consortium was cultured in six different temperatures ranges from 25^{o}C to 50^{o}C with an interval of 5^{o}C. Below and above the optimal temperature, the degradation percentage decreased gradually. A slight decrease in degradation percentage was noted at 50^{o}C where almost 93% degradation was achieved. And below the optimal temperature (i.e. 45^{o}C) the degradation efficacy of the consortium decreased rapidly. At 40^{o}C and 35^{o}C, almost 82% and 81% degradations were obtained respectively. When the temperature decreased further up to 30^{o}C & 25^{o}C, 78.67% and 62.19% degradations were achieved by the consortium respectively (Fig: VIC.1 (a)).

VIC.2.2 Effect of pH:

During the pH study, complete degradation was achieved at pH 8.5 which was found to be the optimal pH for this consortium. Three tests were performed in acidic pH where pH was maintained at 6.5 & 6.0 & 5.5. 88.74% and 80.95% & 79.52% degradations were observed in those acidic pH mediums respectively. In neutral pH (i.e. pH 7), around 90% degradation was observed. Finally, four experiments were carried out in basic pH mediums where pH values were 7.5, 8.0, 8.5 & 9.0 and 92.63%, 94.17%, 100% & 95.66% of degradations were found from those pH mediums respectively (Fig: VIC.1 (b)) indicating the optimal pH at 8.5.

VIC.2.3 Effect of inoculums size:

Inoculums size played a vital role also, in the bioremediation of the bi- solute mixture of Phenol & Catechol. Seven experiments were carried out with seven different ranges of the inoculums, ranging between 2% to 14% with an interval of 2%.

A moderate range of variation was obtained while changing the percentage of inoculums. 68.24% and 74.25% degradations were observed when the inoculums size of the consortium was 2% and 4% respectively. 80.24% and 88.25% degradations were achieved when the inoculums size was increased up to 6% & 8% respectively. When 10% inoculums added to the media, 96.25% degradation was observed and it was increased up to 100% almost, when

12% inoculums was added. But, in case of 14% inoculums, degradation percentage again decreased to around 86% indicating the 12% inoculums size to be the optimum inoculums size for this particular consortium while degrading Phenol & Catechol as a mixed compound (Fig: VIC.1 (c)).

VIC.2.4 Effect of incubation time:

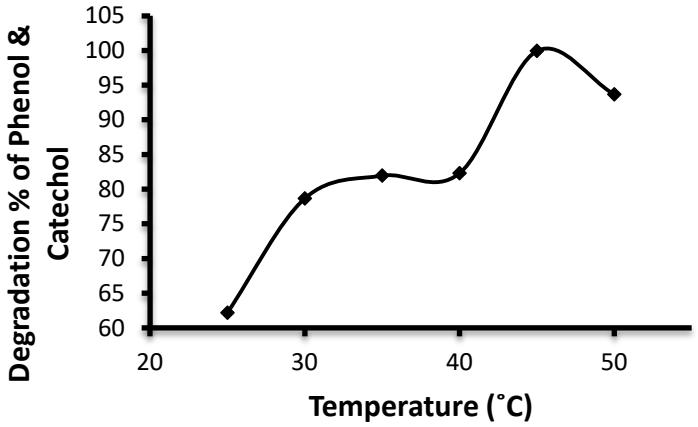
While culturing this consortium, five (5) different times were selected for the study viz. 12 hr, 24 hr, 48 hr, 72 hr & 96 hr. A moderate variation was found during this study. Around 70% degradation of the mixture (Phenol & Catechol) was observed after 12 hours and after 24 hours, it increased only up to ~ 75%. After 48 hours of incubation, 99.92% of degradation was achieved and after 72 and 96 hours of incubation, 96.19% & 95.62% degradations were obtained. So, the degradation percent decreased after 48 hours, where maximum degradation percentage was observed. So, the optimal time for this consortium was found to be 48 hours (Fig: VIC.1 (d)).

VIC.2.5 Effect of media volume:

During this study, a very small scale changes were observed. 95.26% & 96.54% degradations were recorded when the media volume was 200 mL & 400 mL respectively. At 500 mL, 98.24% degradation was found indicating a slight incline in the curve. 99.92% degradation of the bi- solute mixture was noticed at 600 mL volume of the media. Then, the degradation efficacy of the consortium decreased again and reached up to 94.21% when the media volume was 800 mL (Fig: VIC.1 (e)).

VIC.2.6 Effect of initial concentration of the mixture of Phenol & Catechol:

It was one of the most important parameter for the consortium. While deploying the consortium in this study, around 96% & 97% degradations were achieved when the initial conc. of the mixture was maintained as 600 mg/L and 700 mg/L respectively. At 800mg/L initial conc. 98.52% degradation was observed. At 900 mg/L, 99.12% degradation was noticed and in turn, at 1000 mg/L, almost complete degradation was found. When the initial conc. further increased up to 1200 mg/L, 94% degradation was found approximately implying the decrease in the outcome. Hence, the consortium was found to remove maximum amount of the bi- solute mixture of Phenol & Catechol when the initial conc. was 1000 mg/L (500 mg/L of Phenol and 500 mg/L of Catechol) (Fig: VIC.1 (f)).



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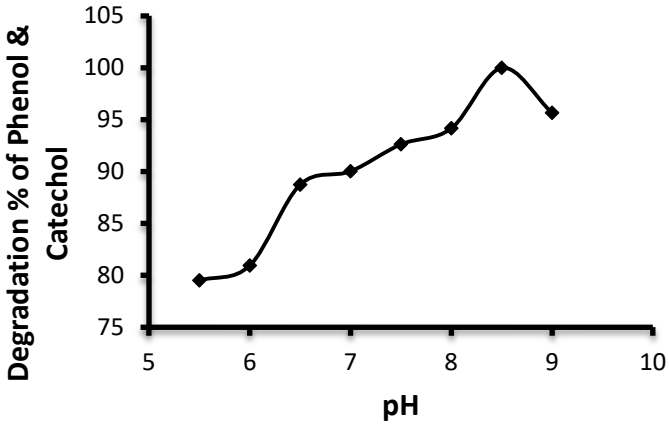

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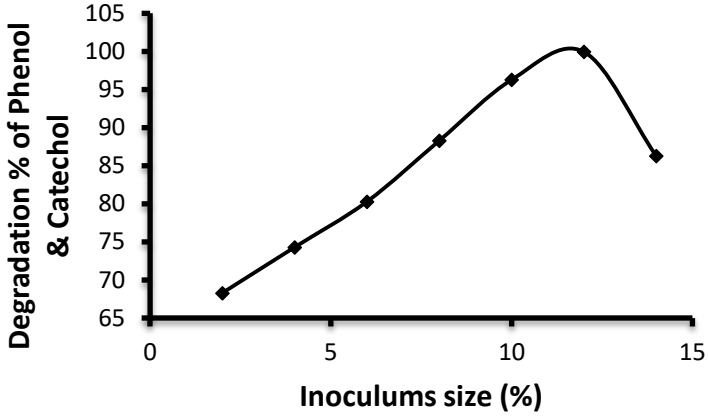

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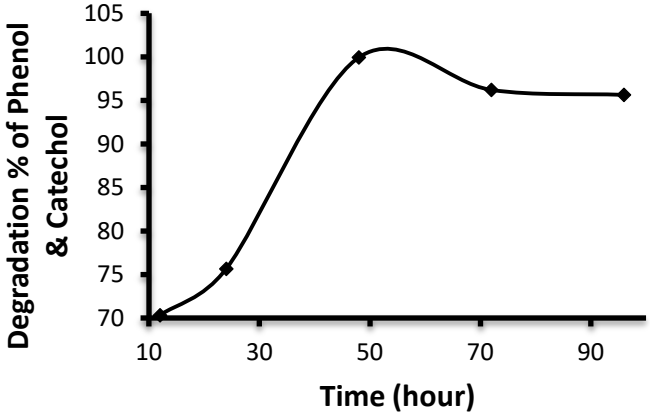

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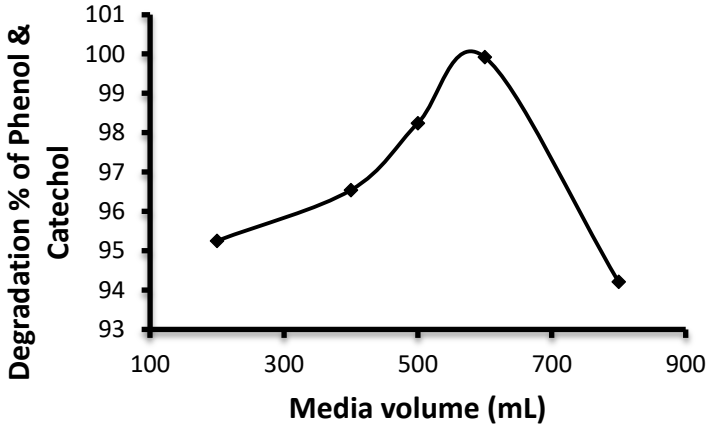

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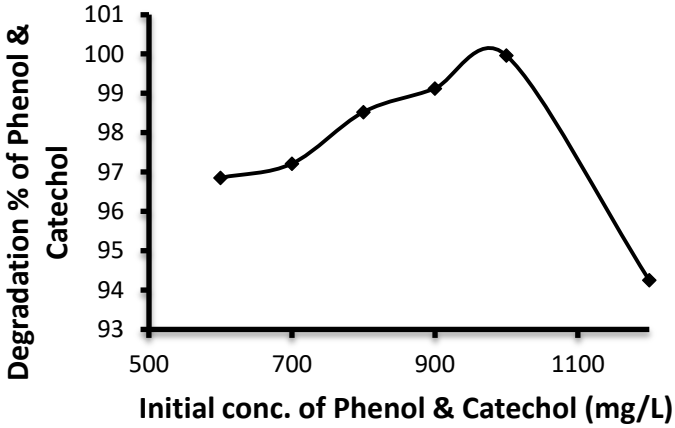


(f)

Fig VIC.1: Effect in the degradation efficacy of the consortium in six different parameters; (a) Temperature (b) pH (c) Inoculums size (d) Time (e) Media volume & (f) Initial conc. of bi-solute mixture (Phenol & Catechol)

Sub chapter VII D

Bioremediation of Phenol & 4-Chloro Phenol & Catechol as a tri- solute mixture by involving a microbial consortium: optimisation of parameters in batch reactor

VID.1 Materials and Methods:

VID.1.1 Materials:

As the mixture of **Phenol & 4- Chloro Phenol & Catechol** was to be degraded; a bacterial consortium was prepared by involving all six bacterial strains, isolated earlier. Those strains are: *Brevibacillus formosus* strain NRRL NRS- 863, *Pseudomonas otitidis* strain MCC10330, *Bacillus timonensis* strain 10403023, *Bacillus cereus* strain K1, *Bacillus Psudomucoides* strain NBRC 101232 and *Bacillus paramycoides* strain MCCC 1A04098. Isolation process of these strains has been described in the chapter II, section IIA.1.5.

Phenol, 4- Chloro Phenol & Catechol solutions were prepared synthetically in the laboratory. Conc. of the stock solutions was maintained as 10 g/L in case of both.

VID.1.2 Experimental set up:

Same as mentioned in the chapter III, section IIIA.1.2.

VID.1.3 Analytical Method:

Percentage of degradation of the tri- solute mixture (Phenol & 4- Chloro Phenol & Catechol) from the waste water was measured in the UV-VIS spectrophotometer at 510 nm wavelength followed by 4- Amino Antipyrene method as suggested by Yang, R.D., et al. 1975. For that purpose a standard curve as well as a standard equation was prepared with the known concentrations of this tri-solute mixture (i.e. Phenol & 4- Chloro Phenol & Catechol) and the residual content of the tri- solute mixture in an unknown sample was calculated from that equation. Details of this process has been mentioned in chapter II, section IIA.1.7.

VID.2 Results and Discussions:

In case of percent of inoculums, total percent of inoculums of the all six bacterial strains was taken into account where percent of each of the six strains was maintained equally (1:1:1:1:1:1 ratio). Similarly in case of initial conc. of the mixture to be removed i.e. **Phenol, 4- Chloro Phenol and Catechol**, total initial conc. (mg/L) of the three compounds was used where individual initial conc. of the compounds was kept equal (1:1:1 ratio).

VID.2.1 Effect of temperature:

The microbial consortium was cultured in six different temperatures ranging between 25^{o}C to 50^{o}C with an interval of 5^{o}C to find out the most suitable one. 45^{o}C temperature was found to be the most favourable temperature for this consortium while removing the tri- solute mixture. 99.82% degradation was achieved at this temperature. Below this temperature, the degradation efficacy of the consortium decreased. At 40^{o}C, 96.5% degradation was obtained and at 35^{o}C, 88.08% degradation was achieved. At 30^{o}C, almost 86.2% degradation was found. And the lowest degradation was recorded at 25^{o}C where 82.5% degradation was gained. Similarly, when the temperature further increased from the optimum temperature, decrease in the degradation percentage was occurred. 90.48% degradation was achieved at 50^{o}C temperature (Fig: VID.1 (a)).

VID.2.2 Effect of pH:

99.88% degradation was found at the optimal pH value i.e. 8.0. Degradation efficacy of the consortium rapidly decreased while increasing the pH. Around 88% & 74% degradations were recorded when the pH of the media increased up to 8.5 & 9.0 respectively. Besides, degradation percent decreased gradually when the pH of the media decreased. 96.24% & 90.25% degradations were achieved when the pH was 7.5 & 7.0 respectively. Degradation efficacy of the consortium further decreased up to 85.2% and 81.5% when the pH was maintained as 6.5 and 6.0. At extreme acidic pH i.e. 5.5, 79.24% degradation was gained. (Fig: VID.1 (b)).

VID.2.3 Effect of inoculums size:

Six different sizes of inoculums were selected ranging between 3% to 18% with an interval of 3%. A moderate range of variation was obtained while changing the percentage of the inoculums. 74.43% and 79.86% degradations were observed when the inoculums size of the consortium was 3% and 6% respectively. 86.67% and 95.47% degradations were achieved when the inoculums size was increased up to 9% & 12% respectively. When 15% inoculums were added to the media, 99.8% degradation was observed and it was decreased again up to 94.14% when the inoculums size further increased up to 18%. Hence 15% inoculums size (2.5% inoculums of each of the six bacterial strains were added) was found to be the

optimum inoculums size for this consortium while degrading the tri- solute mixture of Phenol & 4- Chloro Phenol & Catechol (Fig: VID.1 (c)).

VID.2.4 Effect of incubation time:

Like that of the previous experiments, five (5) different times were selected for the study viz. 12 hr, 24 hr, 48 hr, 72 hr & 96 hr. A wide range of variation was found during this study. Only around 58% degradation of the tri- solute mixture was observed after 12 hours and after 24 hours, it increased only up to 72.49%. After 48 hours of incubation, 99.85% of degradation was achieved and in turn, after 72 and 96 hours of incubation, 97.41% & 95.24% degradations were obtained indicating a negative effect after a certain period of time. So, the optimal time for this consortium to degrade the tri- solute mixture was found to be 48 hours (Fig: VID.1 (d)).

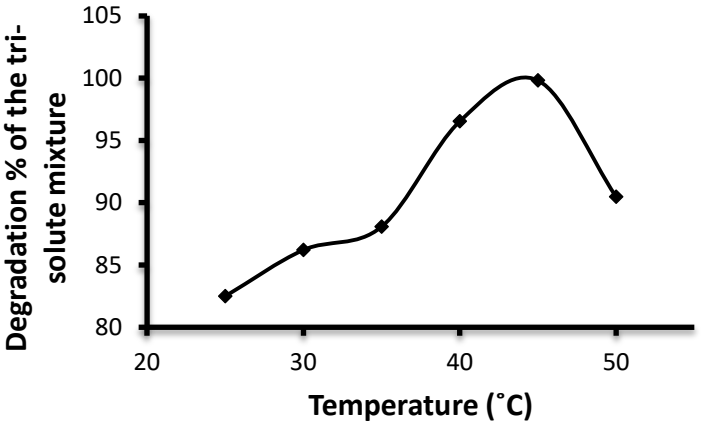
VID.2.5 Effect of media volume:

Like that of the previous studies, here also five different volumes of the media were deployed to check the effect of media volume on the degradation efficacy of the consortium. A very small range of variation was noticed in case of the outcomes. Around 97% & 98% degradations were achieved when the volume of the culture media was 200 mL and 400 mL respectively. At 500 mL of the media volume, 99.31% degradation was achieved. Beyond that volume, the degradation efficacy of the consortium slightly increased up to 99.88% when the media volume was 600 mL. At the volume of 800 mL, again the output decreased up to 92.15%. Although there was not much variation in the outcomes, 600 mL media volume was optimized in case of this consortium for further studies (Fig: VID.1 (e)).

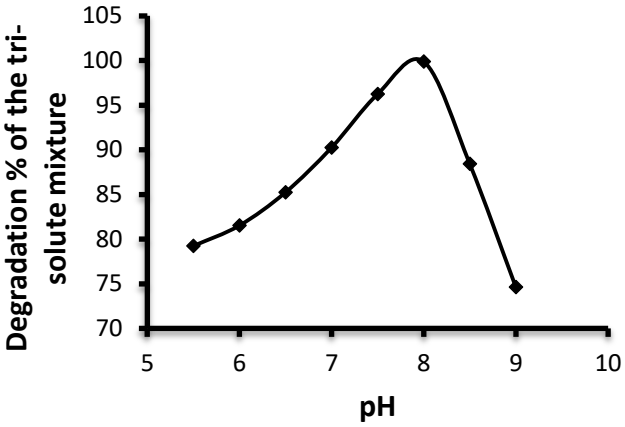
VID.2.6 Effect of initial concentration of the tri- solute mixture (Phenol & 4- Chloro Phenol & Catechol):

It was found to be very vital parameter. Seven tests were performed in seven different initial conc. of the tri- solute mixtures. Around 96.2% & 96.8% degradations were achieved when the initial conc. of the tri- solute mixture was maintained as 750 mg/L and 900 mg/L respectively. Then the degradation efficacy of the consortium fluctuated to some extent. At 1050mg/L initial conc. 97.52% degradation was observed. When the initial conc. of the mixture further increased up to 1200 mg/L & 1500 mg/L, again 98.57% & 99.75%

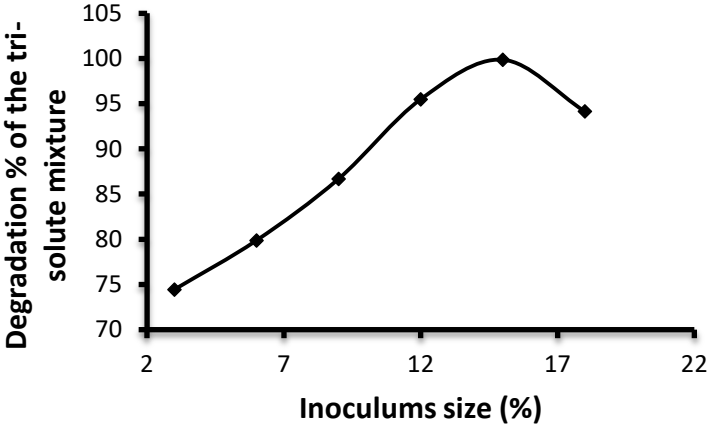
degradations were found respectively which were better than that of the previous initial concentrations. But at the initial conc. of 1800 mg/L and 2100 mg/L, around 87% & 72% degradations were obtained indicating a rapid fall in the degradation percentage. So, the consortium was found to remove maximum amount of the tri- solute mixture when the initial conc. of the mixture was 1500 mg/L (500 mg/L of Phenol & 500 mg/L of 4- Chloro Phenol & 500 mg/L of Catechol) (Fig: VID.1 (f)).



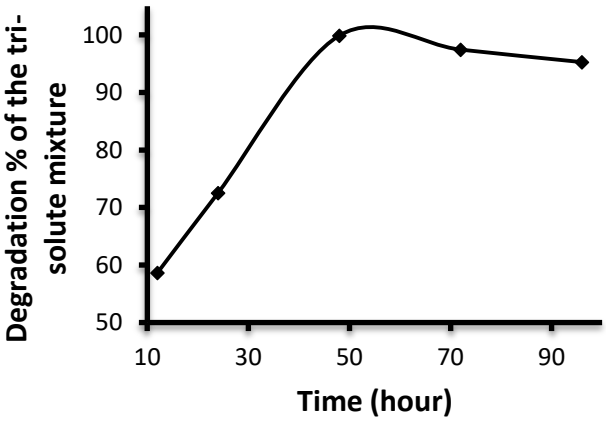
(a)



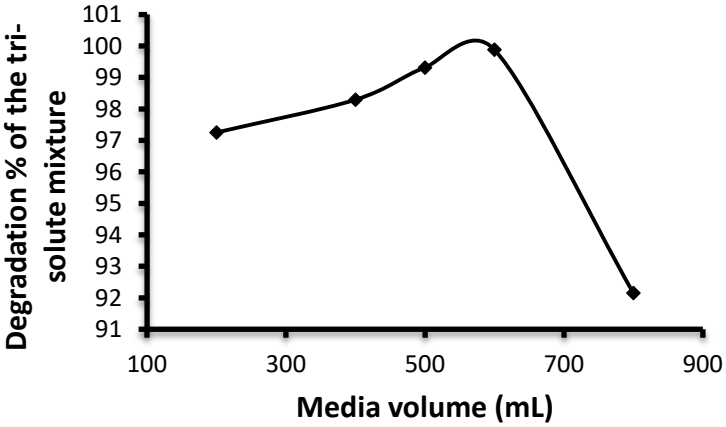
(b)



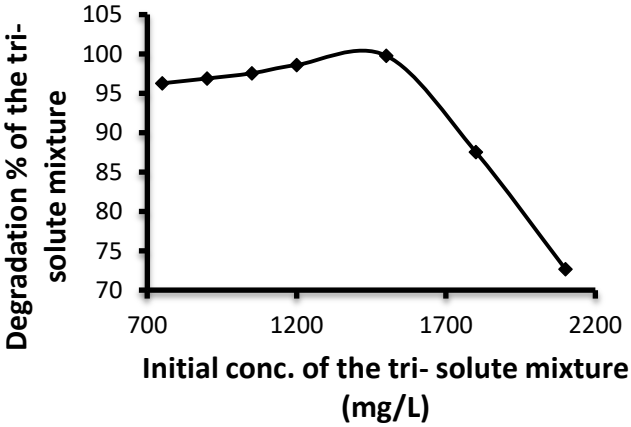
(c)



(d)



(e)



(f)

Fig VID.1: Effect in the degradation efficacy of the consortium in six different parameters; (a) Temperature (b) pH (c) Inoculums size (d) Time (e) Media volume & (f) Initial conc. of tri-solute mixture (Phenol & 4- Chloro Phenol & Catechol)

Conclusions:

From the previous studies of optimization of the single strains, it was found that the physico – chemical parameters are species specific in case of all the six isolated strains. In the current study, four different types of consortiums were prepared with the help of those six strains. While optimizing the parameters of these consortiums, no similarity was found between the previously optimized parameters (in case of single strains) and the current optimized parameters (in case of the consortiums). But many similarities were found in between the present optimal parameters of the four consortiums. In case of temperature, all the four consortiums were found to be optimized at 45^{o}C. All the consortiums found to exhibit their best efficacy at basic pH. Incubation time was found to be the most suitable at 48 hours in case of all the four consortiums. Also in case of media volume, 600 mL volume was found to be favourable for all the consortiums. In case of the inoculums size, 12% inoculums (3percent inoculums of each of the four strains of the consortiums) were optimized in case of the first three consortiums, deployed to degrade the bi- solute mixture. 15% inoculums size (2.5% inoculums of each of the six strains of the consortium) was optimized in case of the fourth consortium, deployed to remove the tri- solute mixture (chapter VID). Finally, in case of the initial conc. of the Phenolic mixtures, 1000 mg/L conc. of the bi- solute mixtures (500 mg/L conc. of each of the compounds) were found to be favourable for the first three consortiums whereas, 1500 mg/L conc. of the tri- solute mixture (500 mg/L conc. of each of the compounds) was found to be most suitable in case of the fourth consortium.

However, in this chapter, only manual optimizations were performed in case of degradation of the mixtures of the Phenolic compounds. Statistical analysis of these optimized parameters will be discussed in the next chapter.

Chapter VII

Optimization of process parameters in case of Bi- & Tri- solutes via Response Surface Methodology (RSM)

Introduction:

In the previous chapter (i.e. in Chapter VI), it was displayed that how biodegradation of the mixture of Phenolic substances by some bacterial consortiums, depends upon some physico – chemical or biological parameters. Though, several studies were conducted in the previous chapter, to identify the perfect values of the parameters, it was necessary to optimize those parameters statistically. Moreover, it was also important to find out the interaction effects between those parameters via 3D curves. That's why Response Surface Methodology (RSM) was involved in case of these microbial consortiums also. As four different bacterial consortiums were prepared to degrade the four different mixtures of the Phenolic compounds (described elaborately in chapter VI), four RSMs were deployed to optimize the parameters of the four consortiums separately.

Earlier, a lot of studies have been conducted on RSM. Some of them have been stated in chapter IV in brief.

Like that of the chapter IV & VI, here also six parameters were taken into account. Those were: temperature, pH, residence time, media volume, inoculums size of the consortium and initial conc. of the Phenolic mixture.

Sub chapter VIIA

Optimization of parameters for the consortium, involved to degrade the mixture of Phenol & 4- Chloro Phenol

VIIA.1 Materials and Methods:

VIIA.1.1 Materials:

Four different strains (*Brevibacillus formosus* strain NRRL NRS- 863, *Pseudomonas otitidis* strain MCC10330, *Bacillus timonensis* strain 10403023 and *Bacillus cereus* strain K1) were utilized to make a microbial consortium.

Phenol & 4- Chloro Phenol were prepared synthetically in the laboratory. The conc. of the solutions was maintained as 10 g/L.

VIIA.1.2 Experimental set up:

In case of designing the percent of inoculums, total percent of inoculums of the all four bacterial strains was taken into account where percent of each of the four strains was maintained equally (1:1:1:1 ratio). Similarly in case of initial conc. of the mixture to be removed i.e. Phenol and 4- Chloro Phenol, total initial conc. (mg/L) of the two compounds was used for the design where individual initial conc. of the compounds was kept equal (1:1 ratio).

Rest of the portion is same as mentioned in the chapter III, section IIIA.1.2.

VIIA.1.3 Analytical Method:

Same as described in chapter VI, section VIA.1.3

VIIA.1.4 Experimental design:

The popular second order Central Composite Design (CCD) was used in case of the experimental design. The CCD based RSM with six independent factors at five levels full factorial was applied using design expert 13.0.5.0. statistical tool. Six (6) independent variables (Table VIIA.1) are Z1 (A): 5.5 – 11.5; Z2 (B): 35^{o} – 55^{o}C; Z3 (C): 200 – 1000 mL; Z4 (D): 0 – 96 hours; Z5 (E): 600 – 1400 mg/L and Z6 (F): 0 – 24%. These six independent variables were coded at five levels between -2 and +2 based on preliminary experimental outputs. The experiment designed with six factors, resulted into ten (10) replicates at the design centre to evaluate pure error and carried in randomised order. In the design, the response can be related to choose factors by quadratic model. The nature of the process can be explained by the following linear quadratic equation:

$$Y = ß_0 + \sum_{i=1} ß_i x_i + \sum_{i=1} ß_{ii} x_i^2 + \sum_{i=1} \sum_{j=1} ß_{ij} x_i x_j + C \qquad (7.1)$$

Here, y stands for process response, i and j are the index number of the patterns, $ß_0$ is the intercept term, $ß_i$ is the linear main effect, $ß_{ii}$ is the quadratic effect, where as $ß_{ij}$ is the interactive effect. C stands for the random error between the predicted and actual values. ANOVA was performed to reveal the relationship in between process variables and response. Coefficient of determination R^2 indicated the quality of fit of the polynomial model. Adjusted R^2 and statistical importance was deducted by F test. The desired goal was to remove the mixture of Phenol and 4- Chloro Phenol at minimum time limit.

Table VIIA.1: Independent variables with coded levels

Independent variable	Symbol	Coded levels				
		-2	-1	0	+1	+2
pH of bacterial media	Z1	5.5	7	8.5	10	11.5
Temperature (^oC)	Z2	35	40	45	50	55
Volume of bacterial media(mL)	Z3	200	400	600	800	1000
Residence time(hr)	Z4	0	24	48	72	96
Initial conc. of the mixture (mg/L)	Z5	600	800	1000	1200	1400
Inoculums percentage (percent)	Z6	0	6	12	18	24

VIIA.1.5 HPLC analysis:

HPLC analysis was performed in order to detect the individual degradation of the two compounds i.e. Phenol& 4- Chloro Phenol. It was done only in case of the sample, obtained as per centre point of RSM design. The chromatogram image (VIIA.9) depicted satisfactory degradation of the two Phenolic compounds individually.

System: MDLC system

Pump used: Duel binary pump

Detector used: Duel λ absorbance detector

Pump controller module: ii

Software used: EMPOWER 2

Mobile phase used. Pump A (0.1percent TFA in H_2O).

Pump B (90percent acetonitrile)

Run type: Gradient run

Table: VIIA.2: Run in the two pumps

Time (min)	A (percent)	B (percent)	Total flow
0	100	0	1ml/min
2	100	0	
2.5	80	20	
4	80	20	
4.5	70	30	
8	70	30	
8.5	80	20	
12	80	20	
12.5	100	0	
15	100	0	

Detection wave length: 280 nm & 254 nm

Column used: C_{18} reverse phase column. Dimension of the column was 4.6 × 250 mm. particle size 5 µm.

VIIA.2 Results and Discussions:

VIIA.2.1 Fitting of the model and Statistical analysis:

Total 86 experiments were performed as per the design matrix and the only one response was percent of degradation of the mixture (Phenol+ 4- Chloro Phenol). Table VIIA.3 displayed both the predicted and experimental/actual values of percent of degradation of the mixture of Phenol & 4- Chloro Phenol. Second order and linear polynomial equations were fitted to the actual data to obtain the regression equation. To reveal the suitable model, sequential model sum of squares and model summary statistics were deducted (Table VIIA.4). The Sequential

P-value for the quadratic model is less than 0.0001; Maximum predicted R^2 and adjusted R^2 values were 0.9686 and 0.9842 respectively for the percent of degradation of the bi- solute mixture. Cubic model found to be aliased; here the sequential P- value was> 0.05. So, finally the quadratic model was chosen for further determination of the percent of degradation of the mixture (Phenol & 4- Chloro Phenol). The following equation clearly showed this:

percent of degradation of the mixture of Phenol & 4- Chloro Phenol (Y): $94.02+ 0.4150Z1 + 0.2144Z2 – 0.2111Z3 +1.56Z4 - 0.3656Z5+6.13Z6 +0.5222Z1Z2 – 0.3763Z1Z3 – 1.09Z1Z4+ 0.0466Z1Z5 - 0.1813Z1Z6 - 0.2734Z2Z3 - 0.3856Z2Z4 + 0.0650Z2Z5 – 0.2834Z2Z6 - 0.1991Z3Z4 + 0.0622Z3Z5 + 0.6994Z3Z6 - 0.1294Z4Z5 + 0.2003Z4Z6 - 0.5691Z5Z6 + 5.01Z1^2 + 5.12Z2^2+ 5.14Z3^2 – 12.87Z4^2 + 5.15Z5^2 – 10.71Z6^2$ (7.2)

Negative coefficients for the model components Z3, Z5, Z1Z3, Z1Z4, Z1Z6, Z2Z3, Z2Z4, Z2Z6, Z3Z4, Z4Z5,Z5Z6, $Z4^2$ and $Z6^2$ exhibit negative impacts on the percent of degradation of the mixture while positive coefficients Z1, Z2, Z4, Z6, Z1Z2, Z1Z5, Z2Z5, Z3Z5, Z3Z6, Z4Z6, $Z1^2$, $Z2^2$, $Z3^2$, and $Z5^2$ have positive effects on the of degradation of the mixture. Moreover, the coefficients, whose values are near 0, reveal lower relative intensity. Z1Z5, Z2Z5, Z3Z5 belong to this class which could not affect percent of degradation of the mixture so much. On the other hand, the coefficients, whose values are higher, have intense effect on the percent of degradation. Z4, Z1Z4, $Z1^2$, $Z2^2$, $Z3^2$, $Z4^2$, $Z5^2$, and $Z6^2$ have intense effects on the response.

Table VIIA.3: Experimental design matrix for the treatment of wastewater by involving a microbial consortium to remove Phenol & 4- Chloro Phenol

Run order	Space type	Z1: pH	Z2: Temperature (°C)	Z3: Medium Volume (mL)	Z4: Residence Time(hr)	Z5: Initial Concentration of mixture (mg/L)	Z6: inoculums size (percent)	Y: percent of degradation of the mixture		
								Experimental value	Predicted value	Error
1	Factorial	10	40	400	72	1200	18	93.47	96.50	-3.03
2	Factorial	7	40	400	72	1200	6	87.65	87.36	0.2864
3	Factorial	7	50	400	72	800	18	99.83	99.36	0.4712
4	Centre	8.5	45	600	48	1000	12	99.99	94.02	5.93
5	Factorial	10	50	400	72	1200	18	95.65	97.31	-1.66
6	Factorial	7	40	800	72	1200	18	99.92	100.42	-0.4722
7	Factorial	7	50	400	24	1200	6	87.61	82.94	4.67
8	Factorial	7	50	400	24	1200	18	86.11	92.06	-5.95
9	Centre	8.5	45	600	48	1000	12	99.99	94.02	5.93
10	Centre	8.5	45	600	48	1000	12	99.99	94.02	5.93
11	Factorial	7	40	400	24	800	18	99.08	93.76	5.32
12	Centre	8.5	45	600	48	1000	12	99.99	94.02	5.93
13	Factorial	7	40	400	24	1200	6	86.65	81.54	5.11
14	Factorial	7	50	400	72	1200	6	88.65	87.22	1.43
15	Factorial	10	50	400	72	1200	6	89.76	88.11	1.65
16	Factorial	10	50	400	24	1200	6	99.88	88.21	11.67
17	Axial	8.5	35	600	48	1000	12	99.76	114.09	-14.33
18	Centre	8.5	45	600	48	1000	12	99.99	94.02	5.93
19	Factorial	10	40	400	72	800	18	98.63	98.79	-0.1587
20	Factorial	10	40	800	24	1200	6	86.87	83.22	3.65
21	Factorial	7	40	400	24	800	6	87.54	81.22	6.32
22	Factorial	7	40	400	72	1200	18	98.44	98.42	0.0199
23	Factorial	10	40	400	24	800	18	97.65	96.03	1.62
24	Factorial	10	50	800	24	1200	18	99.98	96.81	3.17
25	Factorial	10	40	800	72	800	18	97.54	99.04	-1.50
26	Factorial	10	40	800	24	1200	18	95.77	95.55	0.2212
27	Factorial	10	40	800	72	1200	18	96.53	97.00	-0.4666
28	Centre	8.5	45	600	48	1000	12	99.99	94.02	5.93
29	Factorial	10	50	800	72	800	18	98.65	98.50	0.1546
30	Factorial	10	50	800	24	1200	6	88.76	85.62	3.14
31	Factorial	7	50	400	72	800	6	88.76	87.16	1.60
32	Factorial	10	40	400	72	1200	6	88.54	86.17	2.37
33	Factorial	7	50	400	24	800	18	94.65	93.76	0.8890
34	Axial	8.5	45	600	48	600	12	99.65	115.36	-15.71
35	Factorial	7	50	800	24	1200	18	98.76	93.77	4.99
36	Factorial	7	40	400	72	800	6	88.43	87.56	0.8671
37	Factorial	7	40	800	72	800	6	87.65	86.52	1.13
38	Factorial	10	50	800	72	1200	6	82.95	84.72	-1.77

39	Centre	8.5	45	600	48	1000	12	99.99	94.02	5.93
40	Factorial	10	50	400	72	800	6	88.65	87.87	0.7833
41	Axial	11.5	45	600	48	1000	12	99.76	114.90	-15.14
42	Factorial	10	50	800	24	800	18	99.91	98.07	1.92
43	Factorial	7	50	800	72	1200	18	95.23	98.05	-2.82
44	Factorial	7	50	400	72	1200	18	99.71	97.14	2.57
45	Factorial	10	40	400	72	800	6	87.65	86.18	1.47
46	Axial	8.5	45	600	96	1000	12	55.43	45.65	9.78
47	Factorial	10	40	400	24	800	6	88.65	84.22	4.43
48	Factorial	10	50	400	24	800	6	89.76	87.45	2.31
49	Factorial	7	40	800	24	800	6	84.76	80.98	3.78
50	Axial	8.5	45	600	48	1400	12	99.96	113.90	-13.92
51	Centre	8.5	45	600	48	1000	12	99.99	94.02	5.93
52	Factorial	7	40	800	72	800	18	98.78	102.65	-3.87
53	Axial	8.5	45	1000	48	1000	12	99.76	114.15	-14.39
54	Factorial	10	50	800	72	800	6	87.76	84.22	3.54
55	Factorial	10	40	800	24	800	6	88.76	82.47	6.29
56	Axial	8.5	45	600	0	1000	12	0	39.41	-39.41
57	Factorial	7	40	800	24	1200	18	97.65	94.60	3.05
58	Factorial	7	40	800	24	800	18	96.78	96.30	0.4751
59	Factorial	7	40	400	72	800	18	99.16	100.90	-1.74
60	Factorial	7	50	800	24	800	18	98.88	95.22	3.66
61	Factorial	7	50	400	24	800	6	88.76	82.36	6.40
62	Factorial	10	50	400	72	800	18	97.87	99.34	-1.47
63	Axial	8.5	45	200	48	1000	12	99.76	115.00	-15.24
64	Factorial	7	50	800	72	1200	6	87.87	85.33	2.54
65	Factorial	7	50	800	72	800	6	88.65	85.02	3.63
66	Centre	8.5	45	600	48	1000	12	99.99	94.02	5.93
67	Factorial	10	40	400	24	1200	6	87.54	84.73	2.81
68	Factorial	10	50	400	24	800	18	99.95	98.12	1.83
69	Axial	8.5	45	600	48	1000	24	72.76	63.46	9.30
70	Factorial	10	50	800	24	800	6	88.65	84.60	4.05
71	Centre	8.5	45	600	48	1000	12	99.99	94.02	5.93
72	Factorial	10	40	800	72	800	6	87.54	83.63	3.91
73	Factorial	7	40	400	24	1200	18	93.26	91.80	1.46
74	Factorial	7	50	800	72	800	18	97.13	100.02	-2.89
75	Factorial	10	50	400	24	1200	18	99.97	96.61	3.36
76	Factorial	7	40	800	24	1200	6	86.65	81.54	5.11
77	Factorial	10	40	800	24	800	18	98.75	97.07	1.68
78	Factorial	10	40	400	24	1200	18	96.65	94.26	2.39
79	Axial	8.5	45	600	48	1000	0	0	38.93	-38.93
80	Factorial	10	50	800	72	1200	18	94.49	96.71	-2.22
81	Factorial	10	40	800	72	1200	6	87.64	83.87	3.77
82	Factorial	7	40	800	72	1200	6	88.52	86.57	1.95
83	Axial	8.5	55	600	48	1000	12	99.65	114.95	-15.30
84	Axial	5.5	45	600	48	1000	12	98.76	113.24	-14.48
85	Factorial	7	50	800	24	800	6	85.16	81.02	4.14
86	Factorial	7	50	800	24	1200	6	86.31	81.85	4.46

Table VIIA.4: Adequacy of the models tested for the degradation of the mixture

Sequential Model Sum of Squares						
Source	**Sum of Squares**	**df**	**Mean Square**	**F-value**	**p-value**	**Remarks**
Mean vs. Total	7.181E+05	1	7.181E+05			
Linear vs. Mean	2911.02	6	485.17	2.06	0.0679	
2FI vs. Linear	183.61	15	12.24	0.0424	1.0000	
Quadratic vs. 2FI	**12347.34**	**6**	**2057.89**	**19.51**	**< 0.0001**	**Suggested**
Cubic vs. Quadratic	2823.46	26	108.59	1.05	0.4384	Aliased
Residual	3293.99	32	102.94			
Total	7.397E+05	86	8601.02			

Model Summary Statistics						
Source	**Std. Dev.**	**R^2**	**Adjusted R^2**	**Predicted R^2**	**PRESS**	**Remarks**
Linear	15.36	0.1350	0.0693	0.0074	21399.02	
2FI	16.99	0.1435	0.1375	0.0125	21829.24	
Quadratic	**10.27**	**0.9763**	**0.9842**	**0.9686**	**21159.07**	**Suggested**
Cubic	10.15	0.8472	0.5942	25.1742	5.643E+05	Aliased

The constant variance assumption was investigated by plotting internally studentized residual vs. predicted values of percent degradation of the mixture of Phenolic compounds i.e. Phenol & 4- Chloro Phenol (Figure VIIA.1). Studentized residuals were deducted by dividing the residuals by their standard deviations displaying a randomly scattered pattern within the detection limits -3 to +3 and so, prediction of model described in the equation no (7.2) for the percent degradation of Phenolic mixture is satisfactory. The normal probability plot of residuals (Figure VIIA.2) for the percent degradation of Phenolic mixture showed a straight line pattern rather than S shaped followed by the points on the plot. As the residuals are distributed normally, transformation of response is not required. Relation between the predicted and experimental values of response has been displayed in Figure VIIA.3. Very little discrepancies were found by the straight trend line pointing a good relationship in between the predicted and experimental values.

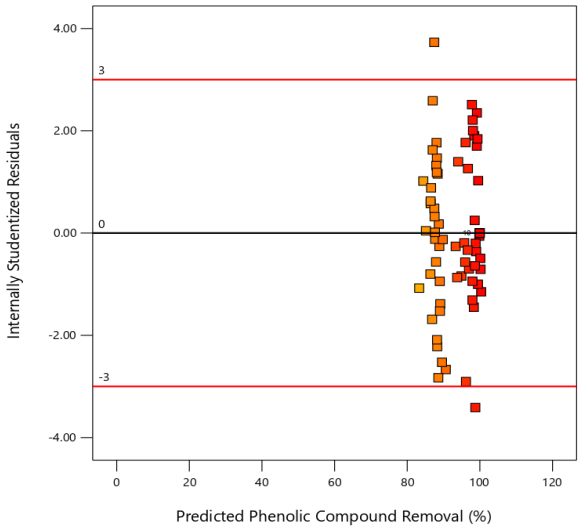


Figure VIIA.1: Internally studentized residuals vs. predicted values

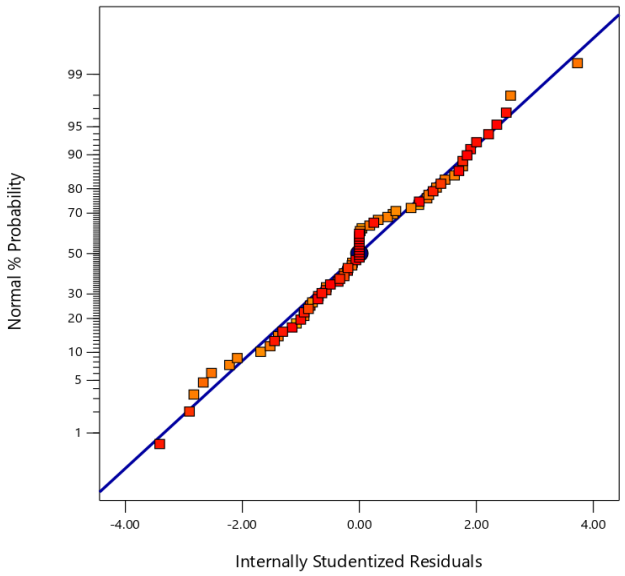


Figure VIIA.2: Internally studentized residuals vs. Normal percent probability

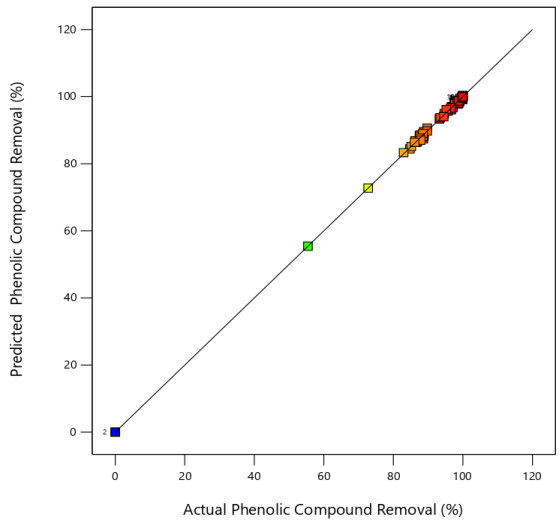


Figure VIIA.3: Actual degradation data vs. predicted data

VIIA.2.2 ANOVA test:

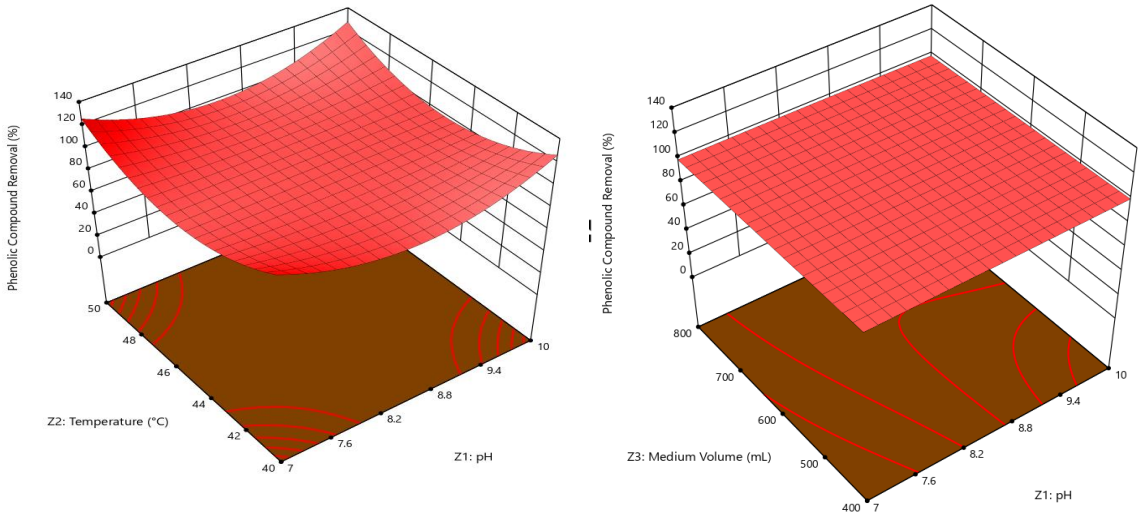
Outcomes of ANOVA for percentage of degradation of the mixture of Phenol & 4- Chloro Phenol have been given in Table VIIA.5. In that table, The Model F-value of 5.42 implies the model is significant. There is only a 0.01% chance that an F-value this large could occur due to noise. P-values less than 0.0500 indicate model terms are significant. In this case Z1, Z2, Z3, Z4, Z5, Z6, Z1Z2, Z1Z4, Z1Z6, Z2Z4, Z2Z6, Z3Z4, Z3Z6, Z4Z5, Z4Z6, Z5Z6, $Z1^2$, $Z2^2$, $Z3^2$, $Z4^2$, $Z5^2$ and $Z6^2$ are significant model terms. Values greater than 0.1000 indicate the model terms are not significant. Z1Z3, Z1Z5, Z2Z3, Z2Z5 and Z3Z5 are not significant. If there are many insignificant model terms (not counting those required to support hierarchy), model reduction may improve the model.

Table VIIA.5: ANOVA of the second order polynomial equation for the degradation of mixture

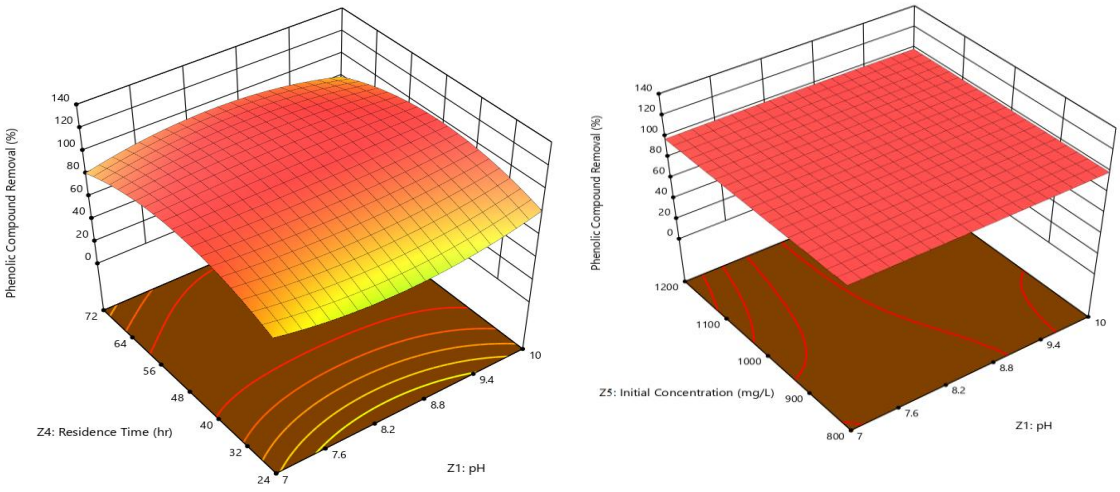
Source	Sum of Squares	df	Mean Square	F-value	p-value	Remarks
Model	15441.97	27	571.92	5.42	< 0.0001	significant
Z1-pH	12.40	1	12.40	0.1176	0.0029	significant
Z2-Temperature	3.31	1	3.31	0.0314	0.0120	significant
Z3-Medium Volume	3.21	1	3.21	0.0304	0.0121	significant
Z4-Residence Time	175.47	1	175.47	1.66	0.0022	significant
Z5-Initial Concentration	9.62	1	9.62	0.0912	0.0137	significant
Z6-Inoculums	2707.01	1	2707.01	25.67	< 0.0001	significant
Z1Z2	17.45	1	17.45	0.1655	0.0257	significant
Z1Z3	9.06	1	9.06	0.0859	0.7705	Not significant
Z1Z4	76.69	1	76.69	0.7271	0.0373	significant
Z1Z5	0.1388	1	0.1388	0.0013	0.9712	Not significant
Z1Z6	2.10	1	2.10	0.0199	0.0282	significant
Z2Z3	4.79	1	4.79	0.0454	0.8321	Not significant
Z2Z4	9.52	1	9.52	0.0902	0.0026	significant
Z2Z5	0.2704	1	0.2704	0.0026	0.9598	Not significant
Z2Z6	5.14	1	5.14	0.0487	0.0260	significant
Z3Z4	2.54	1	2.54	0.0240	0.0087	significant
Z3Z5	0.2475	1	0.2475	0.0023	0.9615	Not significant
Z3Z6	31.30	1	31.30	0.2968	0.0005	significant
Z4Z5	1.07	1	1.07	0.0102	0.0092	significant
Z4Z6	2.57	1	2.57	0.0243	0.0087	significant
Z5Z6	20.73	1	20.73	0.1965	0.0065	significant
$Z1^2$	862.97	1	862.97	8.18	0.0059	significant
$Z2^2$	901.70	1	901.70	8.55	0.0049	significant
$Z3^2$	906.55	1	906.55	8.60	0.0048	significant
$Z4^2$	5693.82	1	5693.82	53.98	< 0.0001	significant
$Z5^2$	911.41	1	911.41	8.64	0.0047	significant
$Z6^2$	3938.91	1	3938.91	37.35	< 0.0001	significant
Residual	6117.45	58	105.47			
Lack of Fit	6117.45	49	124.85			
Pure Error	0.0000	9	0.0000			
Cor Total	21559.42	85				

VIIA.2.3 Effect of pH of the media (Z1):

Effect of pH revealed a significant role in percent degradation of the mixture of Phenol and 4- Chloro Phenol. Percent of degradation of the mixture was maximum (almost 99percent) when the pH value of the culture media was 8.5. Percent degradation was decreased in both situations while the pH value of the media increased and decreased indicating that maximum degradation was occurred at basic condition. Acidic as well as neutral condition is not favourable for the consortium to remove the mixture of Phenol & 4- Chloro Phenol. From the 3D and contour plots, it can be showed that the interaction effects between pH of the media vs. media volume (Z1Z3) and pH of the media vs. initial conc. of the mixture of the Phenolic compounds (Z1Z5) did not possess significant effects on percent degradation of the mixture as the plot is flat shaped (Figure: VIIA.4 (b) & (d)).But interaction effects between pH of the media vs. temperature (Z1Z2), pH of the media vs. residence time (Z1Z4), and pH of the media vs. inoculums percent (Z1Z6) revealed significant effects on the percent degradation of the mixture of Phenol and 4- Chloro Phenol. Plots are convex shaped there (Figure: VIIA.4 (a), (c) & (e)).



(a) (b)



(c) (d)

(e)

Figure VIIA.4: 3-Dimensional surfaces and 2-Dimensional plots of the interaction effects of: (a) pH of the media and temperature (Z1Z2), (b) pH of the media and media vol. (Z1Z3), (c) pH of media and residence time (Z1Z4), (d) pH of the media and initial conc. of the mixture (Z1Z5), (e) pH of the media and inoculums percent (Z1Z6) on the percent of degradation of the Phenolic mixture (Phenol & 4- Chloro Phenol)

VIIA.2.4 Effect of temperature (Z2):

Temperature was found to be very effective factor during this study. Degradation percent of the mixture i.e. Phenol & 4- Chloro Phenol was increased rapidly while temperature reached to 45^{o}C. But below this temperature, degradation percent decreased. Also, while the temperature increased up to 50^{o}C and even 55^{o}C, slight decrease in degradation percent appeared. The interaction effects between temperature vs. media volume (Z2Z3) and

temperature vs. initial conc. of the Phenolic mixture (Z2Z5) found to be insignificant on the response i.e. percent of degradation of the Phenolic mixture. 3D curves found to be flat in shape (Figure: VIIA.5 (a) & (c)). Moreover, p-values of these model terms are 0.8321 and 0.9598 which indicate the insignificance of these terms. On the other hand, temperature vs. residence time (Z2Z4) and temperature vs. inoculums percent (Z2Z6) showed to be very effective on the percent of degradation of the mixture of Phenolic compounds. Here, 3D curves found to be convex shaped (Figure: VIIA.5 (b) & (d)) and the p- values of these model terms also supported the same.

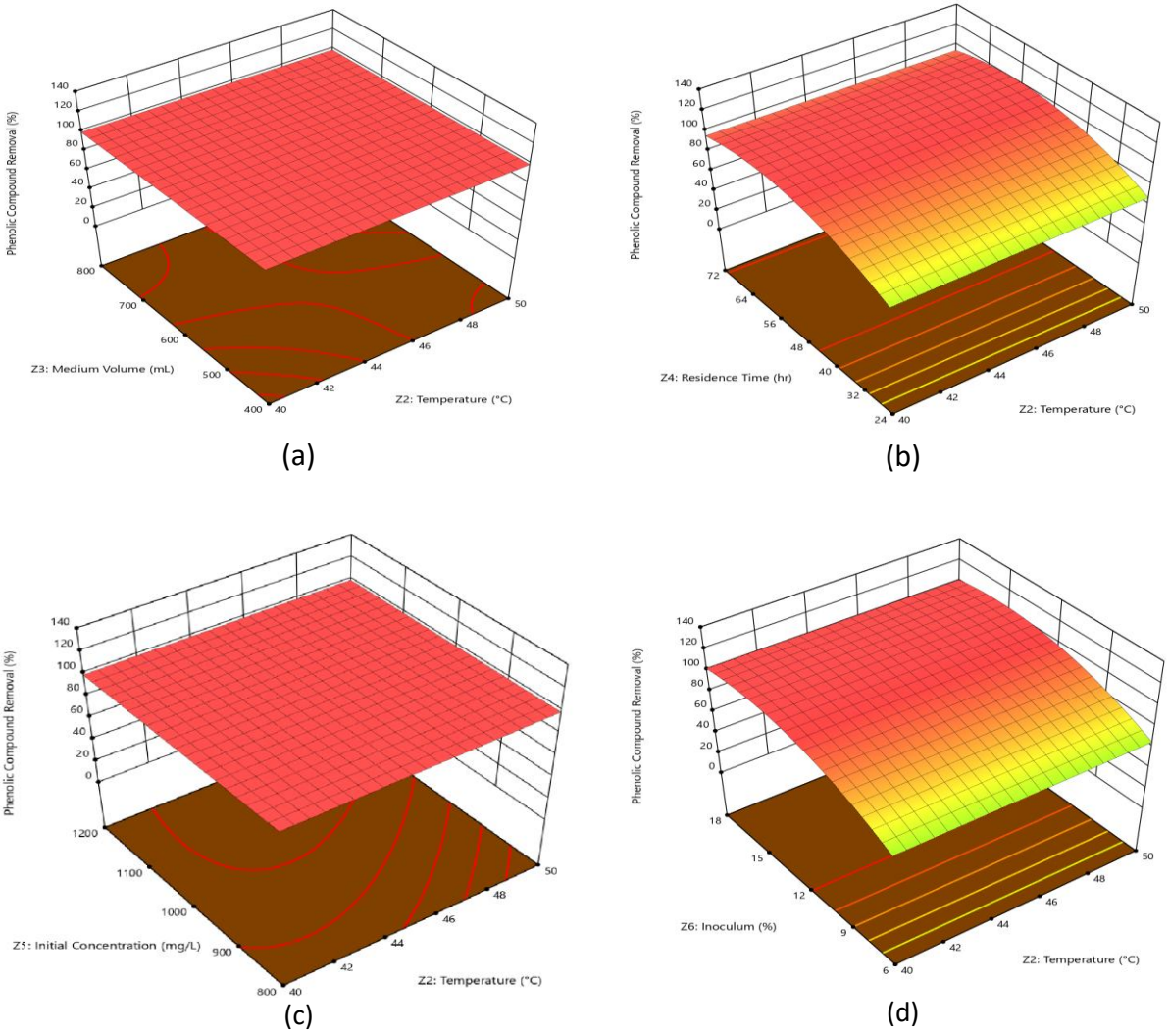
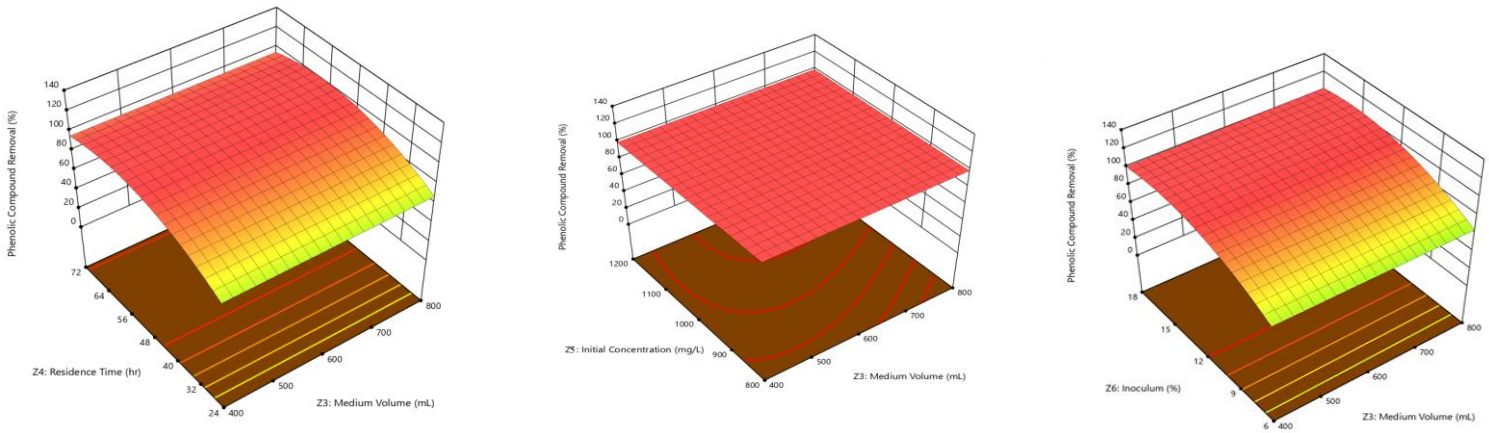


Figure VIIA.5: 3-Dimensional surfaces and 2-Dimensional plots of the interaction effects of: (a) temperature and media vol. (Z2Z3), (b) temperature and residence time (Z2Z4), (c) temperature vs. initial conc. of the mixture (Z2Z5), (d) temperature and inoculums percent (Z2Z6) on the percent of degradation of the mixture (Phenol & 4-Chloro Phenol)

VIIA.2.5 Effect of media volume (Z3):

Volume of the culture media played a pivotal role on degradation percent of Phenol & 4-Chloro Phenol. Percent of degradation of the mixture increased with the increasing volume of the culture media. Up to 600 ml volume, degradation percent increased steadily. But beyond that, when the volume increased up to 800 and even 1000 ml, the degradation percent decreased slightly. Similar effects obtained when the media volume decreased up to 200 ml. In case of interaction effects between media volume vs. residence time (Z3Z4) and media volume vs. inoculums percent (Z3Z6) exhibited significant effects on the degradation percent. Convex shaped 3D curves (Figure: VIIA.6 (a) & (c)) indicated the same. Perhaps the reason behind that was, total inoculums amount remained constant with the increasing media volume. Whereas, the interaction effect between media volume vs. initial conc. of the mixture (Z3Z5) found to be insignificant on the response. P- Value of this model term was found to be 0.9615 indicating its insignificance.



(a) (b) (c)

Figure VIIA.6: 3-Dimensional surfaces and 2-Dimensional plots of the interaction effects of: (a) media vol. and residence time (Z3Z4), (b) media vol. and initial conc. of the mixture (Z3Z5), (c) media vol. and inoculums percent (Z3Z6) on the percent of degradation of the mixture of Phenol & 4- Chloro Phenol

VIIA.2.6 Effect of residence time (Z4):

Residence time played a vital role on the response i.e. percent of degradation. Most of the good responses were achieved from 24 hours onwards (even > 99%). Maximum degradation of the mixture was obtained at 48 hours (~100%). After that time period, no such big changes were appeared. Minimum degradation (0percent) was appeared at 0 hours of residence time. Interaction effect in between residence time vs. initial conc. of the mixture (Z4Z5) and residence time vs. inoculums size of the consortium (Z4Z6) found to be very significant on the percent of degradation of the mixture (Phenol & 4- Chloro Phenol). Both the convex shaped 3D curves (Figure VIIA.7 (a) & (b)) and the p- values of the model terms (Table VIIA.5) indicated the significant effects of both of these interactions.

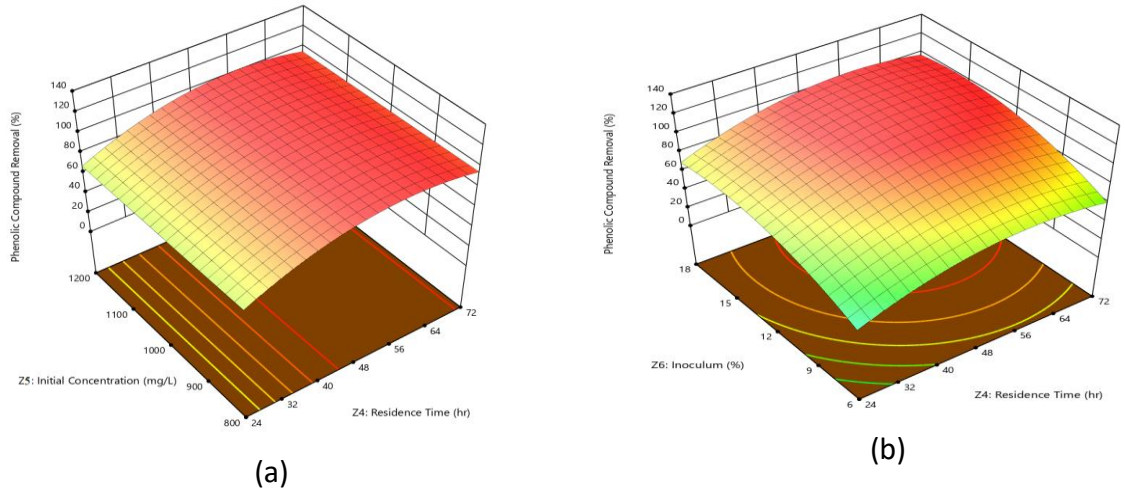


Figure VIIA.7: 3-Dimensional surfaces and 2-Dimensional plots of the interaction effects of: (a) residence time and initial conc. of the mixture (Z4Z5), (b) residence time and inoculums percent (Z4Z6) on the percentage of degradation of the mixture of Phenol & 4- Chloro Phenol

VIIA.2.7 Effect of initial conc. of the mixture of Phenolic compounds and inoculums percent (Z5) & (Z6):

Initial conc. of the mixture (Phenol & 4- Chloro Phenol) and inoculums size of the consortium, both exhibited vital role on the percent of degradation of the mixture. Percent of degradation decreased slightly while initial conc. of the mixture increased. When the conc. of the mixture was 600 mg/L (300 mg/ L Phenol & 300 mg/L 4- Chloro Phenol),it was removed very easily. Almost similar findings were obtained when the initial conc. was 800 mg/L (conc. of each compound was 400 mg/L) and 1000 mg/L (conc. of each compound was 500 mg/L). Optimum degradation was also achieved at the conc. of 1000 mg/L accompanied by suitable pH, temperature and time. When the conc. increased to 1200 mg/L (conc. of each was 600 mg/L), degradation percent slightly decreased. But surprisingly when the initial conc. of the mixture increased further up to 1400 mg/L (conc. of each was 700 mg/L), 99.96% degradation was achieved. Perhaps, this phenomenon was occurred due to the effect of other suitable factors.

Degradation percent increased with the increasing percent of inoculums. 12percent inoculums (3% inoculums of each of the four strains) found to be mostly effective to remove the mixture. Even 12percent inoculums size was found to remove 1400 mg/L mixture of the Phenolic compound (conc. of each was 700 mg/L) completely.

Also, the interaction effect between the initial conc. of the mixture of Phenolic compounds vs. inoculums size (Z5Z6) found to be effective on the response as the curve is convex in nature (Figure VIIA.8).

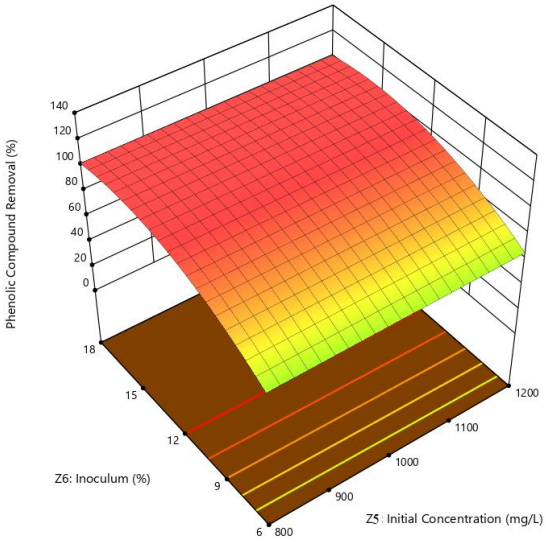


Figure VIIA.8: 3-Dimensional surfaces and 2-Dimensional plots of the interaction effects of: initial conc. of the mixture and inoculums percent (Z5Z6) on the percent of degradation of the mixture of Phenol & 4- Chloro Phenol

VIIA.2.8 Optimization of the operating parameters:

The Response Surface Methodology (RSM) was involved to deduct the optimum conditions for the six independent variables to achieve maximum percent of degradation of the mixture of Phenol & 4-Chlorophenol. Equation (7.2) was defined as objective function for the percent of degradation of the mixture of Phenolic compounds (Phenol & 4- Chloro Phenol) and the independent factors in their ranges were model constraints. Thus the following optimum conditions, achieved for the maximum percent of degradation of the mixture were: 8.5 pH of the bacterial culture media, 45^{o}C temperature, 600 mL volume of the culture media, 48 hours of residence time, 1000 mg/L initial conc. of the mixture (in 1:1 ratio i.e. 500 mg/L Phenol & 500 mg/L 4- Chloro Phenol) and 12percent inoculums size of the consortium (i.e. 3% *Brevibacillus formosus* strain NRRL NRS- 863, 3% *Pseudomonas otitidis* strain MCC10330, 3% *Bacillus timonensis* strain 10403023 and 3% *Bacillus cereus* strain MK789657). 99.99% degradation of the mixture of Phenol and 4- Chloro Phenol was obtained by involving these six favourable parameters. The obtained optimal operating conditions were performed nine more times to validate the predicted values as well to minimise the error and 99.99% degradation was achieved in case of each experimental results indicating the reliability of this model.

VIIA.2.9 Result of HPLC analysis:

From the standard chromatogram of known conc. of Phenol and 4- Chloro Phenol, it can be deduced that the peak of 6.364 AU, represents the residual conc. of Phenol and 6.972 AU represents the residual conc. of 4- Chloro Phenol respectively (Figure VIIA.9). Calculating the area of the chromatograms (both the standard and the sample), it was found that individually 98.84% of Phenol and 97.77% of 4- Chloro Phenol were removed from the bi-solute mixture, while cultured according to the parameters of the centre point of RSM, which almost supported the experimental value, obtained at the centre point of the design matrix (Table VIIA.3).

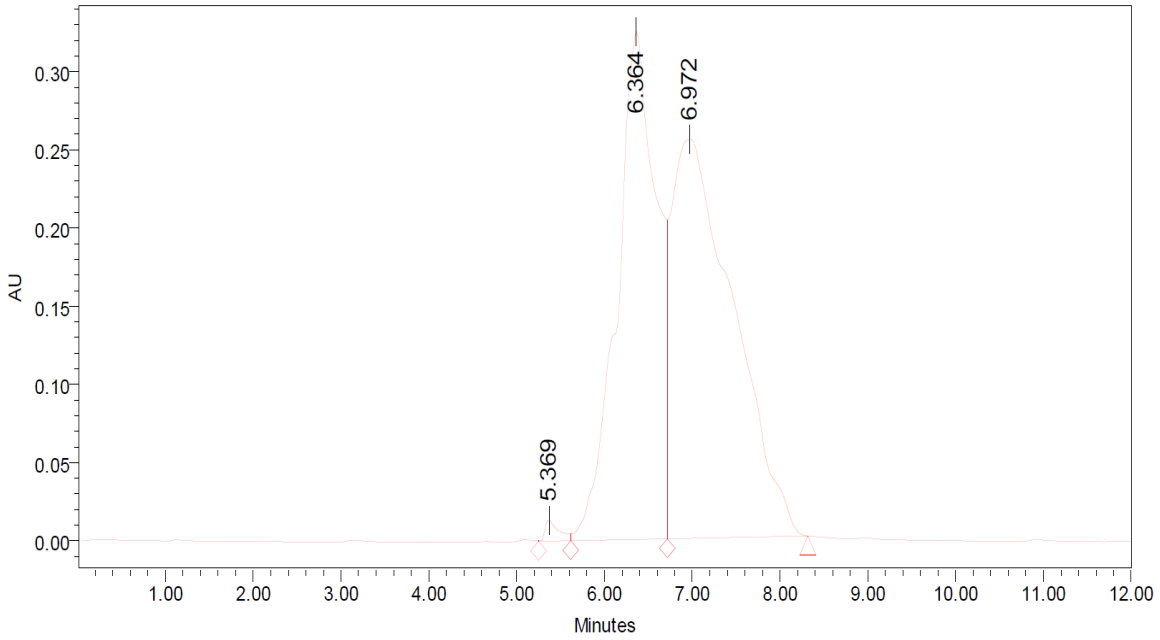


Figure VIIA.9: Chromatogram for the of the sample obtained as per centre point of RSM design

Sub chapter VIIB

Optimization of parameters for the consortium, involved to degrade the mixture of 4- Chloro Phenol & Catechol

VIIB.1 Materials and Methods:

VIIB.1.1 Materials:

Four different strains (*Bacillus timonensis* strain 10403023, *Bacillus cereus* strain K1, *Bacillus pseudomycoides* strain NBRC 101232 and *Bacillus paramycoides* strain MCCC 1A04098) were utilized to make a microbial consortium.

4- Chloro Phenol and Catechol were prepared synthetically in the laboratory. The conc. of the solutions was maintained as 10 g/L.

VIIB.1.2 Experimental set up:

In case of designing the percent of inoculums, total percent of inoculums of the all four bacterial strains was taken into account where percent of each of the four strains was maintained equally (1:1:1:1 ratio). Similarly in case of initial conc. of the mixture to be removed i.e. 4- Chloro Phenol & Catechol, total initial conc. (mg/L) of the two compounds was used for the design where individual initial conc. of the compounds was kept equal (1:1 ratio).

Rest of the portion is same as mentioned in the chapter III, section IIIA.1.2.

VIIB.1.3 Analytical Method:

Same as described in chapter VI, section VIA.1.3

VIIB.1.4 Experimental design:

Six (6) independent variables (Table VIIB.1) are X1 (A): 6.25 – 10.75; X2 (B): 37.5^{o} – 52.5^{o}C; X3 (C): 300 – 900 mL; X4 (D): 12 – 84 hours; X5 (E): 700 – 1300 mg/L and X6 (F): 3 – 21%. These six independent variables were coded at five levels between -1.5 and +1.5 based on preliminary experimental outputs.

Rest of the portion is same as described in sub chapter VIIA, section VIIA.1.4.

Table VIIB.1: Independent variables with coded levels

Independent variable	Symbol	Coded levels				
		-1.5	-1	0	+1	+1.5
pH of bacterial media	X1	6.25	7	8.5	10	10.75
Temperature (^{o}C)	X2	37.5	40	45	50	52.5
Volume of bacterial media(mL)	X3	300	400	600	800	900
Residence time(hr)	X4	12	24	48	72	84
Initial conc. of the mixture (mg/L)	X5	700	800	1000	1200	1300
Inoculums percentage (percent)	X6	3	6	12	18	21

VIIB.1.5 HPLC analysis:

HPLC analysis was performed in order to detect the individual degradation of the two compounds i.e. 4- Chloro Phenol & Catechol. It was done only in case of the sample, obtained as per centre point of RSM design. The chromatogram image (VIIB.9) depicted satisfactory degradation of the two Phenolic compounds individually.

Rest of the portion is same as described in the sub chapter VIIA, section VIIA.1.5.

VIIB.2 Results and Discussions:

VIIB.2.1 Fitting of the model and Statistical analysis:

Total 86 experiments were done as per the design matrix and the only one response was percent of degradation of the mixture (4- Chloro Phenol+ Catechol). Table VIIB.2 displayed both the predicted and experimental/actual values of percent of degradation of the mixture of 4- Chloro Phenol & Catechol. Second order and linear polynomial equations were fitted to the actual data to obtain the regression equation. To reveal the suitable model, sequential model sum of squares and model summary statistics were deducted (Table VIIB.3). The Sequential P-value for the quadratic model is less than 0.0001; Maximum predicted R^2 and adjusted R^2 values were 0.9596 and 0.9604 respectively for the percent of degradation of the mixture. Cubic model found to be aliased; here the sequential P- value was> 0.05. So, finally the quadratic model was chosen for further determination of the percent of degradation of the mixture (4- Chloro Phenol & Catechol).

The following equation clearly showed this:

Percentage of degradation of the mixture of 4- Chloro Phenol & Catechol (Y): $98.23+ 0.7049X1 + 1.50X2 – 0.4112X3 +2.17X4 - 1.65X5+1.25X6 +0.3928X1X2 – 0.0872X1X3 + 0.1700X1X4+ 0.2916X1X5 - 0.0813X1X6+0.4434X2X3 - 0.8156X2X4 - 0.0728X2X5 – 0.3694X2X6 - 0.1725X3X4 + 0.5016X3X5 – 0.5000X3X6 +1.27X4X5 – 1.91X4X6+1.33X5X6 + 0.3524X1^2 + 0.1057X2^2– 0.5032X3^2 – 3.00X4^2 + 0.0813X5^2 – 0.2476X6^2$ (7.3)

Negative coefficients for the model components X3, X5, X1X3, X1X6, X2X4, X2X5, X2X6, X3X4, X3X6,X4X6, $X3^2$, $X4^2$ and $X6^2$ exhibit negative impacts on the percent of degradation of the mixture while positive coefficients X1, X2, X4, X6, X1X2, X1X4, X1X5, X2X3, X3X5, X4X5, X5X6, $X1^2$, $X2^2$, and $X5^2$ have positive effects on the of degradation of the mixture. Moreover, the coefficients, whose values are near 0, reveal lower relative intensity. X1X3, X1X6, X2X5 and $X5^2$ belong to in this class which could not affect percentage of degradation of the mixture so much.

Table VIIB.2: Experimental design matrix for the treatment of wastewater by involving a microbial consortium to remove 4- Chloro Phenol & Catechol

Run order	Space type	X1: pH	X2: Temperature (°C)	X3: Medium Volume (mL)	X4: Residence Time(hr)	X5: Initial Concentration of mixture (mg/L)	X6: inoculums size (percent)	Y: percent of degradation of the mixture		
								Experimental value	Predicted value	Error
1	Factorial	7	50	800	24	1200	18	94.77	95.44	-0.6676
2	Factorial	10	50	400	72	800	6	99.59	101.96	-1.97
3	Factorial	7	40	400	24	800	18	99.13	97.41	1.72
4	Centre	8.5	45	600	48	1000	12	99.99	98.23	1.40
5	Factorial	10	40	800	72	1200	18	96.41	96.83	-0.4175
6	Factorial	10	40	400	24	800	18	94.25	97.13	-2.88
7	Centre	8.5	45	600	48	1000	12	99.99	98.23	1.40
8	Factorial	10	50	800	72	1200	18	98.45	98.98	-0.5268
9	Factorial	10	40	800	24	1200	6	80.56	83.59	-3.03
10	Factorial	10	40	800	72	800	6	96.38	97.58	-1.20
11	Axial	10.75	45	600	48	1000	12	97.93	100.08	-2.15
12	Centre	8.5	45	600	48	1000	12	99.99	98.23	1.40
13	Factorial	10	50	400	24	800	18	99.87	101.05	-1.18
14	Factorial	10	40	400	72	800	6	99.79	99.82	0.1739
15	Factorial	7	50	400	72	800	18	99.29	96.13	3.16
16	Factorial	10	50	400	72	1200	18	98.67	99.43	-0.7587
17	Factorial	10	50	800	24	1200	18	98.95	97.54	1.41
18	Factorial	7	50	800	72	1200	6	97.88	96.43	1.45
19	Centre	8.5	45	600	48	1000	12	99.99	98.23	1.40
20	Factorial	10	40	400	24	800	6	93.13	91.89	1.24
21	Factorial	7	50	800	24	1200	6	86.76	88.05	-1.29
22	Centre	8.5	45	600	48	1000	12	99.99	98.23	1.40
23	Factorial	7	40	800	24	800	18	94.03	94.22	-0.1892
24	Axial	8.5	45	600	48	1000	21	97.87	99.55	-1.68
25	Factorial	7	40	800	72	800	6	99.81	97.22	2.59
26	Factorial	7	40	800	72	800	18	89.78	93.15	-3.37
27	Factorial	7	40	400	24	800	6	90.17	91.85	-1.68
28	Factorial	7	40	400	72	800	18	97.25	97.03	0.2201
29	Factorial	7	40	400	24	1200	18	93.41	92.78	0.6261
30	Factorial	10	50	800	24	800	18	97.88	99.29	-1.41
31	Factorial	7	50	400	72	1200	6	96.88	94.54	2.34
32	Factorial	10	40	400	72	1200	6	98.42	96.14	2.28
33	Factorial	10	50	800	24	800	6	99.75	97.53	2.22
34	Centre	8.5	45	600	48	1000	12	99.99	98.23	1.40
35	Factorial	7	50	800	24	800	18	95.87	98.35	-2.48
36	Factorial	7	50	400	24	1200	18	96.77	94.85	1.92
37	Factorial	7	50	800	72	800	18	96.25	94.02	2.23
38	Centre	8.5	45	600	48	1000	12	99.99	98.23	1.40
39	Factorial	7	50	400	72	800	6	96.58	99.67	-3.09

40	Centre	8.5	45	600	48	1000	12	99.99	98.23	1.40
41	Centre	8.5	45	600	48	1000	12	99.99	98.23	1.40
42	Factorial	10	50	800	72	800	18	97.75	95.63	2.12
43	Factorial	7	50	400	72	1200	18	95.43	96.30	-0.8683
44	Axial	8.5	45	300	48	1000	12	95.78	97.71	-1.93
45	Centre	8.5	45	600	48	1000	12	99.99	98.23	1.40
46	Factorial	10	50	800	24	1200	6	90.87	90.48	0.3938
47	Axial	8.5	37.5	600	48	1000	12	94.49	96.22	-1.73
48	Factorial	10	40	800	24	800	6	90.25	90.35	-0.1004
49	Axial	8.5	45	600	12	1000	12	86.91	88.23	-1.32
50	Factorial	10	40	800	72	800	18	94.46	93.19	1.27
51	Factorial	7	40	400	72	1200	18	95.73	97.49	-1.76
52	Factorial	7	40	800	24	1200	6	81.65	82.73	-1.08
53	Axial	8.5	45	600	48	1300	12	94.11	95.94	-1.83
54	Factorial	7	40	800	24	1200	18	94.17	91.60	2.57
55	Factorial	7	50	400	24	800	18	99.64	99.77	-0.1304
56	Factorial	10	50	400	24	1200	6	90.63	88.24	2.39
57	Factorial	10	40	400	72	800	18	97.41	97.42	2.58
58	Factorial	10	40	800	24	1200	18	94.68	92.13	2.55
59	Axial	8.5	52.5	600	48	1000	12	98.71	100.71	-2.00
60	Factorial	7	50	400	24	800	6	96.87	95.69	1.18
61	Factorial	10	50	400	24	800	6	99.98	97.30	2.68
62	Factorial	10	50	800	72	800	6	99.99	101.50	-1.51
63	Factorial	7	40	400	72	800	6	98.99	99.10	-0.1082
64	Axial	8.5	45	600	48	1000	3	93.74	95.80	-2.06
65	Factorial	10	50	800	72	1200	6	98.98	99.54	-0.5601
66	Axial	6.25	45	600	48	1000	12	96.38	97.97	-1.59
67	Factorial	7	40	800	72	1200	6	95.83	94.38	1.45
68	Factorial	7	50	800	72	800	6	98.73	99.56	-0.8319
69	Factorial	10	50	400	72	1200	6	98.51	97.99	0.5181
70	Factorial	10	40	800	24	800	18	93.63	93.58	0.0466
71	Factorial	7	40	800	24	800	6	92.38	90.66	1.72
72	Factorial	7	50	400	24	1200	6	82.92	85.46	-2.54
73	Factorial	10	40	400	24	1200	6	81.05	83.12	-2.07
74	Factorial	7	40	400	24	1200	6	82.76	81.92	0.8416
75	Factorial	10	40	400	24	1200	18	94.97	93.66	1.31
76	Axial	8.5	45	600	48	700	12	98.98	100.88	-1.90
77	Factorial	7	40	800	72	1200	18	93.74	95.62	-1.88
78	Factorial	10	40	800	72	1200	6	96.92	95.91	1.01
79	Factorial	7	50	800	72	1200	18	96.83	96.20	0.6348
80	Factorial	10	50	400	72	800	18	98.36	98.09	0.2704
81	Axial	8.5	45	600	84	1000	12	92.33	94.74	-2.41
82	Factorial	10	50	400	24	1200	18	95.63	97.30	-1.67
83	Factorial	7	40	400	72	1200	6	94.37	94.25	0.1153
84	Factorial	7	50	800	24	800	6	97.24	96.27	0.9695
85	Axial	8.5	45	900	48	1000	12	94.68	96.48	-1.80
86	Factorial	10	40	400	72	1200	18	98.51	99.05	-0.5431

Table VIIB.3: Adequacy of the models tested for the degradation of the mixture

Sequential Model Sum of Squares						
Source	**Sum of Squares**	**df**	**Mean Square**	**F-value**	**p-value**	**Remarks**
Mean vs. Total	7.872E+05	1	7.872E+05			
Linear vs. Mean	814.40	6	135.73	10.66	< 0.0001	
2FI vs. Linear	565.36	15	37.69	5.48	< 0.0001	
Quadratic vs. 2FI	**192.67**	**6**	**32.11**	**7.51**	**< 0.0001**	**Suggested**
Cubic vs. Quadratic	143.25	26	5.51	1.69	0.0803	Aliased
Residual	104.62	32	3.27			
Total	7.890E+05	86	9174.74			

Model Summary Statistics						
Source	**Std. Dev.**	**R^2**	**Adjusted R^2**	**Predicted R^2**	**PRESS**	**Remarks**
Linear	3.57	0.4474	0.4054	0.3431	1195.76	
2FI	2.62	0.7580	0.6786	0.6128	704.74	
Quadratic	**2.07**	**0.9838**	**0.9604**	**0.9596**	**601.35**	**Suggested**
Cubic	1.81	0.9425	0.8473	-6.6121	13856.47	Aliased

The constant variance assumption was investigated by plotting internally studentized residual vs. predicted values of percent degradation of mixture of Phenolic compounds i.e. 4- Chloro Phenol & Catechol (Figure VIIB.1). The normal probability plot of residuals (Figure VIIB.2) for the percent degradation of Phenolic mixture showed a straight line pattern rather than S shaped followed by the points on the plot. As the residuals are distributed normally, transformation of response is not required. Relation between the predicted and experimental values of response has been displayed in Figure VIIB.3. Very little discrepancies were found by the straight trend line pointing a good relationship in between the predicted and experimental values.

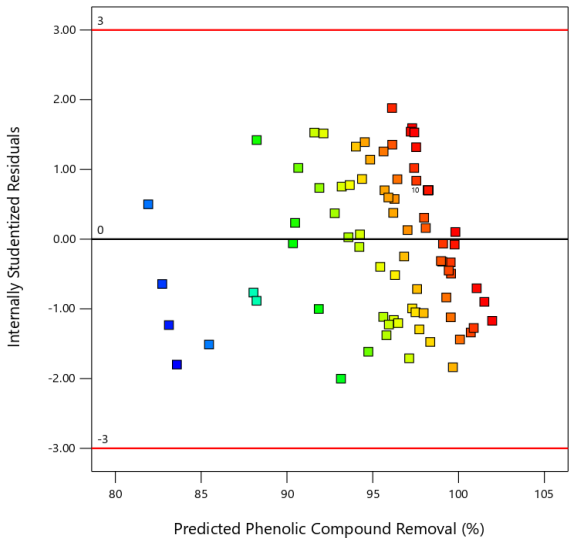


Figure VIIB.1: Internally studentized residuals vs. predicted values

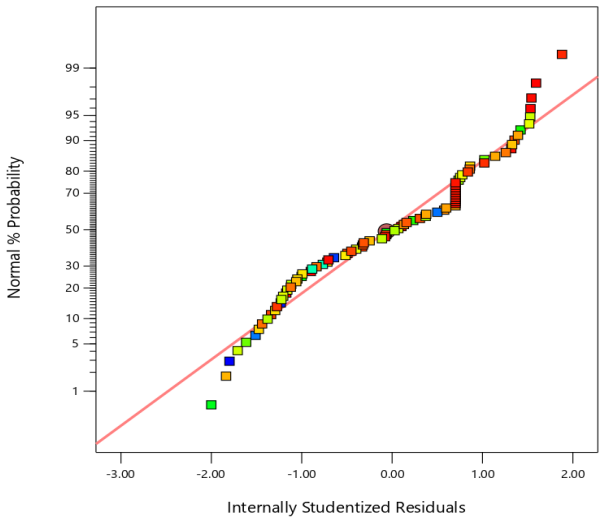


Figure VIIB.2: Internally studentized residuals vs. Normal percent probability

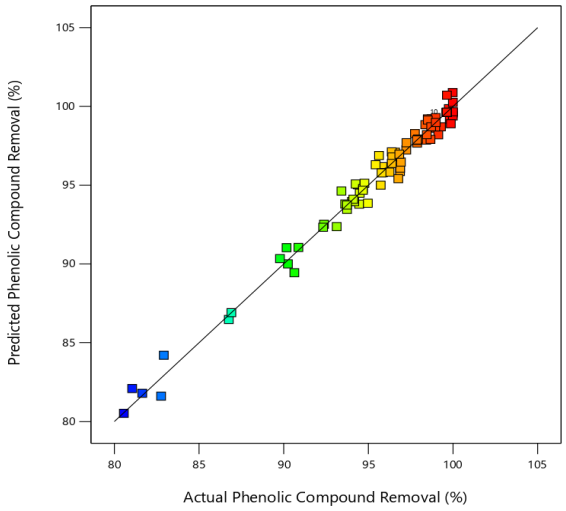


Figure VIIB.3: Actual degradation data vs. predicted data

VIIB.2.2 ANOVA test:

Outcomes of ANOVA for percent of degradation of the mixture of 4- Chloro Phenol & Catechol have been given in Table VIIB.4. In that table, The Model F-value of 13.63 implies the model is significant. There is only a 0.01% chance that an F-value this large could occur due to noise. P-values less than 0.0500 indicate model terms are significant. In this case X1, X2, X3, X4, X5, X6, X1X3, X1X4, X1X5, X1X6, X2X3, X2X4, X2X5, X2X6, X3X4, X3X5, X3X6, X4X5, X4X6, X5X6 and $X4^2$ are significant model terms. Values greater than 0.100 indicate the model terms are not significant. X1X2, $X1^2$, $X2^2$, $X3^2$, $X5^2$ and $X6^2$ are not significant. If there are many insignificant model terms (not counting those required to support hierarchy), model reduction may improve the model.

Table VIIB.4: ANOVA of the second order polynomial equation for the degradation of mixture

Source	Sum of Squares	df	Mean Square	F-value	p-value	Remarks
Model	1572.44	27	58.24	13.63	< 0.0001	significant
X1-pH	34.04	1	34.04	7.96	0.0065	significant
X2-Temperature	153.35	1	153.35	35.88	< 0.0001	significant
X3-Medium Volume	11.58	1	11.58	2.71	0.0051	significant
X4-Residence Time	322.67	1	322.67	75.50	< 0.0001	significant
X5-Initial Concentration	185.96	1	185.96	43.51	< 0.0001	significant
X6-Inoculums	106.81	1	106.81	24.99	< 0.0001	significant
X1X2	9.88	1	9.88	2.31	0.7339	Not significant
X1X3	0.4865	1	0.4865	0.1138	0.0370	significant
X1X4	1.85	1	1.85	0.4328	0.0132	significant
X1X5	5.44	1	5.44	1.27	0.0238	significant
X1X6	0.4225	1	0.4225	0.0989	0.0143	significant
X2X3	12.58	1	12.58	2.94	0.0015	significant
X2X4	42.58	1	42.58	9.96	0.0025	significant
X2X5	0.3393	1	0.3393	0.0794	0.0191	significant
X2X6	8.73	1	8.73	2.04	0.0183	significant
X3X4	1.90	1	1.90	0.4456	0.0171	significant
X3X5	16.10	1	16.10	3.77	0.0071	significant
X3X6	16.00	1	16.00	3.74	0.0079	significant
X4X5	103.73	1	103.73	24.27	< 0.0001	significant
X4X6	232.64	1	232.64	54.44	< 0.0001	significant
X5X6	112.68	1	112.68	26.37	< 0.0001	significant
$X1^2$	1.46	1	1.46	0.3426	0.5606	Not significant
$X2^2$	0.1318	1	0.1318	0.0308	0.8612	Not significant
$X3^2$	2.99	1	2.99	0.6986	0.4067	Not significant
$X4^2$	105.88	1	105.88	24.78	< 0.0001	significant
$X5^2$	0.0779	1	0.0779	0.0182	0.8931	Not significant
$X6^2$	0.7231	1	0.7231	0.1692	0.6823	Not significant
Residual	247.87	58	4.27			
Lack of Fit	247.87	49	5.06			
Pure Error	0.0000	9	0.0000			
Cor Total	1820.31	85				

VIIB.2.3 Effect of pH of the media(X1):

Effect of pH revealed a significant role in percent degradation of the mixture of 4- Chloro Phenol & Catechol. Percent of degradation of the mixture was maximum (above 98%) when the pH value of the culture media was 8.5. Percent degradation was decreased in both situations while the pH value of the media increased and decreased indicating that maximum degradation was occurred at basic condition. Acidic as well as neutral condition is not favourable for the consortium to remove the mixture of 4- Chloro Phenol & Catechol. From the 3D and contour plots, it can be showed that the interaction effects between pH of the media vs. temperature (X1X2) did not possess significant effects on percent degradation of the mixture as the plot is flat shaped (Figure: VIIB.4 (a)).But interaction effects between pH of the media vs. media volume (X1X3), pH of the media vs. residence time (X1X4), pH of the media vs. initial conc. of the mixture of the Phenolic compounds (X1X5) and pH of the media vs. inoculums percent (X1X6) revealed significant effects on the percent degradation of the mixture of 4- Chloro Phenol & Catechol. Plots are convex shaped there (Figure: VIIB.4 (b), (c), (d) & (e)).

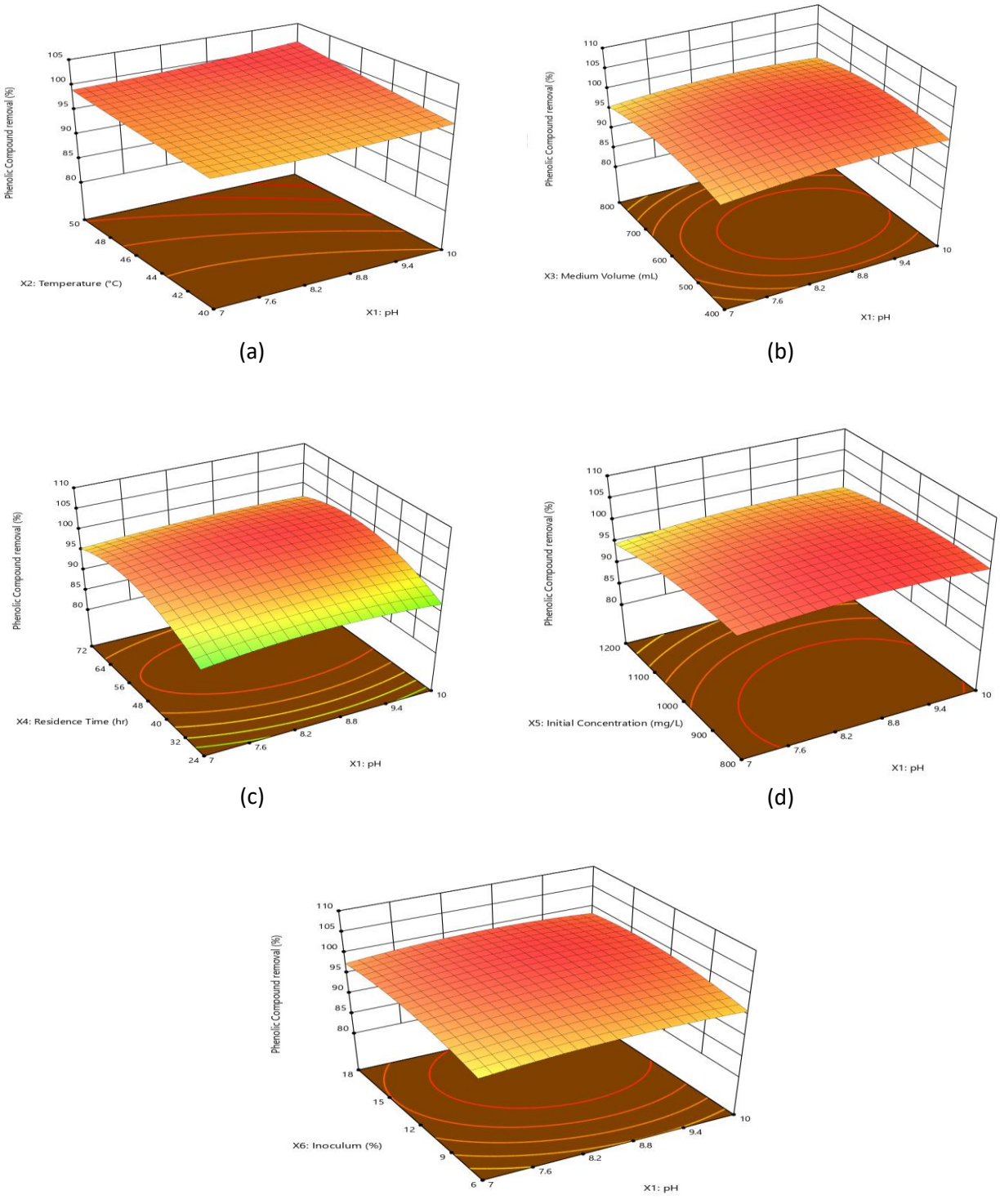


Figure VIIB.4: 3-Dimensional surfaces and 2-Dimensional plots of the interaction effects of: (a) pH of the media and temperature (X1X2), (b) pH of the media and media vol. (X1X3), (c) pH of media and residence time (X1X4), (d) pH of the media and initial conc. of the mixture (X1X5), (e) pH of the media and inoculums percent (X1X6) on the percent of degradation of the Phenolic mixture (4- Chloro Phenol & Catechol)

VIIB.2.4 Effect of temperature (X2):

Temperature was found to be very important factor during this study. Degradation percent of the mixture i.e. 4- Chloro Phenol & Catechol was increased rapidly while temperature reached to 45^{o}C. But below this temperature, degradation percent decreased. Also, when the temperature increased, the degradation efficacy of the consortium decreased. The p- Value of the X2 found to be 0.0065 which strongly indicated its significance on the response. The interaction effects between temperature vs. media volume (X2X3), temperature vs. residence time (X2X4), temperature vs. initial conc. of the Phenolic mixture (X2X5) and temperature vs. inoculums percent (X2X6) all showed to be very effective on the percent of degradation of the mixture of Phenolic compounds (Figure: VIIB.5).

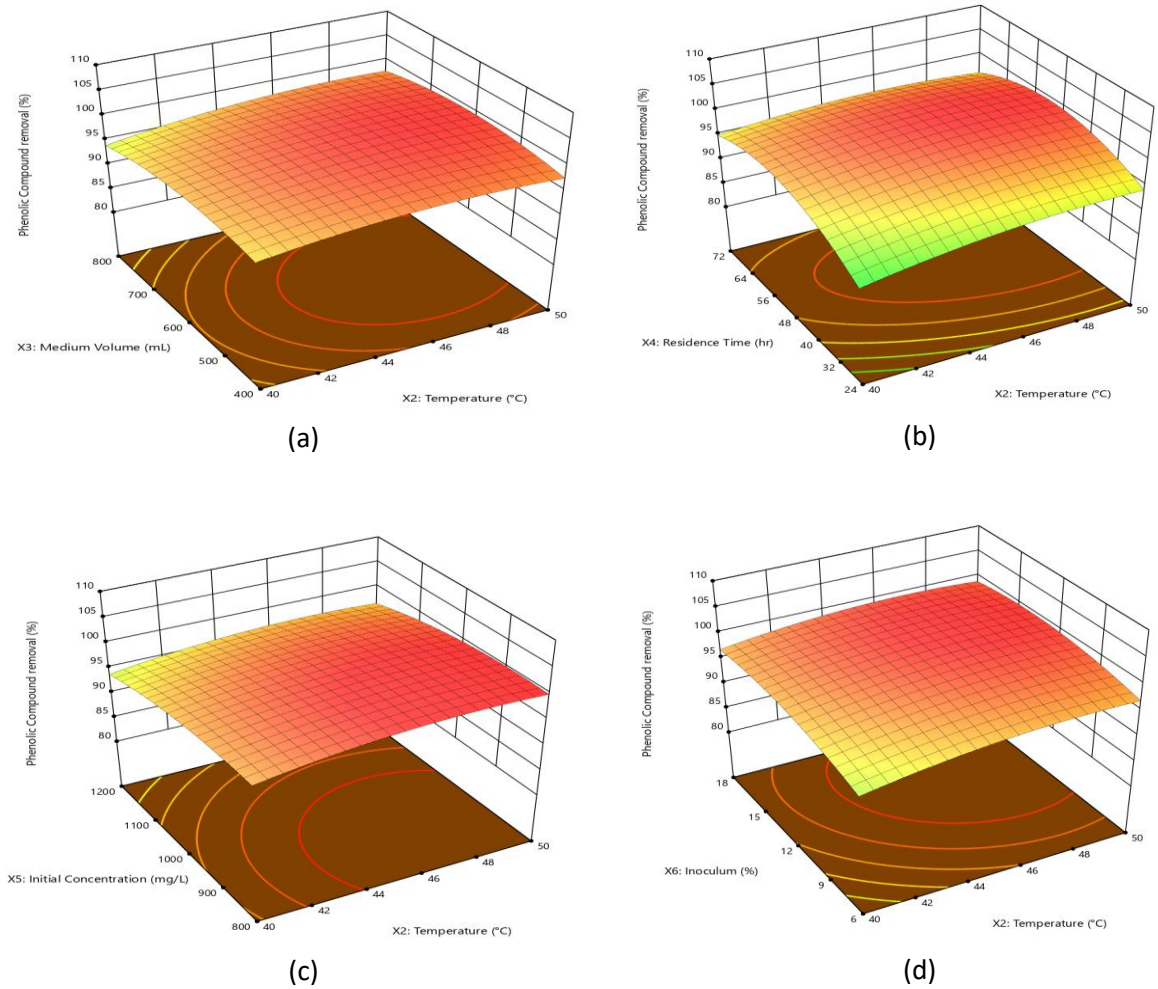
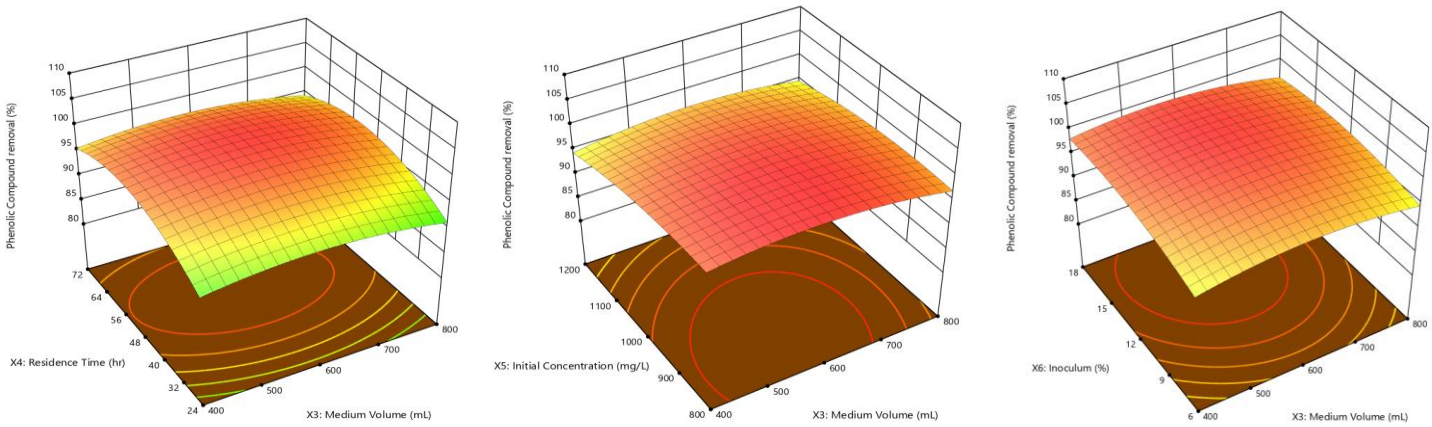


Figure VIIB.5: 3-Dimensional surfaces and 2-Dimensional plots of the interaction effects of: (a) temperature and media vol. (X2X3), (b) temperature and residence time (X2X4), (c) temperature vs. initial conc. of the mixture (X2X5), (d) temperature and inoculums percent (X2X6) on the percent of degradation of the mixture (4- Chloro Phenol & Catechol)

VIIB.2.5 Effect of media volume (X3):

Volume of the culture media played a pivotal role on degradation percent of 4- Chloro Phenol & Catechol. Percent of degradation of the mixture increased with the increasing volume of the culture media. Up to 600 ml volume, degradation percent increased steadily. But beyond that, when the volume increased up to 800 and even 900 ml, the degradation percent decreased slightly. Similar effects obtained when the media volume decreased up to 300 ml. In case of interaction effects between media volume vs. residence time (X3X4), media volume vs. initial conc. of the mixture (X3X5) and media volume vs. inoculums percent (X3X6), all exhibited significant effects on the degradation percent. Convex shaped curves (Figure: VIIB. 6(a), (b) & (c)) indicated the same. The interaction effect between media volume vs. residence time (X3X4) left a very significant role on degradation percent. P-Value of the X3X4 (Table VIIB.4) supported the same.



(a) (b) (c)

Figure VIIB.6: 3-Dimensional surfaces and 2-Dimensional plots of the interaction effects of: (a) media vol. and residence time (X3X4), (b) media vol. and initial conc. of the mixture (X3X5), (c) media vol. and inoculums percent (X3X6) on the percent of degradation of the mixture of 4- Chloro Phenol & Catechol

VIIB.2.6 Effect of residence time (X4):

Residence time played a vital role on the response i.e. percent of degradation. Most of the good responses were achieved from 24 hours onwards (> 90%). Maximum degradation of the mixture was obtained at 48 hours (>95%). After that time period, no such big changes were appeared. Minimum degradation (~86%) was appeared at 12 hours of residence time. Interaction effect in between residence time vs. initial conc. of the mixture (X4X5) and residence time vs. inoculums size of the four bacterial strains (X4X6) found to be very significant on the percent of degradation of the mixture (4- Chloro Phenol & Catechol). Both the figure (VIIB.7 (a) & (b)) and the p- values of these two terms (Table VIIB.4) indicated the same.

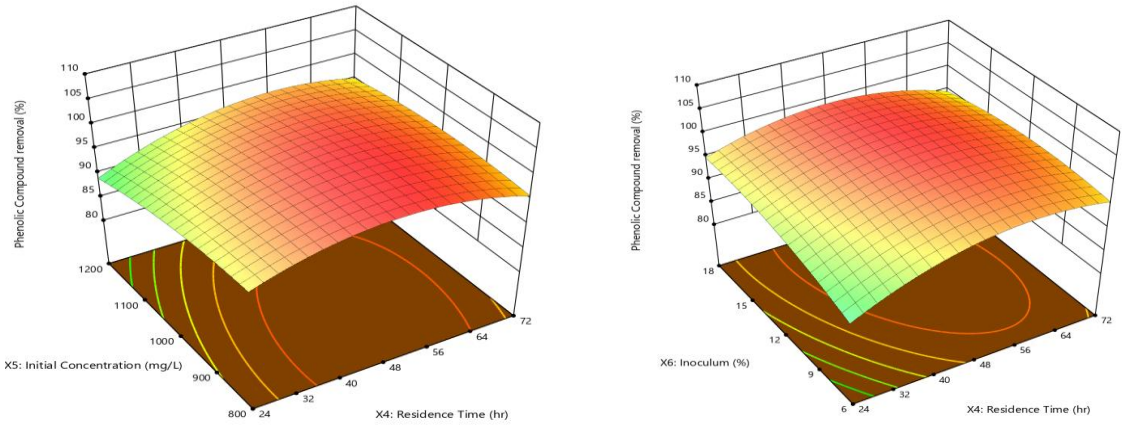


Figure VIIB.7: 3-Dimensional surfaces and 2-Dimensional plots of the interaction effects of: (a) residence time and initial conc. of the mixture (X4X5), (b) residence time and inoculums percent (X4X6) on the percent of degradation of the mixture of 4-Chloro Phenol & Catechol

VIIB.2.7 Effect of initial conc. of the mixture of Phenolic compounds and inoculums percent (X5) & (X6):

Initial conc. of the mixture (4- Chloro Phenol & Catechol) and inoculums size of the four strains both exhibited pivotal role on the percent of degradation of the mixture. Percent of degradation decreased slightly while initial conc. of the mixture increased. When the conc. of the mixture was 700 mg/L (350 mg/L 4- Chloro Phenol & 350 mg/L Catechol),it was removed very easily. Almost similar findings were obtained when the initial conc. was 800 mg/L (conc. of each was 400 mg/L) and 1000 mg/L (conc. of each was 500 mg/L). Optimum degradation was also achieved at the conc. of 1000 mg/L accompanied by suitable pH, temperature and time. But when the conc. increased to 1200 mg/L (conc. of each was 600 mg/L) as well as 1300 mg/L (conc. of each was 650 mg/L), degradation percent slightly decreased.

Degradation percent increased with the increasing percent of inoculums. 12% inoculums (3% inoculums of each of the four strains) found to be mostly effective to remove the mixture. Beyond that, no big changes occurred even after increasing the inoculums percent.

Also, the interaction effect between the initial conc. of the mixture of Phenolic compounds vs. inoculums size found to be effective on the response as the curve is convex in nature (Figure VIIB.8).

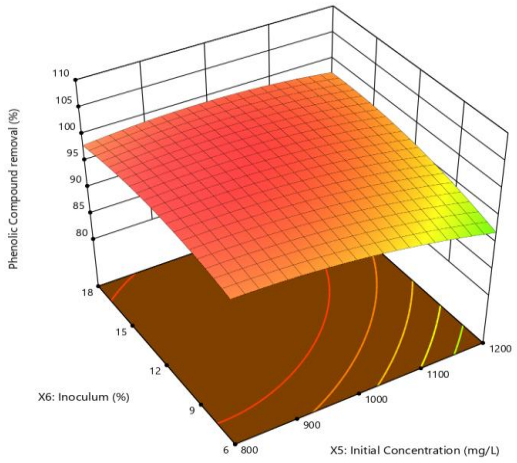


Figure VIIB.8: 3-Dimensional surfaces and 2-Dimensional plots of the interaction effects of: initial conc. of the mixture and inoculums percent (X5X6) on the percent of degradation of the mixture of 4- Chloro Phenol & Catechol

VIIB.2.8 Optimization of the operating parameters:

The Response Surface Methodology (RSM) was involved to deduct the optimum conditions for the six independent variables to achieve maximum percent of degradation of the mixture of 4-Chlorophenol and Catechol. Equation (7.3) was defined as objective function for the percent of degradation of the mixture of Phenolic compounds (4- Chloro Phenol & Catechol) and the independent factors in their ranges were model constraints. Thus the following optimum conditions, achieved for the maximum percent of degradation of the mixture were: 8.5 pH of the bacterial culture media, 45^{o}C temperature, 600 mL volume of the culture media, 48 hours of residence time, 1000 mg/L initial conc. of the mixture (500 mg/L 4-Chloro Phenol & 500 mg/L Catechol) and 12% inoculums size of the consortium (i.e. 3% *Bacillus paramycoides* strain MCCC 1A04098,3% *Bacillus timonensis* strain 10403023, 3% *Bacillus cereus* strain K1 and 3% *Bacillus pseudomycoides* strain NBRC 101232). 99.99percent degradation of the mixture of 4- Chloro Phenol and Catechol was obtained by

involving these six favourable situations. The obtained optimal operating conditions were performed nine more times to validate the predicted values as well to minimise the error and 99.99% degradation was achieved in case of each experimental results, obtaining only 1.76% of error, indicating the reliability of this model.

VIIB.2.9 Result of HPLC analysis:

From the standard chromatogram of known conc. of 4- Chloro Phenol and Catechol, it can be deduced that the peak of 6.382 AU, represents the residual conc. of Catechol and 6.948 AU represents the residual conc. of 4- Chloro Phenol respectively (Figure VIIB.9). Calculating the area of the chromatograms (both the standard and the sample), it was found that individually 99.4% of 4- Chloro Phenol and 96.35% of Catechol were removed from the bi-solute mixture, while cultured according to the parameters of the centre point of RSM, which almost supported the experimental value, obtained at the same point of the design matrix (Table VIIB.2).

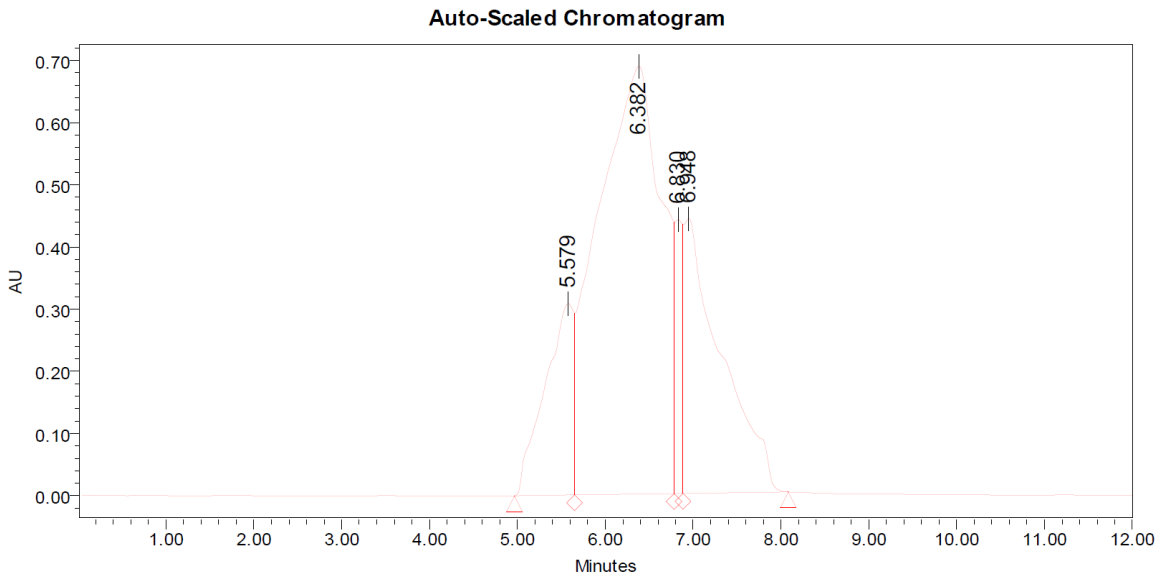


Figure VIIB.9: Chromatogram for the of the sample obtained as per centre point of RSM design

Sub chapter VI.IC

Optimization of parameters for the consortium, involved to degrade the mixture of Phenol & Catechol

VIIC.1 Materials and Methods:

VIIC.1.1 Materials:

Four different strains (*Brevibacillus formosus* strain NRRL NRS- 863, *Pseudomonas otitidis* strain MCC10330, *Bacillus pseudomycoides* strain NBRC 101232 and *Bacillus paramycoides* strain MCCC 1A04098) were utilized to make a microbial consortium.

Phenol and Catechol were prepared synthetically in the laboratory. The conc. of the solutions was maintained as 10 g/L.

VIIC.1.2 Experimental set up:

In case of designing the percent of inoculums, total percent of inoculums of the all four bacterial strains was taken into account where percent of each of the four strains was maintained equally (1:1:1:1 ratio). Similarly in case of initial conc. of the mixture to be removed i.e. Phenol & Catechol, total initial conc. (mg/L) of the two compounds was used for the design where individual initial conc. of the compounds was kept equal (1:1 ratio).

Rest of the portion is same as mentioned in the chapter III, section IIIA.1.2.

VIIC.1.3 Analytical Method:

Same as described in chapter VI, section VIA.1.3

VIIC.1.4 Experimental design:

Six (6) independent variables (Table 1) are Y1 (A): 5.5 – 11.5; Y2 (B): 35^{o} – 55^{o}C; Y3 (C): 300 – 700 mL; Y4 (D): 0 – 96 hours; Y5 (E): 600 – 1400 mg/L and Y6 (F): 0 – 24%. These six independent variables were coded at five levels between -2 and +2 based on preliminary experimental outputs.

Rest of the portion is same as described in sub chapter VIIA, section VIIA.1.4.

Table VIIC.1: Independent variables with coded levels

Independent variable	Symbol	Coded levels				
		-2	-1	0	+1	+2
pH of bacterial media	Y1	5.5	7	8.5	10	11.5
Temperature (^{o}C)	Y2	35	40	45	50	55
Volume of bacterial media(mL)	Y3	300	400	500	600	700
Residence time(hr)	Y4	0	24	48	72	96
Initial conc. of the mixture (mg/L)	Y5	600	800	1000	1200	1400
Inoculums percentage (percent)	Y6	0	6	12	18	24

VIIC.1.5 HPLC analysis:

HPLC analysis was performed in order to detect the individual degradation of the two compounds i.e. Phenol & Catechol. It was done only in case of the sample, obtained as per centre point of RSM design. The chromatogram image (VIIC.9) depicted satisfactory degradation of the two Phenolic compounds individually.

Rest of the portion is same as described in the sub chapter VIIA, section VIIA.1.5.

VIIC.2 Results and Discussions:

VIIC.2.1 Fitting of the model and Statistical analysis:

Total 86 experiments were done as per the design matrix and the only one response was percent of degradation of the mixture (Phenol+ Catechol). Table VIIC.2 displayed both the predicted and experimental/actual values of percent of degradation of the mixture of Phenol & Catechol. Second order and linear polynomial equations were fitted to the actual data to obtain the regression equation. To reveal the suitable model, sequential model sum of squares and model summary statistics were deducted (Table VIIC.3). The Sequential P-value for the quadratic model is less than 0.0001; Maximum predicted R^2 and adjusted R^2 values were 0.9644 and 0.9734 respectively for the percent of degradation of the mixture. Cubic model found to be aliased; here the sequential P- value was> 0.05. So, finally the quadratic model was chosen for further determination of the percent of degradation of the mixture i.e. Phenol and Catechol. The following equation clearly showed this:

percent of degradation of the mixture of Phenol & Catechol (X): 93.88+ 4.50Y1 - 0.1125Y2 – 0.7742Y3 +6.95Y4 - 0.9492Y5+1.37Y6 +0.0466Y1Y2 + 0.4938Y1Y3 - 4.78Y1Y4+ 0.2334Y1Y5 - 0.2347Y1Y6 - 0.4566Y2Y3 - 0.1322Y2Y4 - 0.1625Y2Y5 + 0.4575Y2Y6 - 0.0069Y3Y4 + 1.06Y3Y5 + 0.8491Y3Y6 +1.61Y4Y5 – 0.5322Y4Y6+0.7419Y5Y6 + $4.694Y1^2$ + $4.76Y2^2$+ $4.65Y3^2$ – $10.00Y4^2$ + $4.67Y5^2$ – $13.49Y6^2$ (7.4)

Negative coefficients for the model components Y2, Y3, Y5, Y1Y4, Y1Y6, Y2Y3, Y2Y4, Y2Y5, Y3Y4, Y4Y6, $Y4^2$ and $Y6^2$ exhibit negative impacts on the percent of degradation of the mixture while positive coefficients Y1, Y4, Y6, Y1Y2, Y1Y3, Y1Y5, Y2Y6, Y3Y5, Y3Y6, Y4Y5, Y5Y6, $Y1^2$, $Y2^2$, $Y3^2$ and $Y5^2$ have positive effects on the of degradation of the mixture. Moreover, the coefficients, whose values are near 0, reveal lower relative intensity. Y1Y2 and Y3Y4 belong to this class which could not affect percent of degradation of the mixture so much. On the other hand, $Y4^2$ and $Y6^2$ have higher impacts on the percent degradation of the mixture (Phenol & Catechol).

Table VIIC.2: Experimental design matrix for the treatment of wastewater by involving four different strains to remove Phenol & Catechol

Run order	Space type	Y1: pH	Y2: Temperature (°C)	Y3: Medium Volume (mL)	Y4: Residence Time(hr)	Y5: Initial Concentration of mixture (mg/L)	Y6: inoculums size (percent)	Y: percent of degradation of the mixture		
								Experimental value	Predicted value	Error
1	Factorial	10	50	400	72	800	6	98.24	97.25	0.9949
2	Axial	8.5	45	300	48	1000	12	99.21	114.04	-14.83
3	Factorial	7	50	600	24	800	6	64.03	70.37	-6.34
4	Factorial	10	50	400	24	1200	18	98.74	90.75	7.99
5	Factorial	7	40	400	24	800	18	73.51	77.53	-4.02
6	Factorial	10	40	600	24	1200	18	89.83	93.30	-3.47
7	Factorial	7	40	600	72	800	18	92.35	94.93	-2.58
8	Factorial	7	40	600	72	1200	6	93.67	95.30	-1.63
9	Axial	8.5	45	500	48	1000	24	52.32	42.64	9.68
10	Factorial	10	40	400	72	800	6	98.32	97.32	1.00
11	Factorial	7	40	400	24	1200	6	67.09	68.50	-1.41
12	Factorial	7	50	400	72	1200	6	98.33	95.61	2.72
13	Factorial	10	50	600	24	800	18	98.33	94.48	3.85
14	Factorial	10	50	400	72	800	18	95.63	96.18	-0.5500
15	Factorial	10	40	600	72	1200	18	92.31	100.05	-7.74
16	Factorial	10	40	600	72	800	18	99.07	94.34	4.73
17	Centre	8.5	45	500	48	1000	12	99.38	93.88	5.50
18	Factorial	10	50	400	72	1200	18	96.17	97.01	-0.8360
19	Centre	8.5	45	500	48	1000	12	99.38	93.88	5.50
20	Axial	8.5	45	500	48	1000	0	0	37.17	-37.17
21	Centre	8.5	45	500	48	1000	12	99.38	93.88	5.50
22	Centre	8.5	45	500	48	1000	12	99.38	93.88	5.50
23	Factorial	7	50	400	72	800	18	94.21	98.56	-4.35
24	Factorial	7	50	400	24	1200	18	71.24	73.09	-1.85
25	Factorial	7	50	400	72	1200	18	97.41	98.45	-1.04
26	Centre	8.5	45	500	48	1000	12	99.38	93.88	5.50
27	Factorial	7	40	600	72	800	6	98.27	93.49	4.78
28	Factorial	10	40	600	24	1200	6	99.03	87.70	11.33
29	Factorial	10	50	400	72	1200	6	98.23	95.10	3.13
30	Factorial	7	50	600	72	800	18	91.32	94.68	-3.36
31	Factorial	7	40	400	72	800	6	92.36	98.95	-6.59
32	Axial	8.5	55	500	48	1000	12	99.24	112.70	-13.46
33	Factorial	10	50	600	24	800	6	94.35	90.02	4.33
34	Factorial	7	40	600	24	1200	18	78.24	73.84	4.40
35	Factorial	10	50	400	24	800	18	96.23	96.36	-0.1298
36	Factorial	7	40	600	24	1200	6	75.36	67.31	8.05
37	Centre	8.5	45	500	48	1000	12	99.38	93.88	5.50
38	Factorial	7	50	600	24	1200	18	75.26	73.47	1.79
39	Factorial	7	40	600	24	800	18	78.25	75.50	2.75

40	Factorial	10	50	600	72	800	6	94.35	91.94	2.41
41	Factorial	7	40	400	24	1200	18	76.32	71.64	4.68
42	Factorial	10	50	400	24	1200	6	87.33	86.72	0.6091
43	Axial	8.5	45	500	48	1400	12	98.54	110.68	-12.14
44	Centre	8.5	45	500	48	1000	12	99.38	93.88	5.50
45	Factorial	7	50	600	72	1200	6	94.35	92.57	1.78
46	Factorial	7	50	400	72	800	6	98.24	98.69	-0.4471
47	Centre	8.5	45	500	48	1000	12	99.38	93.88	5.50
48	Factorial	7	50	400	24	800	6	95.32	77.63	17.69
49	Factorial	7	40	600	24	800	6	70.02	71.93	-1.91
50	Factorial	10	50	600	72	800	18	92.35	94.27	-1.92
51	Factorial	7	50	400	24	800	18	78.24	79.63	-1.39
52	Factorial	10	40	400	24	800	18	98.44	94.07	4.37
53	Factorial	7	40	400	72	800	18	94.24	96.99	-2.75
54	Axial	8.5	45	500	96	1000	12	80.24	67.78	12.46
55	Factorial	7	40	400	24	800	6	92.14	77.36	14.78
56	Factorial	10	50	600	72	1200	6	95.08	94.04	1.04
57	Factorial	10	40	400	72	800	18	98.25	94.42	3.83
58	Factorial	10	50	600	24	1200	18	97.74	93.11	4.63
59	Factorial	10	50	600	72	1200	18	95.24	99.33	-4.09
60	Factorial	10	40	600	72	800	6	94.21	93.84	0.3695
61	Factorial	10	40	400	24	1200	18	88.58	89.12	-0.5364
62	Factorial	10	50	600	24	1200	6	96.25	85.68	10.57
63	Factorial	10	50	400	24	800	6	99.13	95.30	3.83
64	Factorial	7	50	600	72	800	6	98.75	91.41	7.34
65	Factorial	10	40	400	24	1200	6	91.25	86.92	4.33
66	Factorial	7	50	600	24	1200	6	64.16	65.10	-0.9414
67	Factorial	10	40	400	72	1200	18	98.15	95.90	2.25
68	Factorial	7	40	400	72	1200	18	97.02	97.53	-0.5124
69	Factorial	7	40	600	72	1200	18	98.33	99.71	-1.38
70	Factorial	10	40	400	24	800	6	98.75	94.84	3.91
71	Factorial	7	50	400	24	1200	6	70.03	68.12	1.91
72	Axial	8.5	35	500	48	1000	12	99.11	113.15	-14.04
73	Factorial	7	50	600	72	1200	18	96.99	98.81	-1.82
74	Factorial	10	40	600	24	800	6	97.77	91.39	6.38
75	Axial	5.5	45	500	48	1000	12	98.52	103.62	-5.10
76	Axial	8.5	45	500	0	1000	12	0	39.96	-39.96
77	Axial	8.5	45	500	48	600	12	99.11	114.47	-15.36
78	Factorial	7	50	600	24	800	18	82.63	75.77	6.86
79	Centre	8.5	45	500	48	1000	12	99.38	93.88	5.50
80	Factorial	7	40	400	72	1200	6	99.01	96.52	2.49
81	Factorial	10	40	600	24	800	18	94.38	94.02	0.3598
82	Axial	8.5	45	700	48	1000	12	98.27	110.94	-12.67
83	Centre	8.5	45	500	48	1000	12	99.38	93.88	5.50
84	Factorial	10	40	600	72	1200	6	98.88	96.59	2.29
85	Axial	11.5	45	500	48	1000	12	99.24	121.64	-22.40
86	Factorial	10	40	400	72	1200	6	98.86	95.83	3.03

Table VIIC.3: Adequacy of the models tested for the degradation of the mixture

Sequential Model Sum of Squares						
Source	**Sum of Squares**	**df**	**Mean Square**	**F-value**	**p-value**	**Remarks**
Mean vs. Total	6.955E+05	1	6.955E+05			
Linear vs. Mean	5185.56	6	864.26	3.32	0.0058	
2FI vs. Linear	1850.30	15	123.35	0.4216	0.9672	
Quadratic vs. 2FI	**11930.36**	**6**	**1988.39**	**16.97**	**< 0.0001**	**Suggested**
Cubic vs. Quadratic	3593.62	26	138.22	1.38	0.1910	Aliased
Residual	3201.29	32	100.04			
Total	7.212E+05	86	8386.49			

Model Summary Statistics						
Source	**Std. Dev.**	**R^2**	**Adjusted R^2**	**Predicted R^2**	**PRESS**	**Remarks**
Linear	16.14	0.2013	0.1406	0.0772	23771.29	
2FI	17.11	0.2731	0.0346	0.1080	22979.77	
Quadratic	**10.82**	**0.9462**	**0.9734**	**0.9644**	**22555.46**	**Suggested**
Cubic	10.00	0.8757	0.6699	-17.9908	4.892E+05	Aliased

The constant variance assumption was investigated by plotting internally studentized residual vs. predicted values of percent degradation of mixture of Phenolic compounds i.e. Phenol & Catechol (Figure VIIC.1). The normal probability plot of residuals (Figure VIIC.2) for the percent degradation of Phenolic mixture showed a straight line pattern rather than S shaped followed by the points on the plot. As the residuals are distributed normally, transformation of response is not required. Relation between the predicted and experimental values of response has been displayed in Figure VIIC.3. Very little discrepancies were found by the straight trend line pointing a good relationship in between the predicted and experimental values.

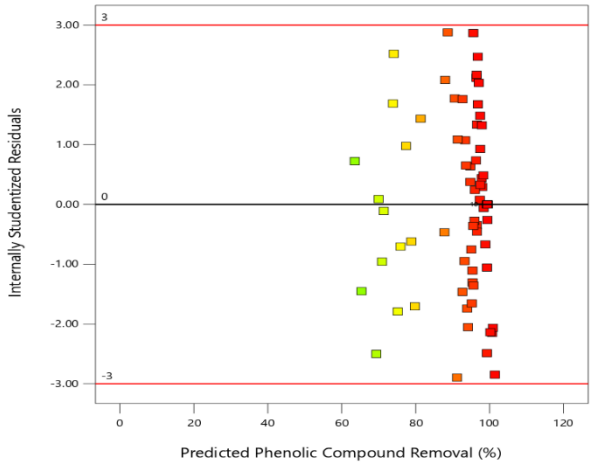


Figure VIIC.1: Internally studentized residuals vs. predicted values

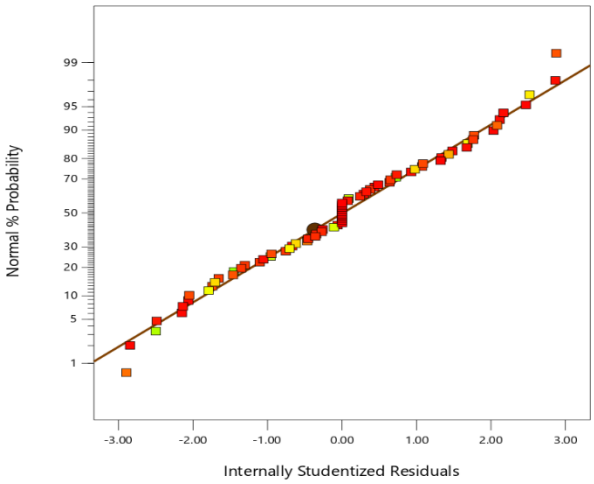


Figure VIIC.2: **Internally studentized residuals vs. Normal percent probability**

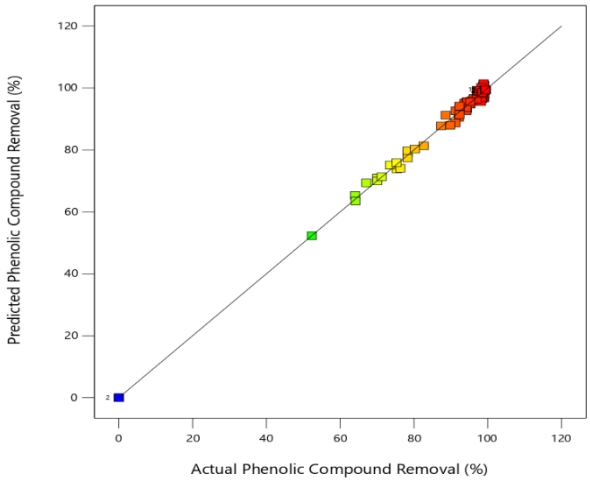


Figure VIIC.3: Actual degradation data vs. predicted data

VIIC.2.2 ANOVA test:

Outcomes of ANOVA for percent of degradation of the mixture of Phenol & Catechol have been given in Table VIIC.4. In that table, The Model F-value of 6.00 implies the model is significant. There is only a 0.01% chance that an F-value this large could occur due to noise. P-values less than 0.0500 indicate model terms are significant. In this case Y1, Y2, Y3, Y4, Y5, Y6, Y1Y2, Y1Y4, Y1Y6, Y2Y4, Y2Y6, Y3Y4, Y3Y6, Y4Y5, Y4Y6, Y5Y6, $Y1^2$, $Y2^2$, $Y3^2$, $Y4^2$,$Y5^2$ and $Y6^2$ are significant model terms. Values greater than 0.1000 indicate the model terms are not significant. Y1Y3, Y1Y5, Y2Y3, Y2Y5 and Y3Y5 are not significant model terms. If there are many insignificant model terms (not counting those required to support hierarchy), model reduction may improve the model.

Table VIIC.4: ANOVA of the second order polynomial equation for the degradation of mixture

Source	Sum of Squares	df	Mean Square	F-value	p-value	Remarks
Model	18966.22	27	702.45	6.00	< 0.0001	significant
Y1-pH	1459.98	1	1459.98	12.46	0.0008	significant
Y2-Temperature	0.9113	1	0.9113	0.0078	0.0350	significant
Y3-Medium Volume	43.15	1	43.15	0.3683	0.0063	significant
Y4-Residence Time	3481.95	1	3481.95	29.72	< 0.0001	significant
Y5-Initial Concentration	64.87	1	64.87	0.5537	0.0098	significant
Y6-Inoculum	134.70	1	134.70	1.15	0.0280	significant
Y1Y2	0.1388	1	0.1388	0.0012	0.9727	significant
Y1Y3	15.60	1	15.60	0.1332	0.7165	Not significant
Y1Y4	1461.15	1	1461.15	12.47	0.0008	significant
Y1Y5	3.49	1	3.49	0.0298	0.8636	Not significant
Y1Y6	3.53	1	3.53	0.0301	0.0029	significant
Y2Y3	13.34	1	13.34	0.1139	0.7370	Not significant
Y2Y4	1.12	1	1.12	0.0095	0.0125	significant
Y2Y5	1.69	1	1.69	0.0144	0.9048	Not significant
Y2Y6	13.40	1	13.40	0.1143	0.0065	significant
Y3Y4	0.0030	1	0.0030	0.0000	0.0260	significant
Y3Y5	71.78	1	71.78	0.6127	0.4369	Not significant
Y3Y6	46.14	1	46.14	0.3938	0.0228	significant
Y4Y5	165.57	1	165.57	1.41	0.0094	significant
Y4Y6	18.13	1	18.13	0.1547	0.0155	significant
Y5Y6	35.22	1	35.22	0.3007	0.0156	significant
$Y1^2$	754.75	1	754.75	6.44	0.0139	significant
$Y2^2$	778.69	1	778.69	6.65	0.0125	significant
$Y3^2$	743.52	1	743.52	6.35	0.0145	significant
$Y4^2$	3437.18	1	3437.18	29.34	< 0.0001	significant
$Y5^2$	750.33	1	750.33	6.40	0.0141	significant
$Y6^2$	6254.09	1	6254.09	53.38	< 0.0001	significant
Residual	6794.91	58	117.15			
Lack of Fit	6794.91	49	138.67			
Pure Error	0.0000	9	0.0000			
Cor Total	25761.13	85				

VIIC.2.3 Effect of pH of the media (Y1):

Effect of pH revealed a significant role in percent degradation of the mixture of Phenol & Catechol. Percent of degradation of the mixture was maximum (almost 99%) when the pH value of the culture media was 8.5. Percent degradation was decreased in both situations while the pH value of the media increased and decreased indicating that maximum degradation was occurred at moderately basic condition. Acidic as well as neutral condition is not favourable for the consortium to remove the mixture of Phenol & Catechol. From the 3D and contour plots, it can be showed that the interaction effects between pH of the media vs. media volume (Y1Y3) and pH of the media vs. initial conc. of the mixture of the Phenolic compounds (Y1Y5) did not possess significant effects on percent degradation of the mixture as the plot is flat shaped (Figure: VIIC.4 (b) & (d)) while the interaction effects between pH of the media vs. temperature (Y1Y2), pH of the media vs. residence time (Y1Y4), and pH of the media vs. inoculums percent (Y1Y6) revealed significant effects on the percent degradation of the mixture of Phenol & Catechol (Figure: VIIC.4 (a), (c) & (e)).

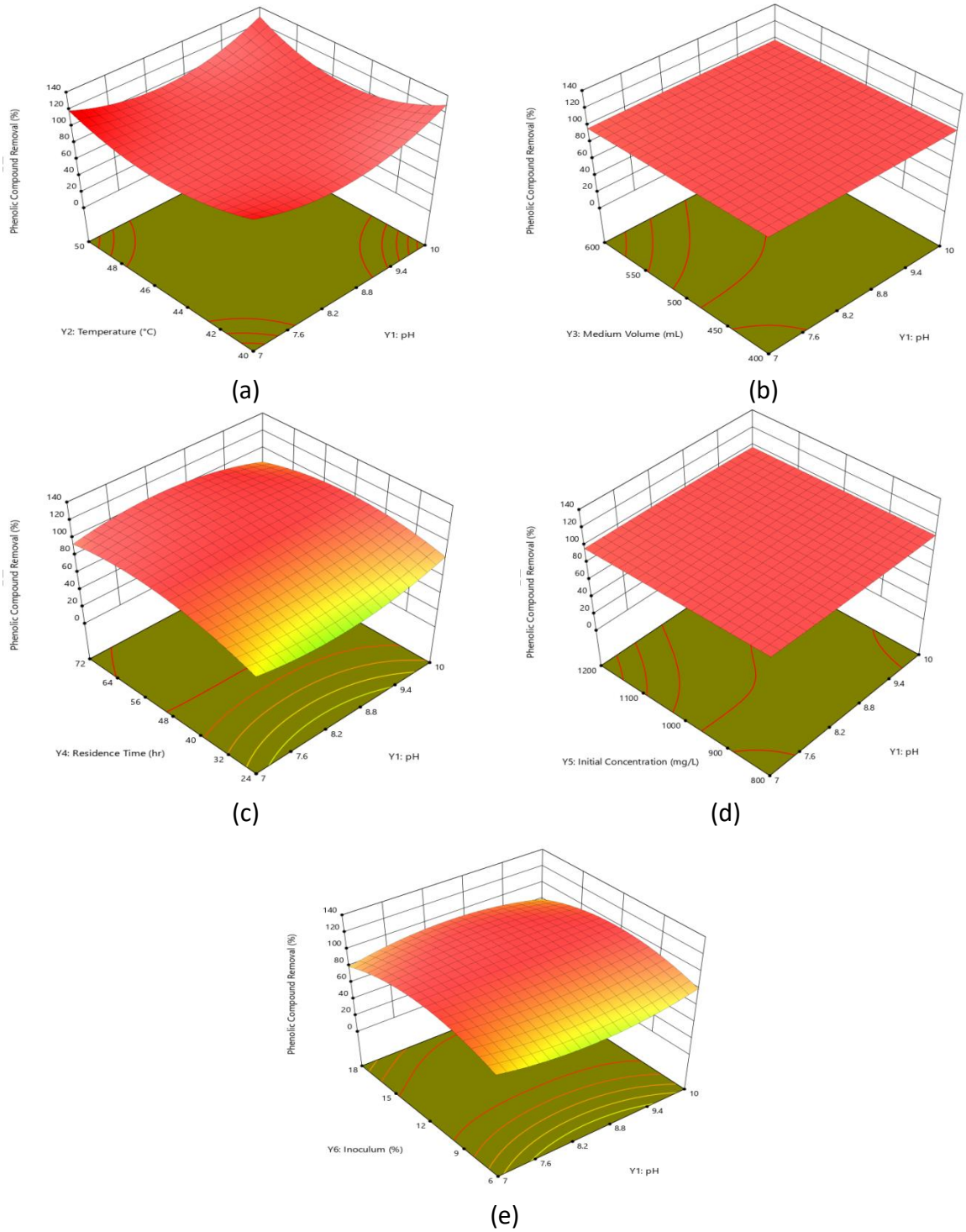


Figure VIIC.4: 3-Dimensional surfaces and 2-Dimensional plots of the interaction effects of: (a) pH of the media and temperature (Y1Y2), (b) pH of the media and media vol. (Y1Y3), (c) pH of media and residence time (Y1Y4), (d) pH of the media and initial conc. of the mixture (Y1Y5), (e) pH of the media and inoculums percent (Y1Y6) on the percent of degradation of the Phenolic mixture (Phenol & Catechol)

VIIC.2.4 Effect of temperature (Y2):

Temperature was found to be very important factor during this study. Degradation percent of the mixture i.e. Phenol & Catechol was increased rapidly while temperature decreased to 45^{o}C. Below and above this temperature, degradation percent decreased slightly. The interaction effects between temperature vs. media volume (Y2Y3) and temperature vs. initial conc. of the Phenolic mixture (Y2Y5) did not exhibit significant effects. 3D curves are flat shaped (Figure: VIIC.5 (a) & (c)). On the other hand, temperature vs. residence time (Y2Y4) and temperature vs. inoculums percent (Y2Y6) showed to be very effective on the percent of degradation of the mixture of Phenolic compounds as the 3D curves are convex shaped (Figure: VIIC.5(b) & (d)).

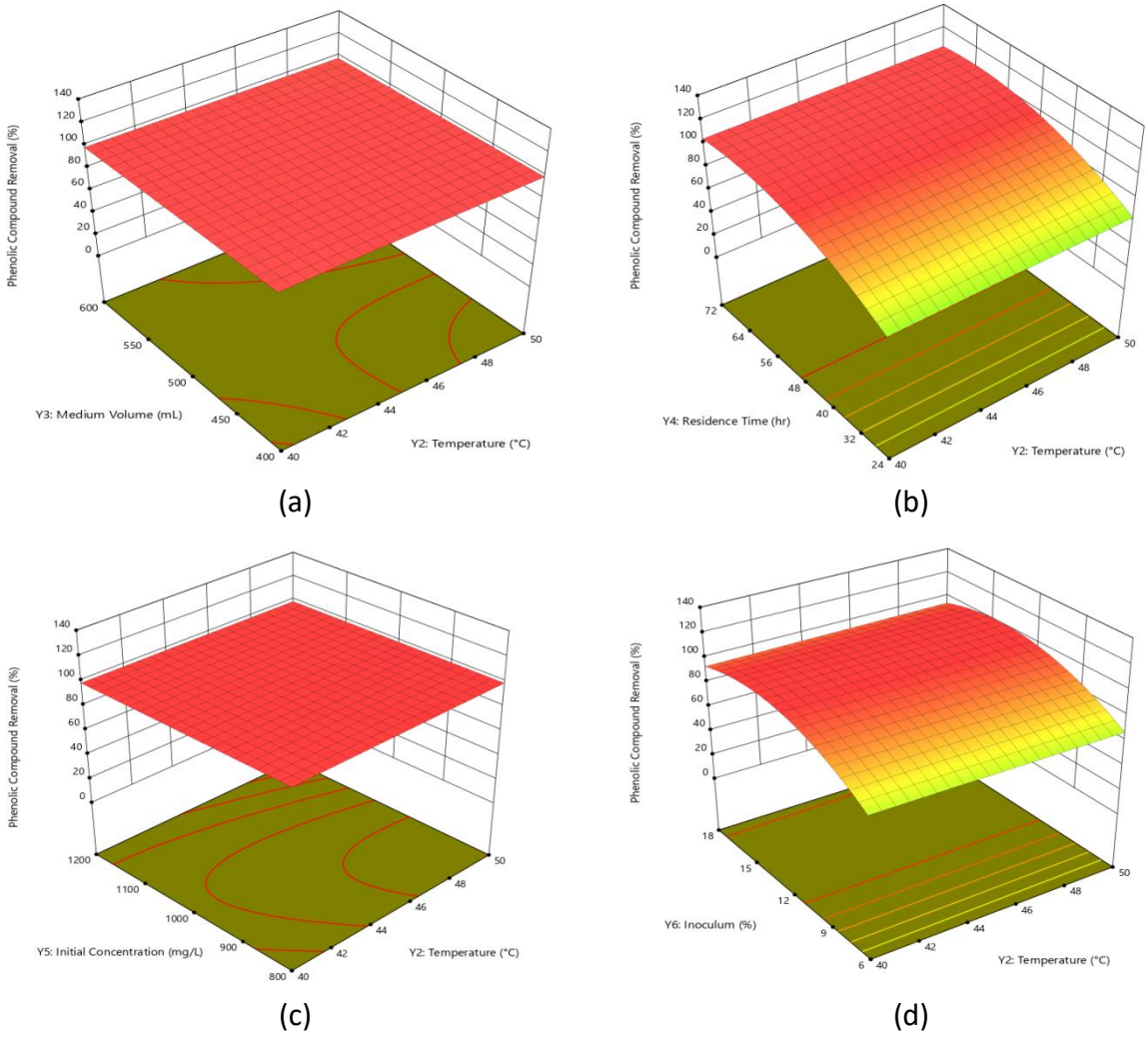


Figure VIIC.5: 3-Dimensional surfaces and 2-Dimensional plots of the interaction effects of: (a) temperature and media vol. (Y2Y3), (b) temperature and residence time (Y2Y4), (c) temperature vs. initial conc. of the mixture (Y2Y5), (d) temperature and inoculums percent (Y2Y6) on the percent of degradation of the mixture (Phenol & Catechol)

VIIC.2.5 Effect of media volume (Y3):

Volume of the culture media played an important role on degradation percent of Phenol & Catechol. Percent of degradation of the mixture increased with the increasing volume of the culture media. Up to 500 ml volume, degradation percent increased steadily. But beyond that, when the volume increased up to 600 and even 700 ml, the degradation percent decreased slightly. Similar effects obtained when the media volume decreased up to 300 ml. In case of interaction effects between media volume vs. residence time (Y3Y4) and media volume vs. inoculums percent (Y3Y6), exhibited significant effects on the degradation percent. Convex shaped curves (Figure: VIIC.6 (a) & (c)) indicated the same. But the interaction between media volume vs. initial conc. of the mixture (Y3Y5) was not found to be effective on the degradation percent as the curve is flat shaped there. Moreover, the p- value of this model (Y3Y5) term was 0.4367 (Table VIIC.4) which denoted its insignificance.

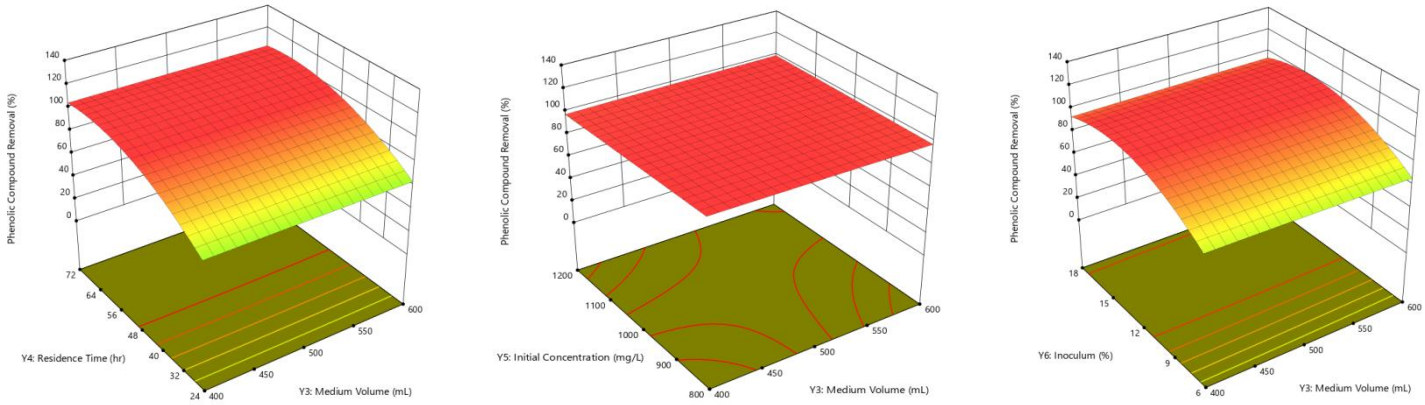


Figure VIIC.6: 3-Dimensional surfaces and 2-Dimensional plots of the interaction effects of: (a) media vol. and residence time (Y3Y4), (b) media vol. and initial conc. of the mixture (Y3Y5), (c) media vol. and inoculums percent (Y3Y6) on the percent of degradation of the mixture of Phenol & Catechol

VIIC.2.6 Effect of residence time (Y4):

Residence time played a vital role on the response i.e. percent of degradation. Most of the good responses were obtained from 24 hours onwards (> 90%). Maximum degradations of

the mixture were obtained at 48 hours (>98%). After that time period, no such big changes were appeared. Surprisingly at 96 hours, the degradation percent decreased to 80.24%. Minimum degradation (0percent) was appeared at 0 hours of residence time where the culture media was withdrawn from the BOD incubator as soon as it was kept in that. Interaction effects in between residence time vs. initial conc. of the mixture (Y4Y5) and residence time vs. inoculums size of the consortium (Y4Y6) found to be very significant on the percent of degradation of the mixture (Phenol & Catechol). Both the figure VIIC.7 (a) & (b)) and the p-values of these two terms (Table VIIC.4) were found to support this.

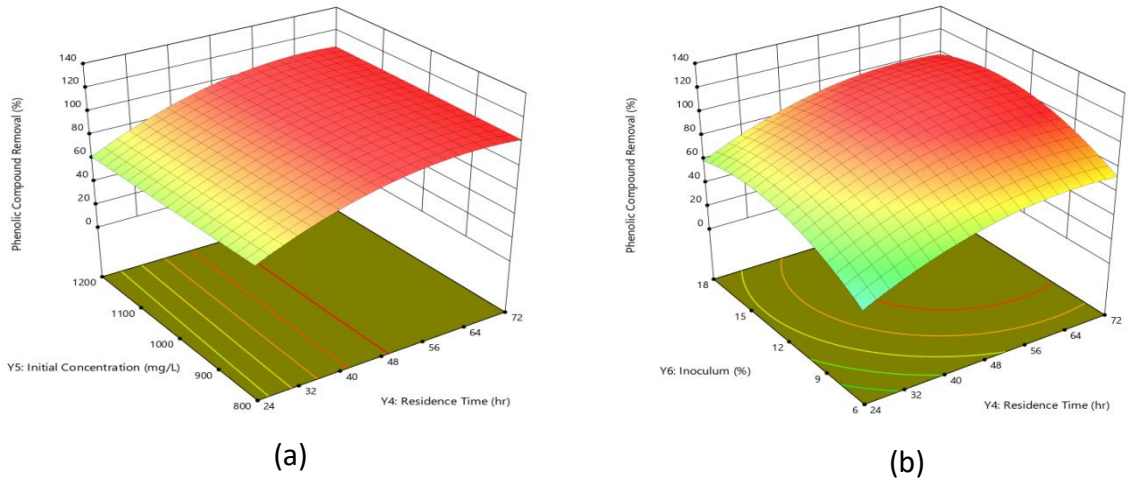


Figure VIIC.7: 3-Dimensional surfaces and 2-Dimensional plots of the interaction effects of: (a) residence time and initial conc. of the mixture (Y4Y5), (b) residence time and inoculums percent (Y4Y6) on the percent of degradation of the mixture of Phenol & Catechol

VIIC.2.7 Effect of initial conc. of the mixture of Phenolic compounds and inoculums percent (Y5) & (Y6):

Initial conc. of the mixture (Phenol & Catechol) and inoculums size of the consortium, both exhibited pivotal role on the percent of degradation of the mixture. Percent of degradation decreased slightly while initial conc. of the mixture increased. When the conc. of the mixture was 600 mg/L (300 mg/L Phenol & 300 mg/L Catechol),it was removed very easily. Almost similar findings were obtained when the initial conc. was 800 mg/L (conc. of each was 400

mg/L) and 1000 mg/L (conc. of each was 500 mg/L). Optimum degradation was achieved at the conc. of 1000 mg/L accompanied by suitable pH, temperature and time. But when the conc. increased to 1200 mg/L (conc. of each was 600 mg/L) degradation percent slightly affected. But when the conc. increased up to 1400 mg/L (conc. of each was 700 mg/L), ~98% degradation was achieved.

Degradation percent increased with the increasing percent of inoculums. 12% inoculums (3% inoculums of each of the four strains) found to be mostly effective to remove the mixture. Beyond that, no big changes occurred even after increasing the inoculums percent.

Also, the interaction effect between the initial conc. of the bi- solute mixture of Phenolic compounds vs. inoculums size found to be very effective on the response as the curve is convex in nature (Figure VIIC.8).

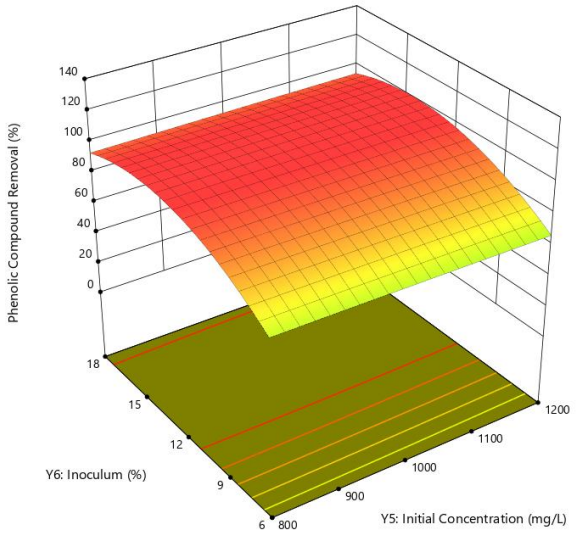


Figure VIIC.8: 3-Dimensional surfaces and 2-Dimensional plots of the interaction effects of: initial conc. of the mixture and inoculums percent (X5X6) on the percent of degradation of the mixture of Phenol & Catechol

VIIC.2.8 Optimization of the operating parameters:

The Response Surface Methodology (RSM) was involved to deduct the optimum conditions for the six independent variables to achieve maximum percent of degradation of the mixture of Phenol and Catechol. Equation (7.4) was defined as objective function for the percent of degradation of the mixture of Phenolic compounds (Phenol & Catechol) and the independent factors in their ranges were model constraints. Thus the following optimum conditions,

achieved for the maximum percent of degradation of the mixture were: 8.5 pH of the bacterial culture media, 45^{o}C temperature, 500 mL volume of the culture media, 48 hours of residence time, 1000 mg/L initial conc. of the mixture (500 mg/L Phenol & 500 mg/L Catechol) and 12% inoculums size of the consortium (i.e. 3% *Bacillus paramycoides* strain MCCC 1A04098, 3% *Bacillus pseudomycoides* strain NBRC 101232, 3% *Brevibacillus formosus* strain NRRL NRS- 863 and 3% *Pseudomonas otitidis* strain MCC10330). 99.38% degradation of the mixture of Phenol and Catechol was obtained by involving these six favourable situations. The obtained optimal operating conditions were performed nine more times to validate the predicted values as well to minimise the error and 99.38percent degradation was achieved in case of each experimental results indicating the reliability of this model.

VIIC.2.9 Result of HPLC analysis:

From the standard chromatogram of known conc. of Phenol and Catechol, it can be deduced that the peak of 6.116 AU, represents the residual conc. of Catechol and 6.646 AU represents the residual conc. of Phenol respectively (Figure VIIC.9). Calculating the area of the chromatograms (both the standard and the sample), it was found that individually 99.85% of Phenol and 99.6% of Catechol were removed from the bi- solute mixture, while cultured according to the parameters of the centre point of RSM, which almost supported the experimental value, obtained at the same point of the design matrix (Table VIIC.2).

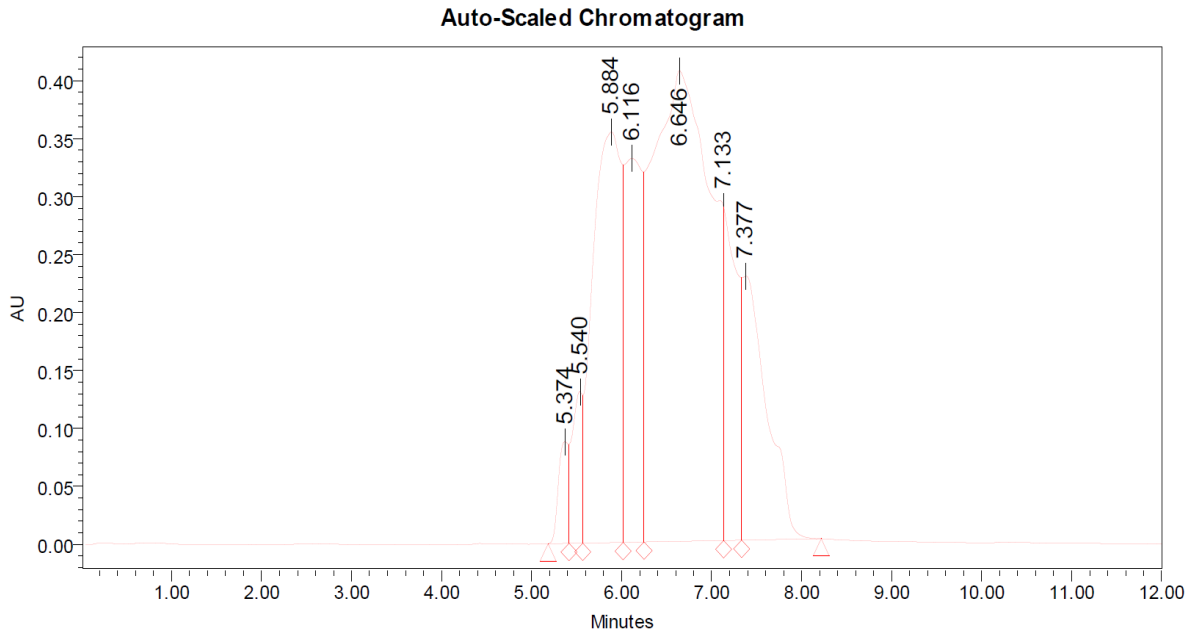


Figure VIIC.9: Chromatogram for the of the sample obtained as per centre point of RSM design

Sub chapter VIID

Optimization of parameters for the consortium, involved to degrade the tri- solute mixture of Phenol & 4- Chloro Phenol & Catechol

VIID.1 Materials and Methods:

VIID.1.1 Materials:

Six different strains (*Brevibacillus formosus* strain NRRL NRS- 863, *Pseudomonas otitidis* strain MCC10330, *Bacillus timonensis* strain 10403023, *Bacillus cereus* strain K1, *Bacillus pseudomycoides* strain NBRC 101232 and *Bacillus paramycoides* strain MCCC 1A04098) were utilized to make a microbial consortium.

VIID.1.2 Experimental set up:

In case of designing the percent of inoculums, total percent of inoculums of the all six bacterial strains was taken into account where percent of each of the six strains was maintained equally (1:1:1:1:1:1 ratio). Similarly in case of initial conc. of the tri- solute mixture to be removed i.e. Phenol & 4- Chloro Phenol & Catechol, total initial conc. (mg/L) of the three compounds was used for the design where individual initial conc. of the compounds was kept equal (1:1:1 ratio).

Rest of the portion is same as mentioned in the chapter III, section IIIA.1.2.

VIID.1.3 Analytical Method:

Same as described in chapter VI, section VIA.1.3

VIID.1.4 Experimental design:

Six (6) independent variables (Table VIID.1) are Q1 (A): 5 – 11; Q2 (B): 37.5^{o} – 52.5^{o}C; Q3 (C): 450 – 750 mL; Q4 (D): 12 – 84 hours; Q5 (E): 1050 – 1950 mg/L and Q6 (F): 10.5 – 19.5%. These six independent variables were coded at five levels between -1.5 and +1.5 based on preliminary experimental outputs.

Rest of the portion is same as described in sub chapter VIIA, section VIIA.1.4.

Table VIID.1: Independent variables with coded levels

Independent variable	Symbol	Coded levels				
		-2	**-1**	**0**	**+1**	**+2**
pH of bacterial media	Q1	5	6	8	10	11
Temperature (^oC)	Q2	37.5	40	45	50	52.5
Volume of bacterial media(mL)	Q3	450	500	600	700	750
Residence time(hr)	Q4	12	24	48	72	84
Initial conc. of the mixture (mg/L)	Q5	1050	1200	1500	1800	1950
Inoculums percentage (percent)	Q6	10.5	12	15	18	19.5

VIID.1.5 HPLC analysis:

HPLC analysis was performed in order to detect the individual degradation of the two compounds i.e. Phenol & Catechol. It was done only in case of the sample, obtained as per centre point of RSM design. The chromatogram image (VIID.9) depicted satisfactory degradation of the two Phenolic compounds individually.

Rest of the portion is same as described in the sub chapter VIIA, section VIIA.1.5.

VIID.2 Results and Discussions:

VIID.2.1 Fitting of the model and Statistical analysis:

Total 86 experiments were done as per the design matrix and the only one response was percent of degradation of the mixture (Phenol+ 4- Chloro Phenol+ Catechol). Table VIID.2 displayed both the predicted and experimental/actual values of percent of degradation of the mixture of Phenol, 4- Chloro Phenol and Catechol. Second order and linear polynomial equations were fitted to the actual data to obtain the regression equation. To reveal the suitable model, sequential model sum of squares and model summary statistics were deducted (Table VIID.3). The Sequential P-value for the quadratic model is less than 0.0001; Maximum predicted R^2 and adjusted R^2 values were 0.9756 and 0.9838 respectively for the percent of degradation of the mixture. Cubic model found to be aliased; here the sequential P-value was> 0.05. So, finally the quadratic model was chosen for further determination of the percent of degradation of the mixture (Phenol, 4- Chloro Phenol & Catechol). The following equation clearly showed this:

percent of degradation of the mixture of Phenol, 4- Chloro Phenol & Catechol (Y):

$$98.07+ 5.51Q1 + 0.5642Q2 – 0.3949Q3 +4.89Q4 - 0.9207Q5+0.9530Q6 +0.0509Q1Q2 – 0.5131Q1Q3 – 5.00Q1Q4 - 0.7044Q1Q5 - 0.6006Q1Q6 - 0.3437Q2Q3 - 0.7062Q2Q4 - 0.8250Q2Q5 – 0.2506Q2Q6 - 0.4184Q3Q4 - 0.5591Q3Q5 + 0.1791Q3Q6 - 0.3766Q4Q5 + 0.3834Q4Q6 + 0.3759Q5Q6 - 4.79Q1^2 - 0.1869Q2^2 - 3.68Q3^2 + 0.0887Q4^2 + 0.8354Q5^2 – 0.7957Q6^2 \quad (7.5)$$

The equation no. (7.5) in terms of coded factors can be used to make predictions about the response for given levels of each factor. By default, the high levels of the factors are coded as +2 and the low levels are coded as -2. The coded equation is useful for identifying the relative impact of the factors by comparing the factor coefficients.

Negative coefficients for the model components Q3, Q5, Q1Q3, Q1Q4, Q1Q5, Q1Q6, Q2Q3, Q2Q4, Q2Q5, Q2Q6, Q3Q4, Q3Q5, Q4Q5, $Q1^2$, $Q2^2$, $Q3^2$ and $Q6^2$ exhibit negative impacts on the percent of degradation of the mixture while positive coefficients Q1, Q2, Q4, Q6, Q1Q2, Q3Q6, Q4Q6, Q5Q6, $Q4^2$ and $Q5^2$ have positive effects on the of degradation of the mixture. Moreover, the coefficients, whose values are near 0, reveal lower relative intensity. Q1Q2 and $Q4^2$ belong to this class which could not affect percent of degradation of the mixture so much. On the other hand, the coefficients, whose values are higher, have intense effect on the percent of degradation. Q1, Q4, Q1Q4, $Q1^2$ and $Q3^2$ have intense effects on the response.

Table VIID.2: Experimental design matrix for the treatment of waste water by involving a mix microbial culture to remove Phenol & 4- Chloro Phenol & Catechol

Run order	Space type	Q1: pH	Q2: Temperature (°C)	Q3: Medium Volume (mL)	Q4: Residence Time(hr)	Q5: Initial Concentration of mixture (mg/L)	Q6: inoculums size (percent)	Y: percent of degradation of the mixture		
								Experimental value	Predicted value	Error
1	Axial	8	52.5	600	48	1500	15	96.23	98.49	-2.26
2	Factorial	10	50	500	72	1800	18	94.08	94.93	-0.8528
3	Factorial	6	50	500	24	1200	12	79.66	74.90	4.76
4	Factorial	10	50	500	72	1200	12	96.58	98.86	-2.28
5	Factorial	6	40	500	72	1200	18	98.92	94.90	4.02
6	Factorial	10	50	700	72	1200	12	97.25	96.28	0.9746
7	Factorial	6	40	700	24	1800	18	80.24	76.20	4.04
8	Factorial	10	50	700	24	1800	12	92.47	92.73	-0.2615
9	Factorial	10	40	700	24	1800	12	88.25	91.92	-3.67
10	Factorial	10	50	500	72	1200	18	97.33	98.72	-1.39
11	Factorial	6	50	700	72	1800	18	96.94	93.34	3.60
12	Axial	8	45	600	84	1500	15	99.35	105.60	-6.25
13	Factorial	10	40	500	72	1800	12	93.81	94.21	-0.4021
14	Factorial	6	50	500	24	1800	12	72.42	73.94	-1.52
15	Factorial	10	40	500	72	1200	18	94.42	97.06	-2.64
16	Factorial	10	50	500	24	1200	18	98.33	97.98	0.3482
17	Factorial	6	40	700	72	1800	12	94.35	91.08	3.27
18	Factorial	6	40	700	24	1800	12	74.16	72.25	1.91
19	Factorial	6	40	700	72	1200	18	96.45	96.47	-0.0161
20	Factorial	10	50	500	24	1800	18	93.07	95.70	-2.63
21	Factorial	6	50	700	24	1800	12	76.42	72.85	3.57
22	Factorial	6	50	500	72	1200	18	97.78	96.35	1.43
23	Factorial	6	50	700	24	1200	12	75.79	76.05	-0.2580
24	Centre	8	45	600	48	1500	15	99.87	98.07	1.80
25	Factorial	6	50	700	24	1800	18	72.9	75.80	-2.90
26	Centre	8	45	600	48	1500	15	99.87	98.07	1.80
27	Axial	8	45	600	48	1500	19.5	94.57	97.70	-3.13
28	Centre	8	45	600	48	1500	15	99.87	98.07	1.80
29	Axial	5	45	600	48	1500	15	75.77	79.01	-3.24
30	Factorial	10	40	700	72	1800	12	90.61	90.77	-0.1604
31	Centre	8	45	600	48	1500	15	99.87	98.07	1.80
32	Factorial	6	50	500	24	1200	18	76.17	75.63	0.5387
33	Factorial	10	40	500	24	1200	12	95.74	94.17	1.57
34	Factorial	6	50	700	24	1200	18	75.32	77.49	-2.17
35	Factorial	10	50	700	72	1200	18	96.77	96.85	-0.0820
36	Factorial	10	40	700	72	1200	12	95.55	94.99	0.5562
37	Factorial	6	40	700	72	1800	18	94.69	96.56	-1.87
38	Factorial	10	40	500	24	1800	18	97.55	94.53	3.02
39	Axial	8	45	600	48	1950	15	95.11	98.56	-3.45

40	Factorial	10	50	500	24	1800	12	96.94	95.87	1.07
41	Factorial	10	50	700	72	1800	18	94.31	90.83	3.48
42	Axial	8	45	750	48	1500	15	85.93	89.19	-3.26
43	Axial	11	45	600	48	1500	15	93.98	95.55	-1.57
44	Factorial	6	40	500	24	1800	18	73.51	75.20	-1.69
45	Factorial	10	40	700	72	1200	18	97.96	96.57	1.39
46	Factorial	6	40	500	72	1200	12	90.71	91.64	-0.9274
47	Factorial	10	50	700	24	1200	18	99.63	97.79	1.84
48	Factorial	6	40	500	72	1800	18	96.11	97.24	-1.13
49	Factorial	10	40	500	24	1800	12	94.25	93.69	0.5572
50	Factorial	10	40	700	72	1800	18	91.11	93.85	-2.74
51	Factorial	10	50	700	72	1800	12	90.64	88.75	1.89
52	Centre	8	45	600	48	1500	15	99.87	98.07	1.80
53	Centre	8	45	600	48	1500	15	99.87	98.07	1.80
54	Factorial	6	40	700	24	1200	18	71.83	74.59	-2.76
55	Centre	8	45	600	48	1500	15	99.87	98.07	1.80
56	Centre	8	45	600	48	1500	15	99.87	98.07	1.80
57	Factorial	6	40	500	72	1800	12	94.81	92.47	2.34
58	Factorial	6	50	500	72	1800	12	88.81	91.62	-2.81
59	Factorial	10	50	700	24	1200	12	99.58	98.75	0.8314
60	Factorial	6	50	700	72	1200	18	96.58	96.54	0.0386
61	Factorial	10	40	500	72	1200	12	99.07	96.20	2.87
62	Axial	8	37.5	600	48	1500	15	94.25	96.80	-2.55
63	Axial	8	45	450	48	1500	15	88.83	90.38	-1.55
64	Factorial	6	40	500	24	1200	12	66.54	69.62	-3.08
65	Factorial	10	40	500	24	1200	18	94.08	93.50	0.5772
66	Factorial	10	50	500	24	1200	12	96.92	99.66	-2.74
67	Factorial	6	50	700	72	1200	12	93.83	93.56	0.2677
68	Factorial	6	50	500	72	1800	18	98.05	95.39	2.66
69	Factorial	6	50	700	72	1800	12	83.61	88.86	-5.25
70	Factorial	10	50	700	24	1800	18	90.95	93.28	-2.33
71	Axial	8	45	600	12	1500	15	92.37	90.93	1.44
72	Factorial	10	40	500	72	1800	18	96.83	96.58	0.2513
73	Factorial	6	50	500	24	1800	18	77.74	76.17	1.57
74	Axial	8	45	600	48	1500	10.5	93.17	94.85	-1.68
75	Factorial	6	40	700	24	1200	12	73.75	72.15	1.60
76	Factorial	10	40	700	24	1800	18	95.03	93.47	1.56
77	Factorial	10	40	700	24	1200	18	97.52	94.69	2.83
78	Factorial	10	50	500	72	1800	12	97.67	93.57	4.10
79	Factorial	6	40	700	72	1200	12	93.38	92.48	0.8955
80	Centre	8	45	600	48	1500	15	99.87	98.07	1.80
81	Factorial	6	40	500	24	1200	18	69.88	71.36	-1.48
82	Centre	8	45	600	48	1500	15	99.87	98.07	1.80
83	Axial	8	45	600	48	1050	15	99.97	101.33	-1.36
84	Factorial	6	40	500	24	1800	12	70.27	71.96	-1.69
85	Factorial	6	50	500	72	1200	12	93.79	94.09	-0.3002
86	Factorial	10	40	700	24	1200	12	91.27	94.64	-3.37

Table VIID.3: Adequacy of the models tested for the degradation of the mixture

Sequential Model Sum of Squares						
Source	**Sum of Squares**	**df**	**Mean Square**	**F-value**	**p-value**	**Remarks**
Mean vs. Total	7.164E+05	1	7.164E+05			
Linear vs. Mean	3872.08	6	645.35	15.16	< 0.0001	
2FI vs. Linear	1817.71	15	121.18	5.02	< 0.0001	
Quadratic vs. 2FI	**1070.83**	**6**	**178.47**	**21.80**	**< 0.0001**	**Suggested**
Cubic vs. Quadratic	227.61	26	8.75	1.13	0.3647	Aliased
Residual	247.17	32	7.72			
Total	7.236E+05	86	8414.32			

Model Summary Statistics						
Source	**Std. Dev.**	**R^2**	**Adjusted R^2**	**Predicted R^2**	**PRESS**	**Remarks**
Linear	6.52	0.5352	0.4999	0.4544	3947.63	
2FI	4.91	0.7864	0.7163	0.6947	2209.28	
Quadratic	**2.86**	**0.9944**	**0.9838**	**0.9756**	**1189.79**	**Suggested**
Cubic	2.78	0.9658	0.9093	-2.2638	23615.22	Aliased

The constant variance assumption was investigated by plotting internally studentized residual vs. predicted values of percent degradation of tri- solute mixture of Phenolic compounds i.e. Phenol & 4- Chloro Phenol & Catechol (Figure VIID.1). The normal probability plot of residuals (Figure VIID.2) for the percent degradation of Phenolic mixture showed a straight line pattern rather than S shaped followed by the points on the plot. As the residuals are distributed normally, transformation of response is not required. Relation between the predicted and experimental values of response has been displayed in Figure VIID.3. Very little discrepancies were found by the straight trend line pointing a good relationship in between the predicted and experimental values.

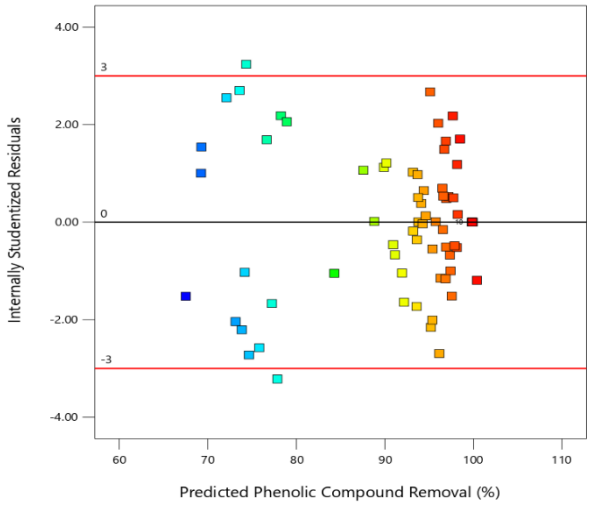


Figure VIID.1: Internally studentized residuals vs. predicted values

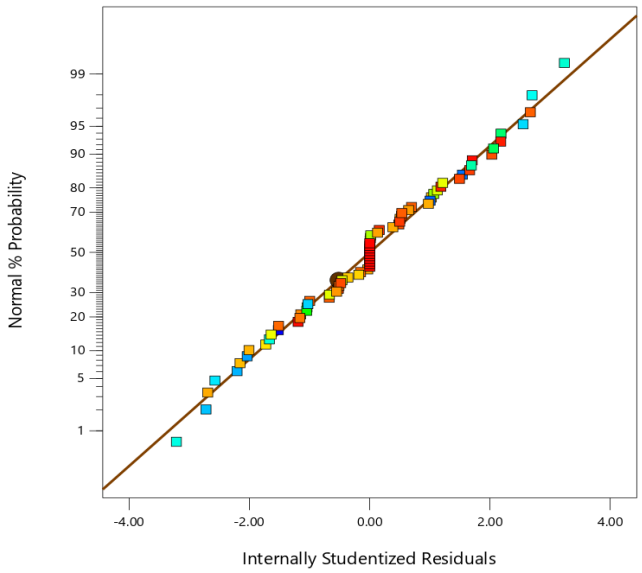


Figure VIID.2: Internally studentized residuals vs. Normal percent probability

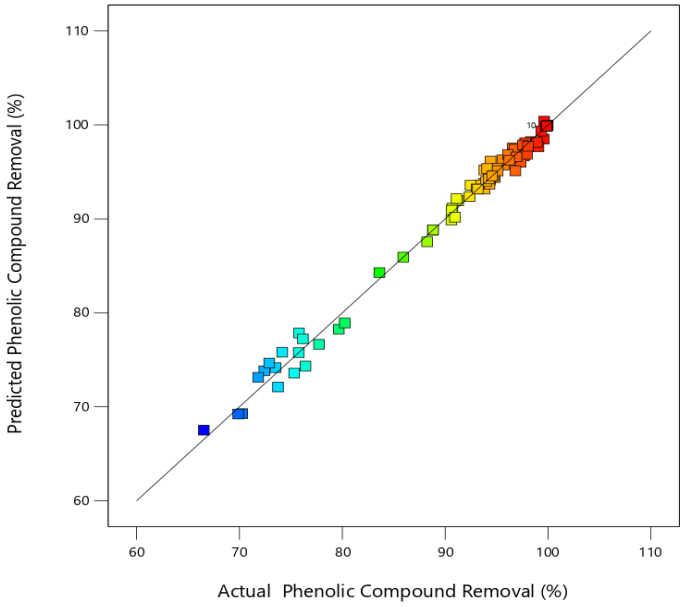


Figure VIID.3: Actual degradation data vs. predicted data

VIID.2.2 ANOVA test:

Outcomes of ANOVA for percent of degradation of the mixture of Phenol, 4- Chloro Phenol & Catechol have been given in Table VIID.4. In that table, The Model F-value of 30.59 implies the model is significant. There is only a 0.01% chance that an F-value this large could occur due to noise. P-values less than 0.0500 indicate model terms are significant. In this case Q1, Q2, Q3, Q4, Q5, Q6, Q1Q2, Q1Q3, Q1Q4, Q1Q5, Q1Q6, Q2Q3, Q2Q4, Q2Q5, Q3Q4, Q3Q5, Q3Q6, Q4Q5, Q4Q6, $Q1^2$ and $Q3^2$, are significant model terms. Values greater than 0.100 indicate the model terms are not significant. Q2Q6, Q5Q6, $Q2^2$, $Q4^2$, $Q5^2$ and $Q6^2$ are not significant. If there are many insignificant model terms (not counting those required to support hierarchy), model reduction may improve the model.

Table VIID.4: ANOVA of the second order polynomial equation for the degradation of mixture

Source	Sum of Squares	df	Mean Square	F-value	p-value	Remarks
Model	6760.63	27	250.39	30.59	< 0.0001	significant
Q1-pH	2080.11	1	2080.11	254.11	< 0.0001	significant
Q2-Temperature	21.81	1	21.81	2.66	0.0081	significant
Q3-Medium Volume	10.68	1	10.68	1.30	0.0180	significant
Q4-Residence Time	1639.20	1	1639.20	200.25	< 0.0001	significant
Q5-Initial Concentration	58.07	1	58.07	7.09	0.0100	significant
Q6-Inoculum size	62.21	1	62.21	7.60	0.0078	significant
Q1Q2	0.1661	1	0.1661	0.0203	0.0072	significant
Q1Q3	16.85	1	16.85	2.06	0.0167	significant
Q1Q4	1598.00	1	1598.00	195.22	< 0.0001	significant
Q1Q5	31.75	1	31.75	3.88	0.0037	significant
Q1Q6	23.09	1	23.09	2.82	0.0184	significant
Q2Q3	7.56	1	7.56	0.9239	0.0105	significant
Q2Q4	31.92	1	31.92	3.90	0.0131	significant
Q2Q5	43.56	1	43.56	5.32	0.0247	significant
Q2Q6	4.02	1	4.02	0.4911	0.4862	Not significant
Q3Q4	11.21	1	11.21	1.37	0.0168	significant
Q3Q5	20.00	1	20.00	2.44	0.0034	significant
Q3Q6	2.05	1	2.05	0.2507	0.0185	significant
Q4Q5	9.08	1	9.08	1.11	0.0067	significant
Q4Q6	9.41	1	9.41	1.15	0.0181	significant
Q5Q6	9.05	1	9.05	1.10	0.2975	Not significant
$Q1^2$	270.96	1	270.96	33.10	< 0.0001	significant
$Q2^2$	0.4117	1	0.4117	0.0503	0.8233	Not significant
$Q3^2$	159.71	1	159.71	19.51	< 0.0001	significant
$Q4^2$	0.0928	1	0.0928	0.0113	0.9156	Not significant
$Q5^2$	8.23	1	8.23	1.01	0.3202	Not significant
$Q6^2$	7.47	1	7.47	0.9122	0.3435	Not significant
Residual	474.78	58	8.19			
Lack of Fit	474.78	49	9.69			
Pure Error	0.0000	9	0.0000			
Cor Total	7235.41	85				

VIID.2.3 Effect of pH of the media (Q1):

Effect of pH revealed a significant role in percent degradation of the mixture of Phenol, 4-Chloro Phenol and Catechol. Percent of degradation of the mixture was maximum (almost 99percent) when the pH value of the culture media was 8. Percent degradation was decreased in both situations while the pH value of the media increased and decreased indicating that maximum degradation was occurred at moderately basic condition. Acidic, neutral and extreme basic conditions are not favourable for the consortium to remove the mixture of Phenol, 4- Chloro Phenol & Catechol. From the 3D and contour plots, it can be showed that the interaction effects between pH of the media vs. temperature (Q1Q2), pH of the media vs. media volume (Q1Q3),) pH of the media vs. residence time (Q1Q4), pH of the media vs. initial conc. of the mixture of the Phenolic compounds (Q1Q5) and pH of the media vs. inoculums percent (Q1Q6), all revealed significant effects on the percent degradation of the tri – solute mixture of Phenol, 4- Chloro Phenol and Catechol. All the 3D Plots are convex shaped (Figure: VIID.4 (a), (b) (c), (d) & (e)).

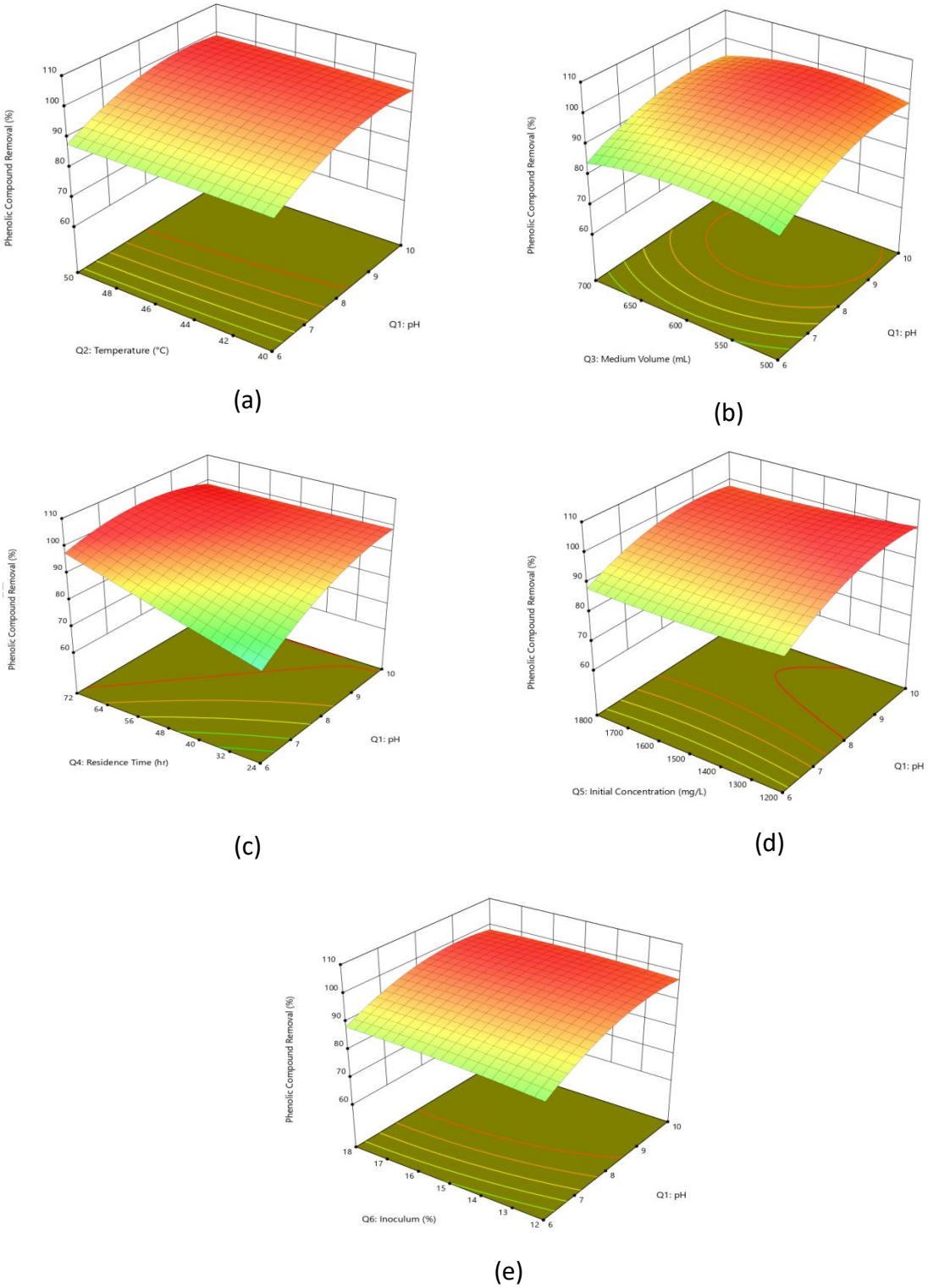
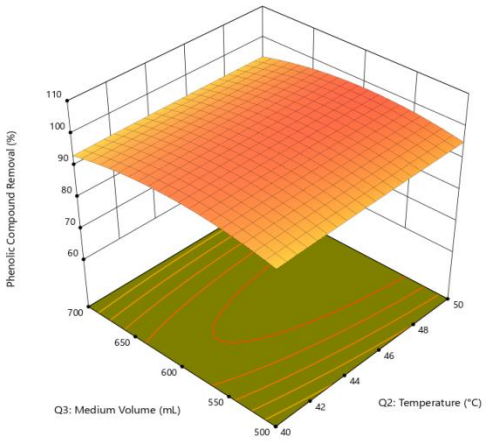


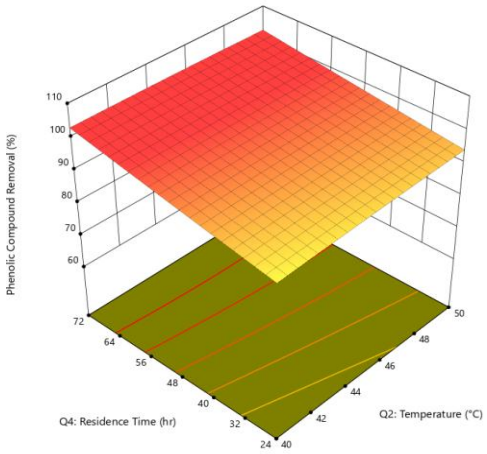
Figure VIID.4: 3-Dimensional surfaces and 2-Dimensional plots of the interaction effects of: (a) pH of the media and temperature (Q1Q2), (b) pH of the media and media vol. (Q1Q3), (c) pH of media and residence time (Q1Q4), (d) pH of the media and initial conc. of the mixture (Q1Q5), (e) pH of the media and inoculums percent (Q1Q6) on the percent of degradation of the Phenolic mixture (Phenol & 4- Chloro Phenol & Catechol)

VIID.2.4 Effect of temperature (Q2):

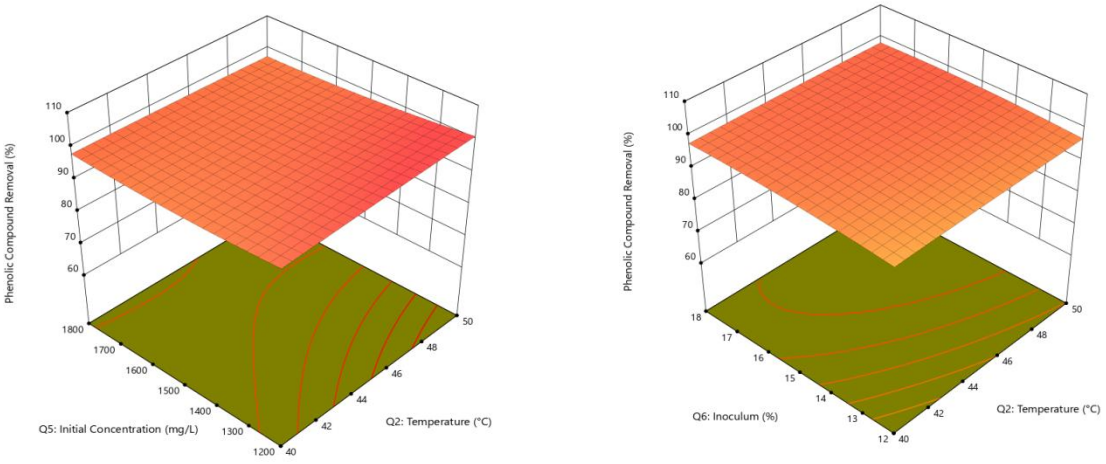
Temperature was found to be very effective factor during this study. Degradation percent of the tri- solute mixture (i.e. Phenol, 4- Chloro Phenol & Catechol) was increased rapidly while temperature reached to 45^{o}C. But below this temperature, degradation percent decreased. Also, while the temperature increased up to 50^{o}C and even 52.5^{o}C, decrease in degradation percent appeared. The interaction effects between temperature vs. media volume (Q2Q3), temperature vs. residence time (Q2Q4) and temperature vs. initial conc. of the Phenolic mixture (Q2Q5) found to be significant on the response i.e. percent of degradation of the Phenolic mixture. 3D curves found to be convex shaped here to some extent (Figure: VIID.5 (a), (b) & (c)). On the other hand, and temperature vs. inoculums percent (Q2Q6) showed to be insignificant on the percent of degradation of the mixture of Phenolic compounds. 3D curve found to be flat in shape (Figure: VIID.5 (d)). Moreover, p-value of the model term is 0.4862 which indicates the insignificance of this model term (Table VIID.4).



(a)



(b)

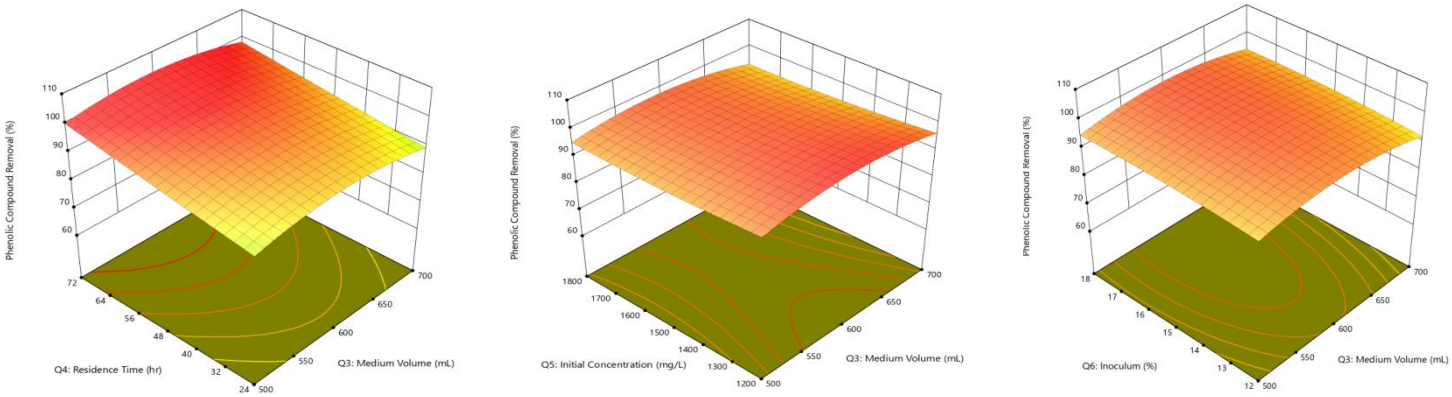


(c) (d)

Figure VIID.5: 3-Dimensional surfaces and 2-Dimensional plots of the interaction effects of: (a) temperature and media vol. (Q2Q3), (b) temperature and residence time (Q2Q4), (c) temperature vs. initial conc. of the mixture (Q2Q5), (d) temperature and inoculums percent (Q2Q6) on the percent of degradation of the mixture (Phenol & 4-Chloro Phenol & Catechol)

VIID.2.5 Effect of media volume (Q3):

Volume of the culture media played a pivotal role on degradation percent of Phenol, 4-Chloro Phenol & Catechol. Percent of degradation of the mixture increased with the increasing volume of the culture media. Up to 600 ml volume, degradation percent increased steadily. But beyond that, when the volume increased up to 700 and even 750 ml, the degradation percent decreased slightly. A sudden decrease in degradation percent was noticed (up to ~88percent) when the media volume decreased up to 450 ml. In case of the interaction effects between media volume vs. residence time (Q3Q4), media volume vs. initial conc. of the mixture (Q3Q5) and media volume vs. inoculums percent of the consortium (Q3Q6) exhibited significant effects on the degradation percent. Convex shaped 3D curves (Figure: VIID.6 (a), (b) & (c)) indicated the same. P- Values of these model terms were found to be below 0.0500 (TableVIID.4) indicating their significant effects on the response.



(a) (b) (c)

Figure VIID.6: 3-Dimensional surfaces and 2-Dimensional plots of the interaction effects of: (a) media vol. and residence time (Q3Q4), (b) media vol. and initial conc. of the mixture (Q3Q5), (c) media vol. and inoculums percent (Q3Q6) on the percent of degradation of the mixture of Phenol & 4- Chloro phenol & Catechol

VIID.2.6 Effect of residence time (Q4):

Residence time played a vital role on the response i.e. percent of degradation. Most of the good responses were achieved in between 24 hours to 72 hours (even > 90%). Maximum degradation of the tri- solute mixture was obtained at 48 hours (~99percent). After that time period, a slight decrease in degradation percent appeared. Interaction effects in between residence time vs. initial conc. of the mixture (Q4Q5) and residence time vs. inoculums size of the consortium of bacterial strains (Q4Q6) found to be very significant on the percent of degradation of the mixture (Phenol, 4- Chloro Phenol & Catechol). Both the convex shaped 3D curves (Figure VIID.7 (a) & (b)) and the p- values of the model terms (Table VIID.4) indicated the significant effects of both of these interactions.

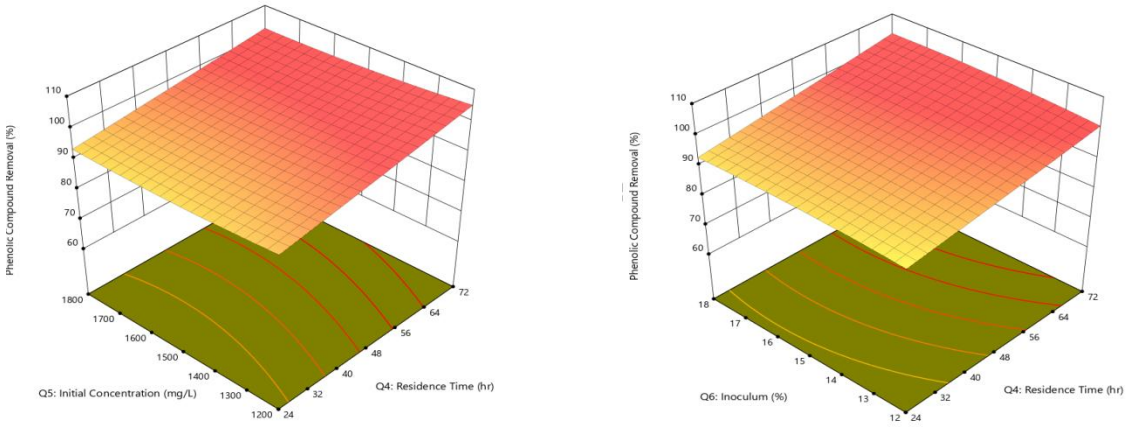


Figure VIID.7: 3-Dimensional surfaces and 2-Dimensional plots of the interaction effects of: (a) residence time and initial conc. of the mixture (Q4Q5), (b) residence time and inoculums percent (Q4Q6) on the percent of degradation of the mixture of Phenol & 4- Chloro Phenol & Catechol

VIID.2.7 Effect of initial conc. of the mixture of Phenolic compounds and inoculums percent (Q5) & (Q6):

Initial conc. of the mixture (Phenol & 4- Chloro Phenol & Catechol) and inoculums size of the consortium of six bacterial strains both exhibited vital role on the percent of degradation of the mixture. Percent of degradation decreased slightly while initial conc. of the mixture increased. When the conc. of the mixture was 1050 mg/L (350 mg/ L Phenol, 350 mg/L 4-Chloro Phenol & 350 mg/L Catechol),it was removed very easily (almost 100percent degradation was achieved). Slight decrease in degradation percent was obtained when the initial conc. was 1200 mg/L (conc. of each compound was 400 mg/L) and 1500 mg/L (conc. of each compound was 500 mg/L). Optimum degradation was also achieved at the conc. of 1500 mg/L accompanied by suitable pH, temperature and time. When the conc. increased to 1800 mg/L (conc. of each was 600 mg/L), degradation percent slightly decreased. And when the initial conc. of the mixture increased further up to 1950 mg/L (conc. of each was 650 mg/L), 95.11% degradation was achieved.

Degradation percent increased with the increasing percent of inoculums. 15% inoculums (2.5% inoculums of each of the six strains) found to be mostly effective to remove the tri-solute mixture when it was 1500 mg/L. When inoculums size was increased up to 18%, no more changes occurred. Even 19.5% inoculums size was able to remove 94.57% of the tri-

solute mixture of the Phenolic compounds when the initial conc. of the mixture was 1500 mg/L (conc. of each was 500 mg/L).

But, the interaction effect between the initial conc. of the mixture of Phenolic compounds vs. inoculums size (Q5Q6) found to be insignificant on the response as the curve is flat in nature (Figure VIID.8). P-value of the model term (Q5Q6) found to be 0.2975 (Table VIID.4) which indicated the same.

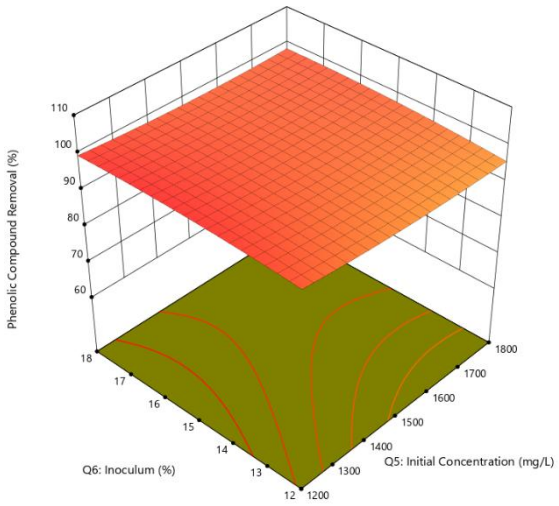


Figure VIID.8: 3-Dimensional surfaces and 2-Dimensional plots of the interaction effects of: initial conc. of the mixture and inoculums percent (Q5Q6) on the percent of degradation of the mixture of Phenol & 4- Chloro Phenol & Catechol

VIID.2.8 Optimization of the operating parameters:

The Response Surface Methodology (RSM) was involved to deduct the optimum conditions for the six independent variables to achieve maximum percent of degradation of the mixture of Phenol & 4-Chlorophenol & Catechol. Equation (7.5) was defined as objective function for the percent of degradation of the mixture of Phenolic compounds (Phenol & 4- Chloro Phenol & Catechol) and the independent factors in their ranges were model constraints. Thus the following optimum conditions, achieved for the maximum percent of degradation of the mixture were: 8. pH of the bacterial culture media, 45^{o}C temperature, 600 mL volume of the culture media, 48 hours of residence time, 1500 mg/L initial conc. of the mixture (in 1:1:1

ratio i.e. 500 mg/L Phenol, 500 mg/L 4- Chloro Phenol & 500 mg/L Catechol) and 15% inoculums size of the consortium (i.e. 2.5% *Brevibacillus formosus* strain NRRL NRS- 863, 2.5% *Pseudomonas otitidis* strain MCC10330, 2.5% *Bacillus timonensis* strain 10403023, 2.5% *Bacillus cereus* strain MK789657, 2.5% *Bacillus pseudomycoides* strain NBRC 101232 and 2.5% *Bacillus paramycoides* strain MCCC 1A04098). 99.87% degradation of the mixture of Phenol & 4- Chloro Phenol & Catechol was obtained by involving these six favourable parameters. The obtained optimal operating conditions were performed nine more times to validate the predicted values as well to minimise the error and 99.87% degradation was achieved in case of each experimental results indicating the reliability of this model.

VIID.2.9 Result of HPLC analysis:

From the standard chromatogram of known conc. of Phenol, 4- Chloro Phenol and Catechol, it can be deduced that the peak of 6.156 AU represents the residual conc. of Catechol, 6.464 AU represents the residual conc. of Phenol and 7.065 AU represents 4- Chloro Phenol respectively (Figure VIID.9). Calculating the area of the chromatograms (both the standard and the sample), it was found that individually 98.96% of Phenol, 96.81% of 4- Chloro Phenol and 98.60% of Catechol were removed from the tri- solute mixture, while cultured according to the parameters of the centre point of RSM, which almost supported the experimental value, obtained at the same point of the design matrix (Table VIID.2).

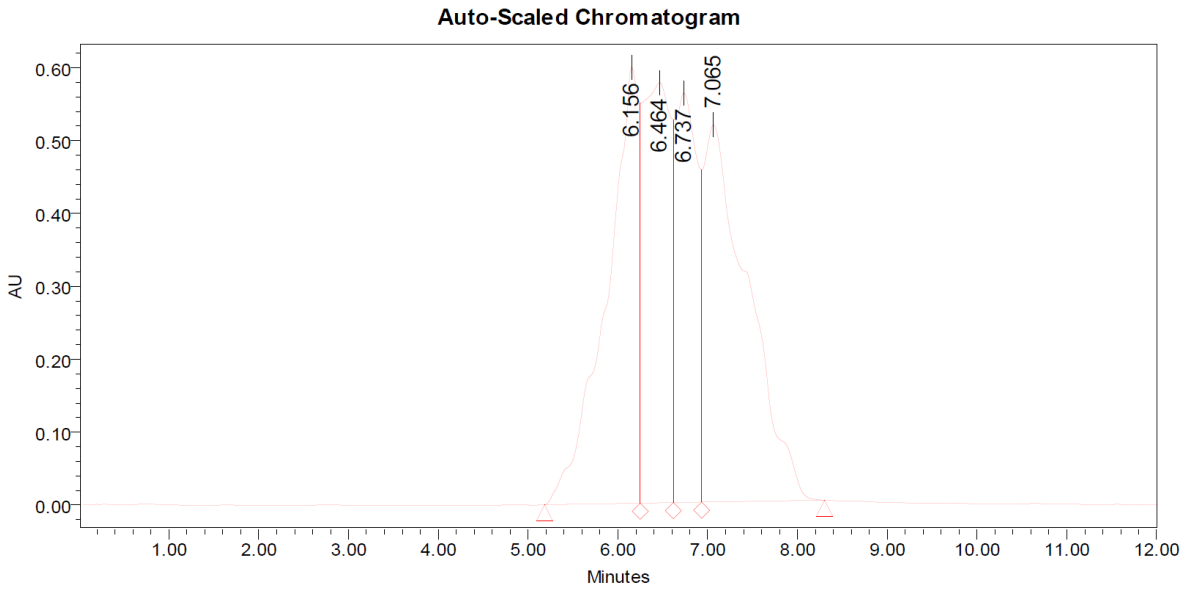


Figure VIID.9: Chromatogram for the of the sample obtained as per centre point of RSM design

Conclusions:

The Response Surface Methodology (RSM), was applied to optimize the process parameters in case of all the four different types of consortiums. The developed statistical as well as mathematical model is able to provide a comprehensive exploration of the cross- factor interactive effects of the independent variables (six parameters) on the response. Maximum cross-factors were found to be significant and some of them were very much significant on the response i.e. degradation percentage of bi- solute mixture of Phenol & 4- Chloro Phenol, 4- Chloro Phenol & Catechol, Phenol & Catechol and tri- solute mixture of Phenol & 4- Chloro Phenol & Catechol whichever was applicable. The proposed models, explaining the treatment of pharmaceutical waste water by involving four different consortiums , were all found to be suitable for the future studies to design a bioreactor, modelling. To check the individual degradation percentages of the bi- or tri- solute mixtures, HPLC analysis was also deployed in case of the centre point matrix in all cases and all those chromatograms found to successfully validate the RSM results where total degradation was considered instead of checking individual degradations. Experiment in pilot- scale is recommended for the future study. In the next to next chapter, design and fabrication of a bioreactor has been described elaborately.

Chapter VIII

Determination of growth kinetics of the consortiums while degrading the Phenolic substances as a mixture

Introduction:

From the previous chapter (chapter VII), the optimization of the operating parameters were determined by Response Surface Methodology (RSM) in case of the all the four consortiums. In some favourable parameters, up to a certain concentration of toxic Phenolic substances as bi- solute mixtures (Phenol & 4- Chloro Phenol, 4- Chloro Phenol & Catechol and Phenol & Catechol) or tri- solute mixture (Phenol & 4- Chloro Phenol & Catechol) can be degraded by involving those microbial consortiums. The details of those consortiums and the mixtures of Phenolic substances degraded by them have elaborately been discussed in chapter VII. As at high concentrations, Phenolic substances exhibit inhibition effects on the cultures (Hill and Robinson; 1975 & Yang and Humphrey; 1975), the mixture of Phenolic compounds will exhibit the same against the consortiums also. Numerous processes that impact the entire microbial growth process have been linked to the suppression of microbes by substrates (Edward, 1970). So, deduction of the substrate inhibition has become an important factor in case of biodegradation of the toxic Phenolic mixtures in culture media. A large variety of mathematical models have been proposed by the scientists to calculate the inhibitory effects on the growth kinetics of the consortiums. Out of them, one of the most widely used model is Haldane model. According to Goudar et al., 2000, Haldane model is the most common equation to describe the growth inhibition kinetics of microbes.

Sub chapter VIIIA

Determination of growth kinetics of Phenol & 4- Chloro Phenol (as a mixture) degrading consortium and inhibitory effect of this bi- solute mixture

VIIIA.1 Materials and Methods:

VIIIA.1.1 Materials:

Same as mentioned in chapter VI, section VIA.1.1.

VIIIA.1.2 Experimental set up:

The five operating parameters in case of the microbial consortium were as follows: pH: 8.5, Temperature: 45^{o}C, Media volume: 600 mL, Time: 48 hours & Inoculums size: 12percent (3percent inoculums of each of the four bacterial strains). All of these operating parameters were obtained at the centre point of RSM while optimizing the parameters in case of this consortium (chapter VII). The consortium was cultured at seven different initial conc. of the bi- solute mixture i.e. Phenol & 4- Chloro Phenol. Individual initial conc. of Phenol & 4- Chloro Phenol was kept equal in each case (in 1:1 ratio). Initial conc. of the bi- solute mixture in the seven mediums were maintained as 50 mg/L (25 mg/L Phenol & 25 mg/L 4- Chloro Phenol),100 mg/L, 200 mg/L, 400 mg/L, 600 mg/L, 800 mg/L and 1000 mg/L (500 mg/L Phenol & 500 mg/L 4- Chloro Phenol) along with a control. The highest initial conc. of the mixture was maintained as 1000 mg/L as this conc. was obtained at the centre point of the RSM (chapter VII). Periodically after 3 hours, all the samples were analyzed for biomass and degradation percentage of the bi- solute mixture (Phenol & 4- Chloro Phenol).

Rest of the portion is same as described in the chapter V, section VA.1.2.

VIIIA.1.3 Analytical Method:

The method of determination of residual bi- solute mixture in the media was same as mentioned in chapter VI, section VIA.1.3. Cell mass was analyzed at 600 nm wavelengths in spectrophotometer.

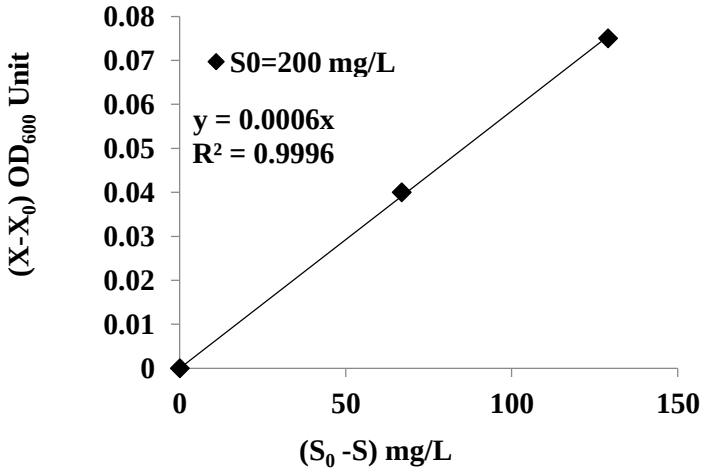
VIIIA.1.4 Calculations:

Same as described the chapter V, section VA.1.4.

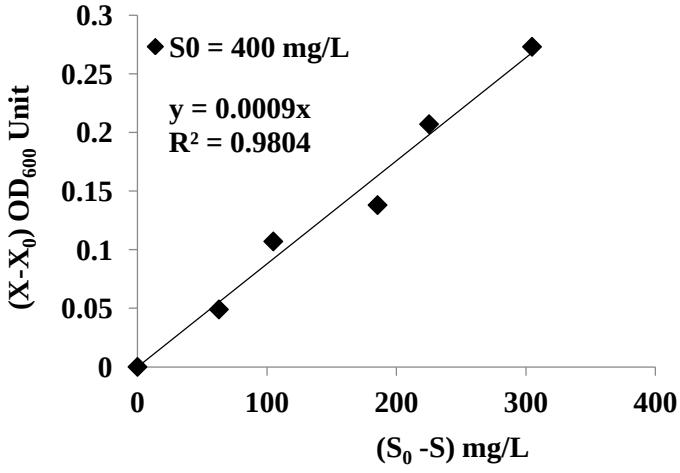
VIIIA.2 Results and Discussions:

VIIIA.2.1 Degradation of bi- solute mixture (Phenol & 4- Chloro Phenol) & biomass production:

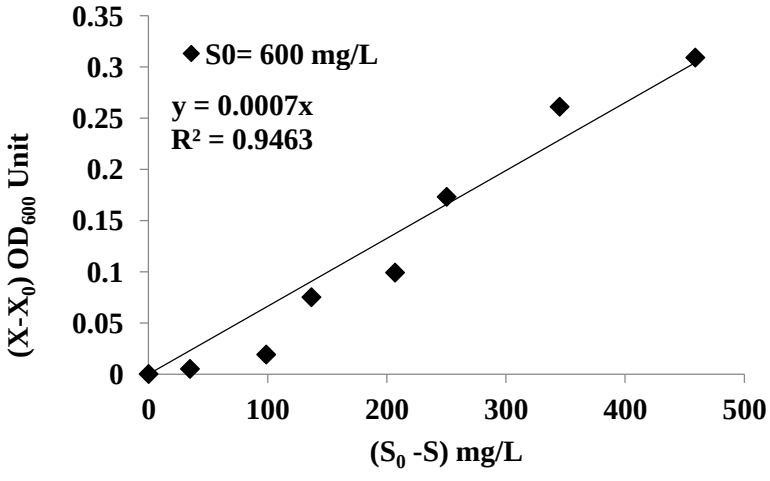
A bacterial consortium was utilized in this study. Incubation time varied with respect to the initial conc. of bi- solute mixture. 48 hours of residence time was taken by this consortium for complete degradation of 1000 mg/L of the bi- solute mixture. When the initial conc. of the mixture was low, it was removed within very short time span. The consortium completely removed 50 mg/L and 100 mg/L of the bi- solute mixture within just 3 hours. And thereby, 200 mg/L, 400 mg/L, 600 mg/L and 800 mg/L of the mixture was degraded by this consortium within 9 hours, 18 hours, 24 hours and 48 hours respectively. The effects of initial conc. of the mixture on the growth of the consortium with respect to that of the biomass yield have been shown in Figure: VIIIA.1.The yield of biomass per unit mass of consumed mixture i.e. Phenol & 4- Chloro Phenol ($Y_{x/s}$) have been displayed in the Table VIIIA.1. Maximum yield of 0.001 OD units. L/mg was achieved by the consortium when initial conc. was 800 mg/L.



(a)



(b)



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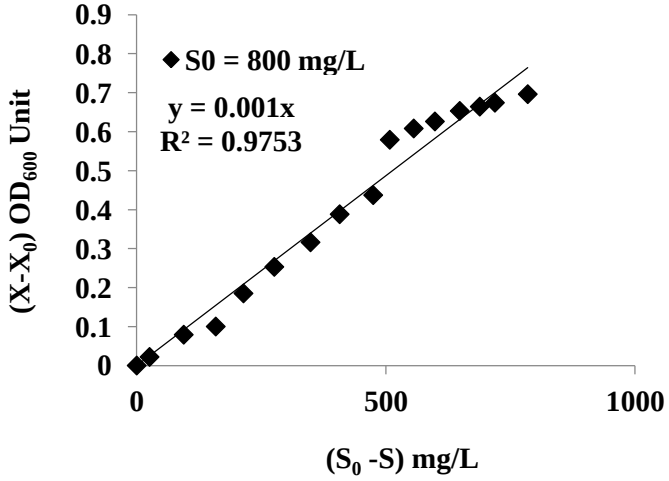

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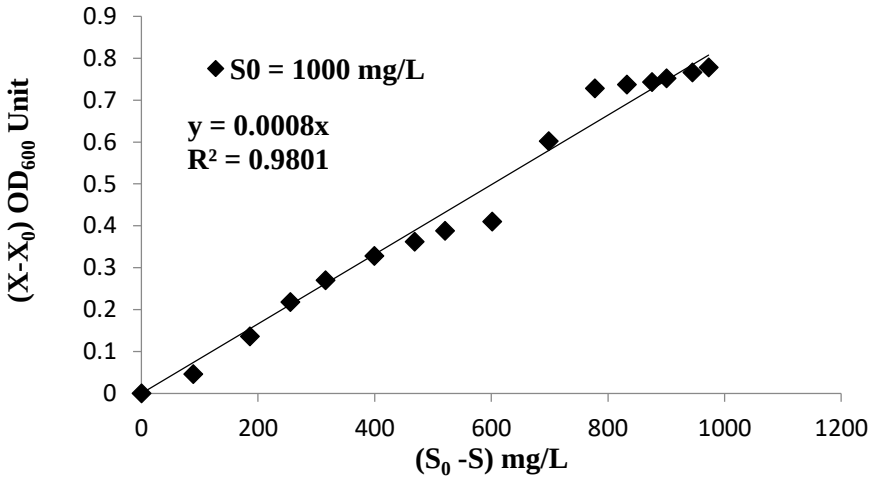


(e)

Figure VIIIA.1: Yield coefficients for the consortium; growth on the mixture of Phenol & 4- Chloro Phenol

Table VIIIA.1: Yield coefficients for the for the growth of consortium on the bi- solute mixture (Phenol & 4- Chloro Phenol) determined from the plots

S_0 (mg/L)	Yield coefficients ($Y_{x/s}$) [l_{600} units. L/mg]
200	0.0006
400	0.0009
600	0.0007
800	0.001
1000	0.0008

Here in this study, yield coefficients were not deducted for 50 mg/L and 100 mg/L initial conc. of the mixture. The time duration of the microbial growth and degradation of the mixture for 800 mg/L and 1000 mg/L have been shown in Figure VIIIA.2. The growth profile of the consortium both in the highest conc. of the bi- solute mixture and control medium, has been displayed in Figure VIIIA.3. The highest OD value and the incubation time to reach the stationary phase depend upon the initial conc. of the mixture. The highest optical density (λ_{600}) of 0.786 was attained by the consortium when the conc. of the mixture was 1000 mg/L. The corresponding highest OD value of 0.779 was obtained by the consortium at the same initial conc. of the bi- solute mixture. The growths had exponential and stationary phase with increasing lag phase. Duration of the lag phase increased with the increasing initial conc. of the mixture and in turn, increasing the incubation time to attain the complete degradation of the mixture.

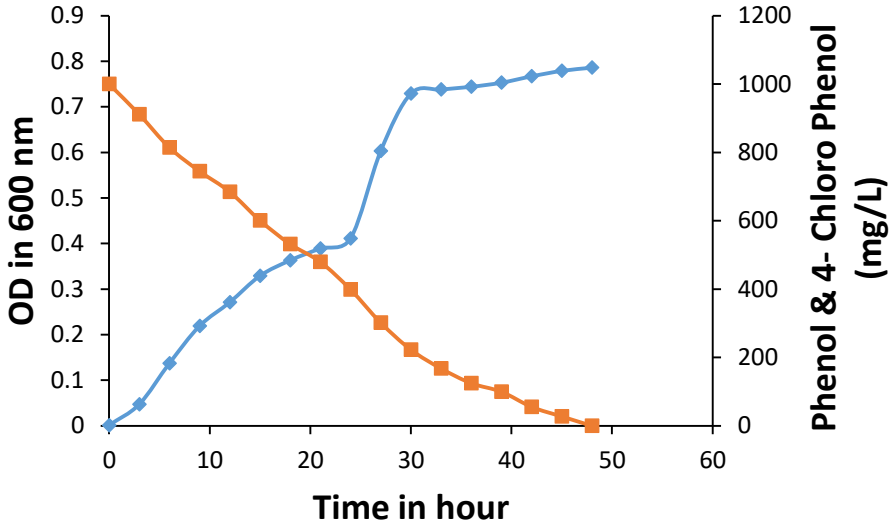


Figure VIIIA.2: Time duration of the microbial growth and degradation of the bi-solute mixture (Phenol & 4- Chloro Phenol)

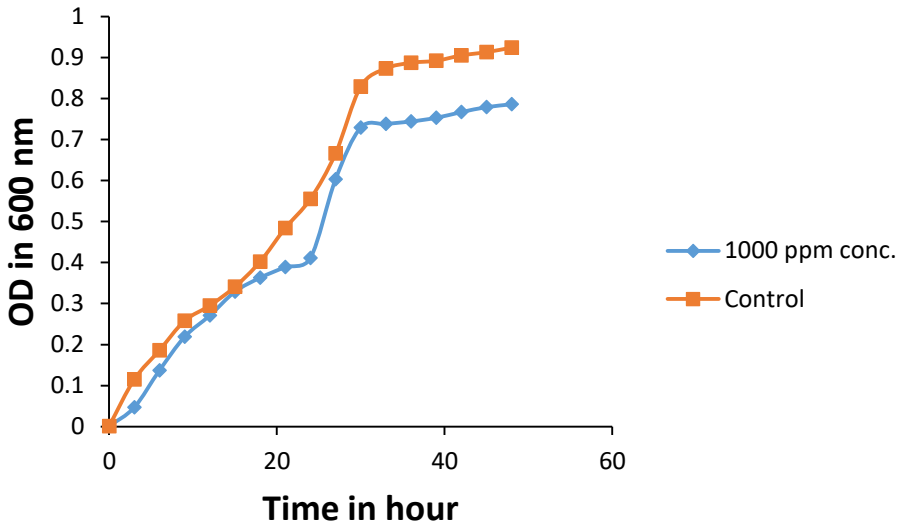
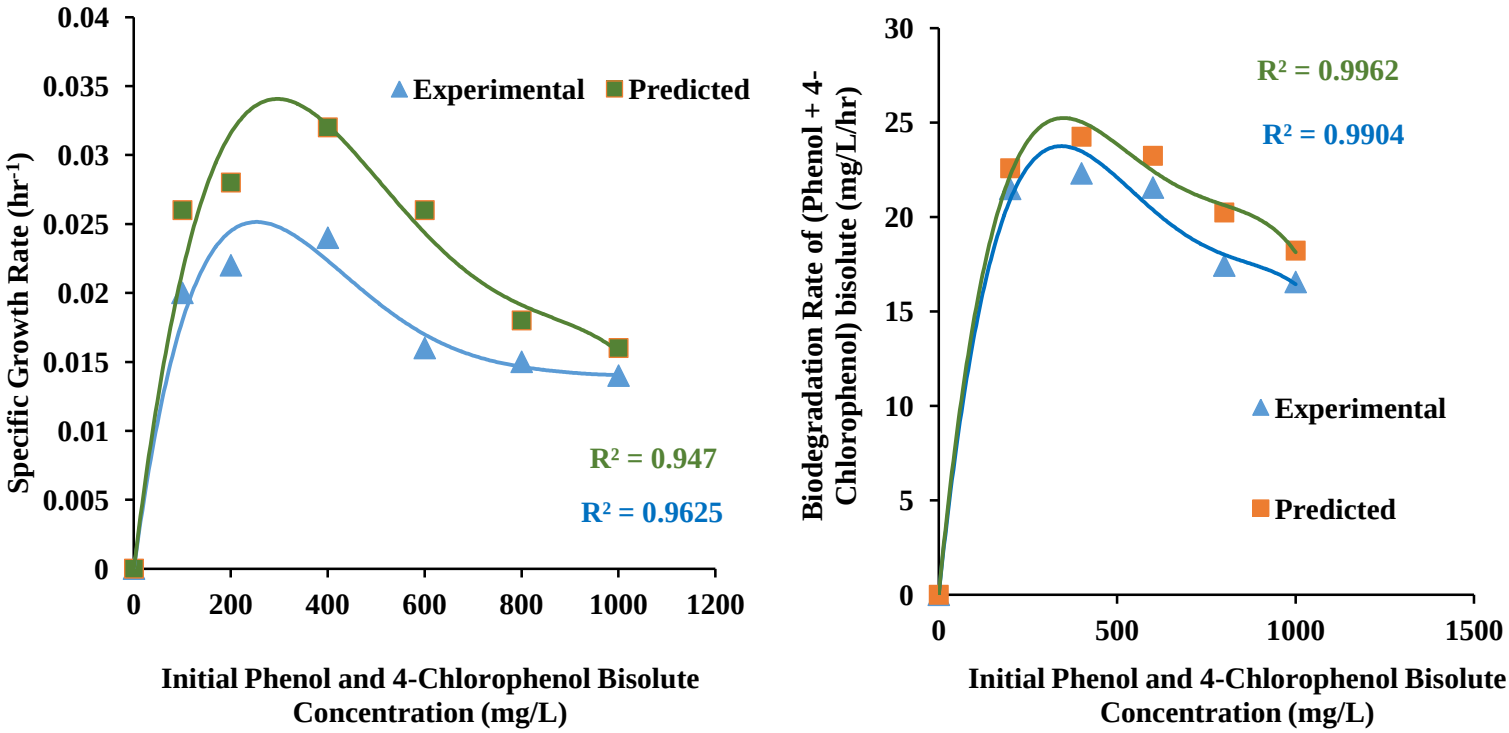


Figure VIIIA.3: The growth profile of the consortium in the highest conc. of the bi-solute mixture and control medium

VIIIA.2.2 Kinetics of degradation of the mixture and production of biomass:

The growth and degradation data might be explained by an integrated Haldane's substrate inhibition model with R^2 values more than 0.9, according to the progress curve analysis. The pattern of substrate inhibition was followed by the growth of the consortium and the biodegradation of the mixture of Phenol & 4- Chloro Phenol. The growth rate and degradation of the mixture was initially increased when the concentration of the mixture increased. After that, both the rates decreased subsequently with the increasing conc. of the mixture. The Haldane model described overall growth of the consortium (R^2 = 0.9625) good at the S_0 range of 0 – 1000 mg/L. The kinetic parameters of the Haldane model have been displayed in Table VIII.1. Haldane model with R^2 values of 0.9904 for the consortium described the overall degradation rates very well.



(a) (b)

Fig VIIIA.4: Experimental and predicted specific growth and degradation rate of the consortium during the biodegradation of bi- solute mixture

Sub chapter VIIIB

Determination of growth kinetics of 4- Chloro Phenol & Catechol (as a mixture) degrading consortium and inhibitory effect of this bi- solute mixture

VIIIB.1 Materials and Methods:

VIIIB.1.1 Materials:

Same as mentioned in chapter VI, section VIB.1.1.

VIIIB.1.2 Experimental set up:

The five operating parameters in case of the microbial consortium were as follows: pH: 8.5, Temperature: 45^{o}C, Media volume: 600 mL, Time: 48 hours & Inoculums size: 12percent (3percent inoculums of each of the four bacterial strains). All of these operating parameters were obtained at the centre point of RSM while optimizing the parameters in case of this consortium (chapter VII). The consortium was cultured at seven different initial conc. of the bi- solute mixture i.e. 4- Chloro Phenol & Catechol. Individual initial conc. of 4- Chloro Phenol & Catechol was kept equal in each case (in 1:1 ratio). Initial conc. of the bi- solute mixture in the seven mediums were maintained as 50 mg/L (25 mg/L 4- Chloro Phenol & 25 mg/L Catechol),100 mg/L, 200 mg/L, 400 mg/L, 600 mg/L, 800 mg/L and 1000 mg/L (500 mg/L 4- Chloro Phenol & 500 mg/L Catechol) along with a control. The highest initial conc. of the mixture was maintained as 1000 mg/L as this conc. was obtained at the centre point of the RSM (chapter VII). Periodically after 3 hours, all the samples were analyzed for biomass and degradation percentage of the bi- solute mixture (4- Chloro Phenol & Catechol).

Rest of the portion is same as described in the chapter V, section VA.1.2.

VIIIB.1.3 Analytical Method:

The method of determination of residual bi- solute mixture in the media was same as mentioned in chapter VI, section VIB.1.3. Cell mass was analyzed at 600 nm wavelengths in spectrophotometer.

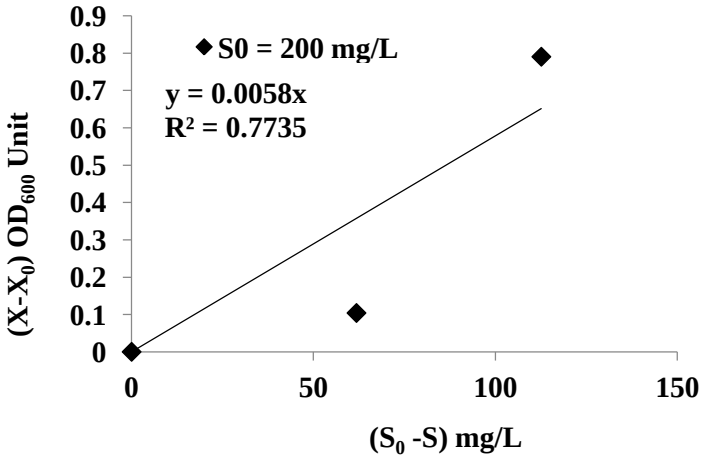
VIIIB.1.4 Calculations:

Same as described the chapter V, section VA.1.4.

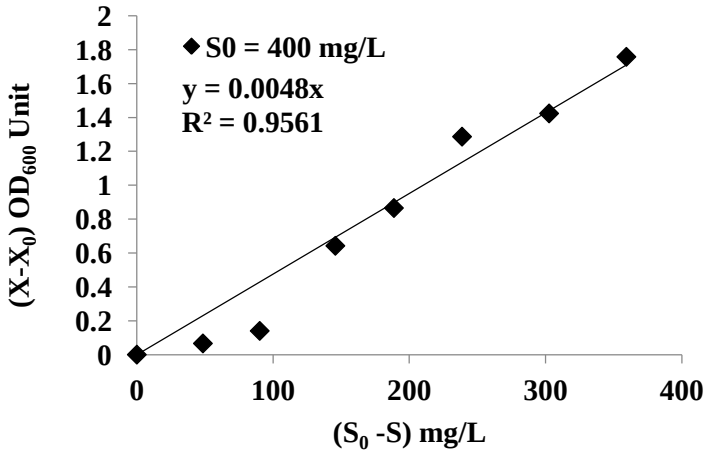
VIIIB.2 Results and Discussions:

VIIIB.2.1 Degradation of bi- solute mixture (4- Chloro Phenol & Catechol) & biomass production:

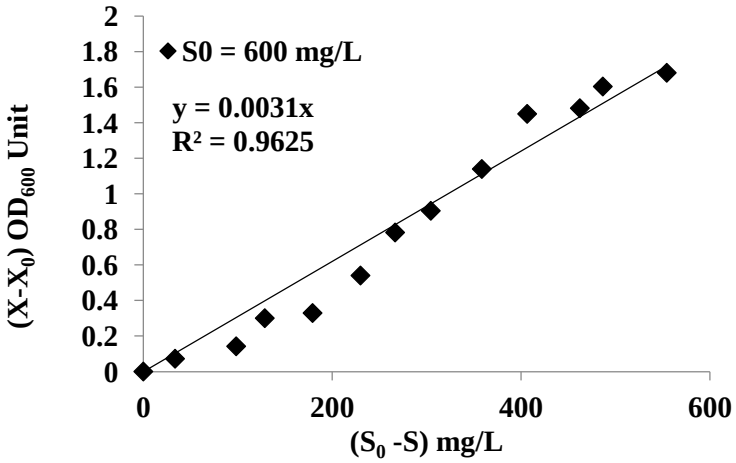
A bacterial consortium was utilized in this study. Incubation time varied with respect to the initial conc. of bi- solute mixture. 48 hours of residence time was taken by this consortium for complete degradation of 1000 mg/L of the bi- solute mixture. When the initial conc. of the mixture was low, it was removed within very short time span. The consortium completely removed 50 mg/L and 100 mg/L of the bi- solute mixture within just 3 hours. And thereby, 200 mg/L, 400 mg/L, 600 mg/L and 800 mg/L of the mixture was degraded by this consortium within 9 hours, 24 hours, 39 hours and 48 hours respectively. The effects of initial conc. of the mixture on the growth of the consortium with respect to that of the biomass yield have been shown in Figure: VIIIB.1.The yield of biomass per unit mass of consumed mixture i.e. 4- Chloro Phenol & Catechol ($Y_{x/s}$) have been displayed in the Table VIIIB.1. Maximum yield of 0.0058 OD units. L/mg was achieved by the consortium when initial conc. was 200 mg/L.



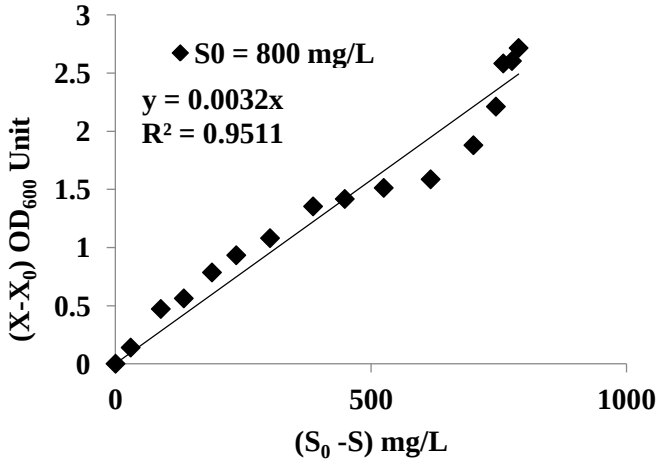
(a)



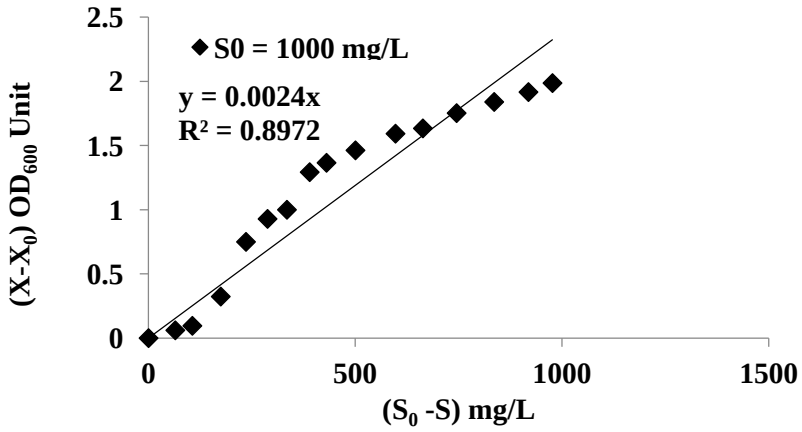
(b)



(c)



(d)



(e)

Figure VIIIB.1: Yield coefficients for the consortium; growth on the mixture of 4- Chloro Phenol & Catechol

Table VIIIB.1: Yield coefficients for the for the growth of consortium on the bi- solute mixture (4- Chloro Phenol & Catechol) determined from the plots

S_0 (mg/L)	Yield coefficients ($Y_{x/s}$) [l_{600} units. L/mg]
200	0.0058
400	0.0048
600	0.0031
800	0.0032
1000	0.0024

Here in this study, yield coefficients were not deducted for 50 mg/L and 100 mg/L initial conc. of the mixture. The time duration of the microbial growth and degradation of the mixture for 800 mg/L and 1000 mg/L have been shown in Figure VIIIB.2. The growth profile of the consortium both in the highest conc. of the bi- solute mixture and control medium has been displayed in Figure VIIIB.3. The highest OD value and the incubation time to reach the stationary phase depend upon the initial conc. of the mixture. The highest optical density (λ_{600}) of 2.91 was attained by the consortium when the conc. of the mixture was 800 mg/L. The corresponding highest OD value of 2.717 was obtained by the consortium at the same initial conc. of the bi- solute mixture. The growths had exponential and stationary phase with increasing lag phase. Duration of the lag phase increased with the increasing initial conc. of the mixture and in turn, increasing the incubation time to attain the complete degradation of the mixture.

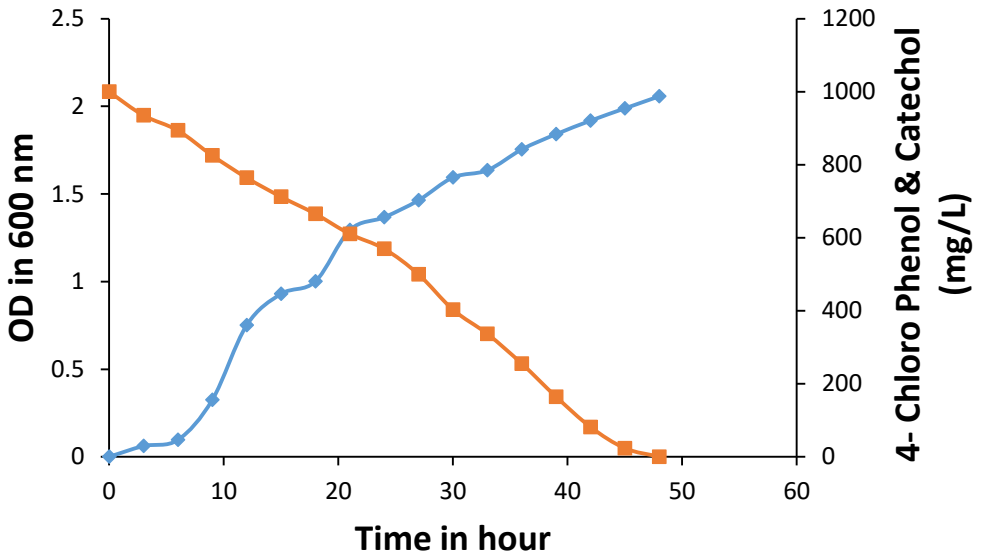


Figure VIIIB.2: Time duration of the microbial growth and degradation of the bi-solute mixture (4- Chloro Phenol & Catechol)

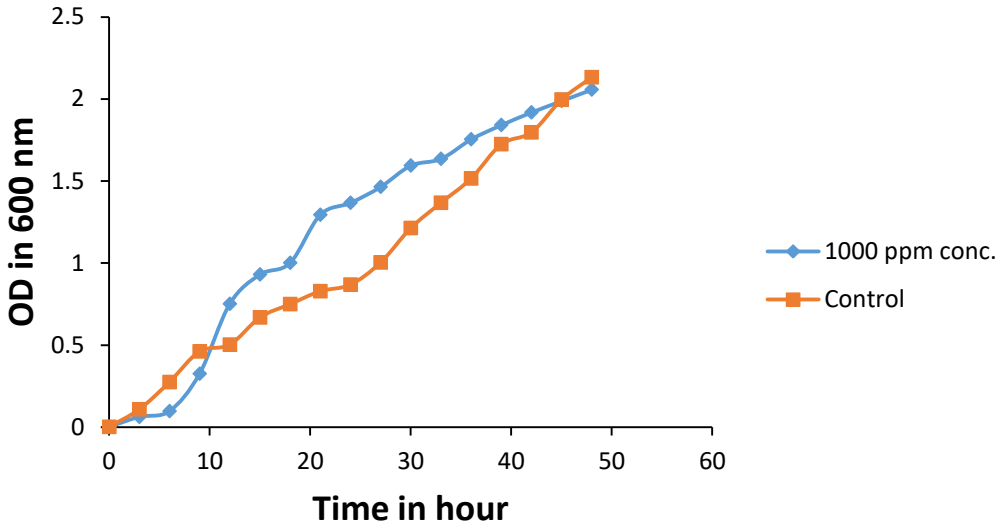
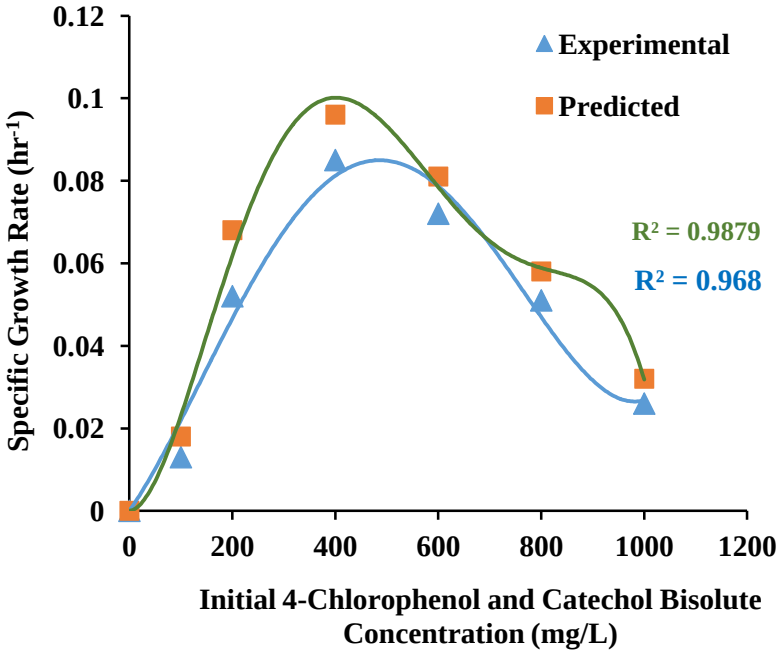


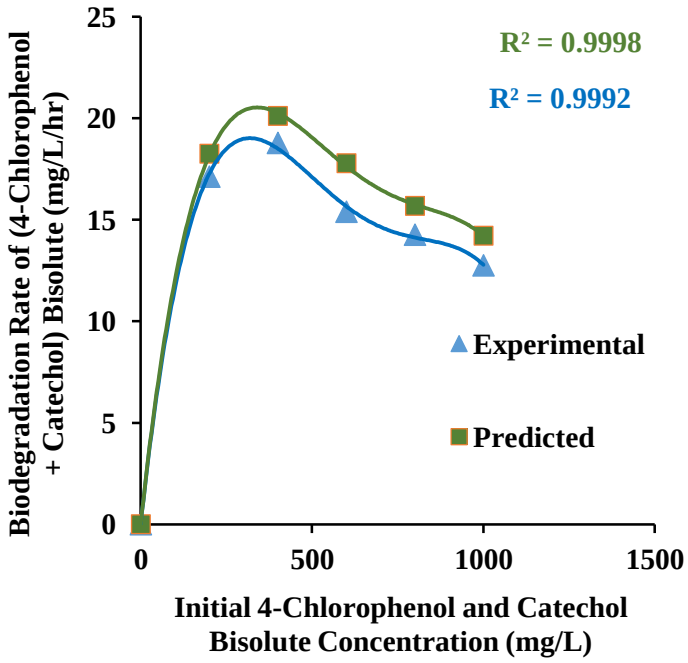
Figure VIIIB.3: The growth profile of the consortium in the highest conc. of the bi-solute mixture and control medium

VIIIB.2.2 Kinetics of degradation of the mixture and production of biomass:

The growth and degradation data might be explained by an integrated Haldane's substrate inhibition model with R^2 values more than 0.9, according to the progress curve analysis. The pattern of substrate inhibition was followed by the growth of the consortium and the biodegradation of the mixture of 4- Chloro Phenol & Catechol. The growth rate and degradation of the mixture was initially increased when the concentration of the mixture increased. After that, both the rates decreased subsequently with the increasing conc. of the mixture. The Haldane model described overall growth of the consortium (R^2 = 0.968) good at the S_0 range of 0 – 1000 mg/L. The kinetic parameters of the Haldane model have been displayed in Table VIII.1. Haldane model with R^2 values of 0.9992 for the consortium described the overall degradation rates very well.



(a)



(b)

Fig VIIIB.4: Experimental and predicted specific growth and degradation rate of the consortium during the biodegradation of bi- solute mixture

Sub chapter VIIIC

Determination of growth kinetics of Phenol & Catechol (as a mixture) degrading consortium and inhibitory effect of this bi- solute mixture

VIIIC.1 Materials and Methods:

VIIIC.1.1 Materials:

Same as mentioned in chapter VI, section VIC.1.1.

VIIIC.1.2 Experimental set up:

The five operating parameters in case of the microbial consortium were as follows: pH: 8.5, Temperature: 45^{o}C, Media volume: 500 mL, Time: 48 hours & Inoculums size: 12percent (3percent inoculums of each of the four bacterial strains). All of these operating parameters were obtained at the centre point of RSM while optimizing the parameters in case of this consortium (chapter VII). The consortium was cultured at seven different initial conc. of the bi- solute mixture i.e. Phenol & Catechol. Individual initial conc. of Phenol & Catechol was kept equal in each case (in 1:1 ratio). Initial conc. of the bi- solute mixture in the seven mediums were maintained as 50 mg/L (25 mg/L Phenol & 25 mg/L Catechol),100 mg/L, 200 mg/L, 400 mg/L, 600 mg/L, 800 mg/L and 1000 mg/L (500 mg/L Phenol & 500 mg/L Catechol) along with a control. The highest initial conc. of the mixture was maintained as 1000 mg/L as this conc. was obtained at the centre point of the RSM (chapter VII). Periodically after 3 hours, all the samples were analyzed for biomass and degradation percentage of the bi- solute mixture (Phenol & Catechol).

Rest of the portion is same as described in the chapter V, section VA.1.2.

VIIIC.1.3 Analytical Method:

The method of determination of residual bi- solute mixture in the media was same as mentioned in chapter VI, section VIC.1.3. Cell mass was analyzed at 600 nm wavelengths in spectrophotometer.

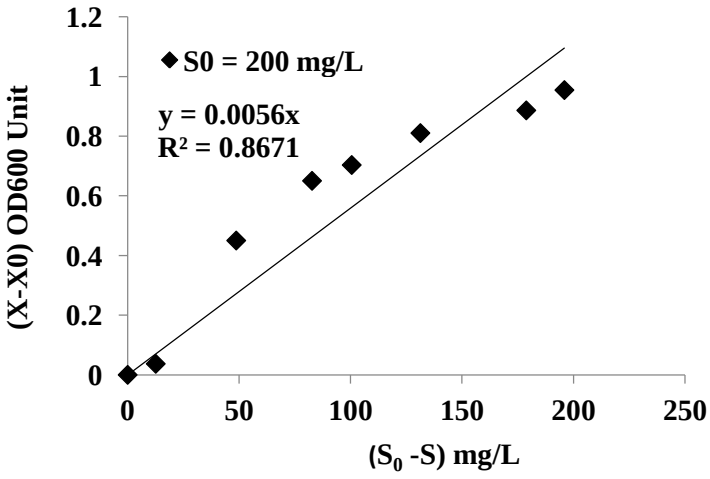
VIIIC.1.4 Calculations:

Same as described the chapter V, section VA.1.4.

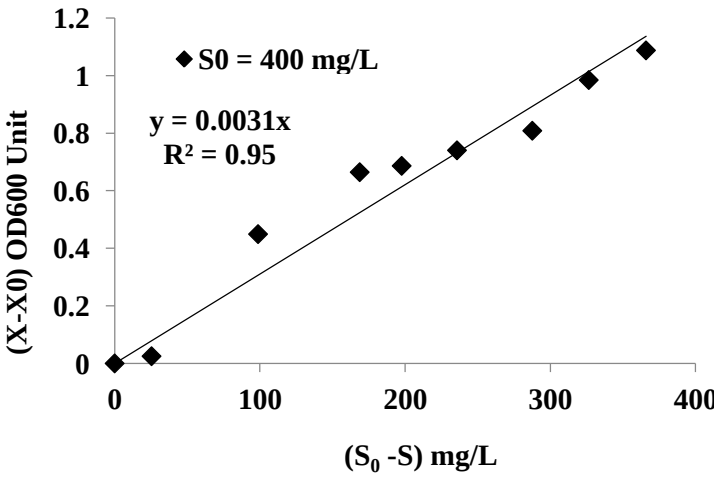
VIIIC.2 Results and Discussions:

VIIIC.2.1 Degradation of bi- solute mixture (Phenol & Catechol) & biomass production:

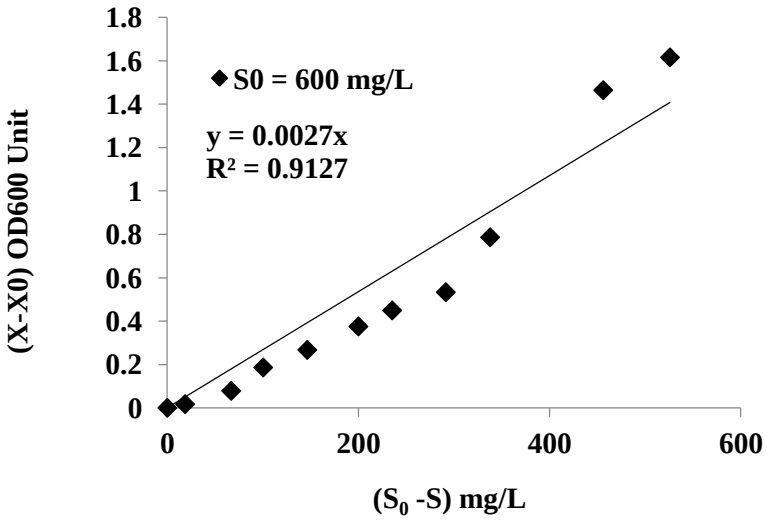
A bacterial consortium was utilized in this study. Incubation time varied with respect to the initial conc. of bi- solute mixture. 48 hours of residence time was taken by this consortium for complete degradation of 1000 mg/L of the bi- solute mixture. When the initial conc. of the mixture was low, it was removed within very short time span. The consortium completely removed 50 mg/L and 100 mg/L of the bi- solute mixture within just 3 hours. And thereby, 200 mg/L, 400 mg/L, 600 mg/L and 800 mg/L of the mixture was degraded by this consortium within 24 hours, 27 hours, 33 hours and 48 hours respectively. The effects of initial conc. of the mixture on the growth of the consortium with respect to that of the biomass yield have been shown in Figure: VIIIC.1.The yield of biomass per unit mass of consumed mixture i.e. Phenol & Catechol ($Y_{x/s}$) have been displayed in the Table VIIIC.1. Maximum yield of 0.056 OD units. L/mg was achieved by the consortium when initial conc. was 200 mg/L.



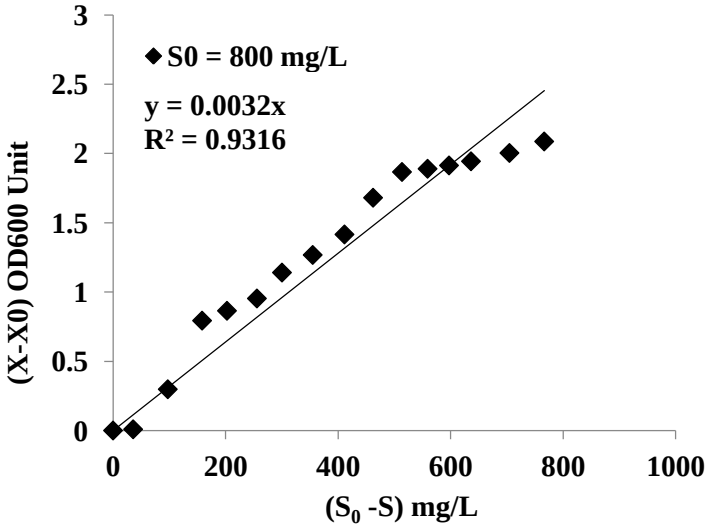
(a)



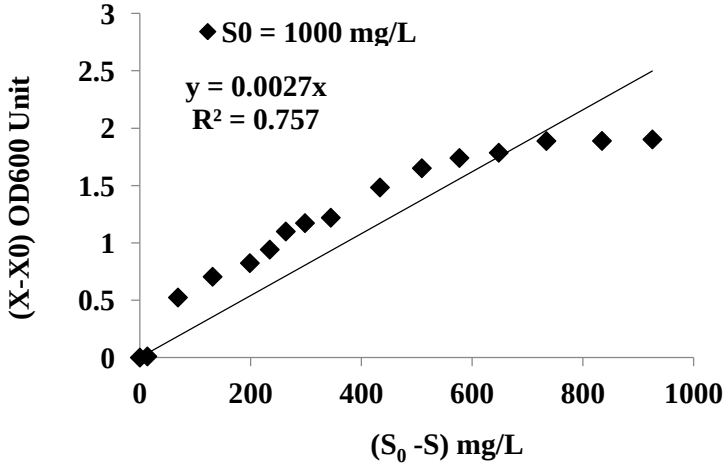
(b)



(c)



(d)



(e)

Figure VIIIC.1: Yield coefficients for the consortium; growth on the mixture of Phenol & Catechol

Table VIIIC.1: Yield coefficients for the for the growth of consortium on the bi- solute mixture (Phenol & Catechol) determined from the plots

S_0 (mg/L)	Yield coefficients ($Y_{x/s}$) [l_{600} units. L/mg]
200	0.0056
400	0.0031
600	0.0027
800	0.0032
1000	0.0027

Here in this study, yield coefficients were not deducted for 50 mg/L and 100 mg/L initial conc. of the mixture. The time duration of the microbial growth and degradation of the mixture for 800 mg/L and 1000 mg/L have been shown in Figure VIIIC.2. The growth profile of the consortium both in the highest conc. of the bi- solute mixture and control medium has been displayed in Figure VIIIC.3. The highest OD value and the incubation time to reach the stationary phase depend upon the initial conc. of the mixture. The highest optical density (λ_{600}) of 2.16 was attained by the consortium when the conc. of the mixture was 800 mg/L. The corresponding highest OD value of 2.087 was obtained by the consortium at the same initial conc. of the bi- solute mixture. The growths had exponential and stationary phase with increasing lag phase. Duration of the lag phase increased with the increasing initial conc. of the mixture and in turn, increasing the incubation time to attain the complete degradation of the mixture.

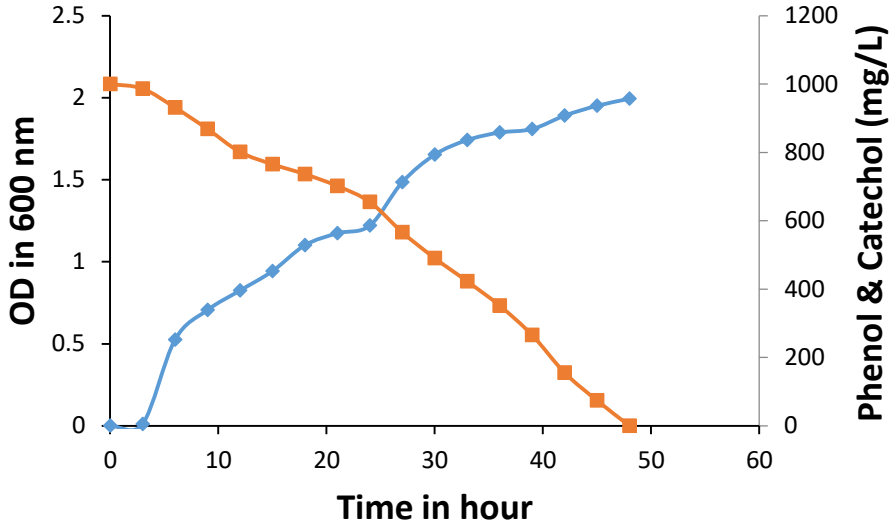


Figure VIIIC.2: Time duration of the microbial growth and degradation of the bi-solute mixture (Phenol & Catechol)

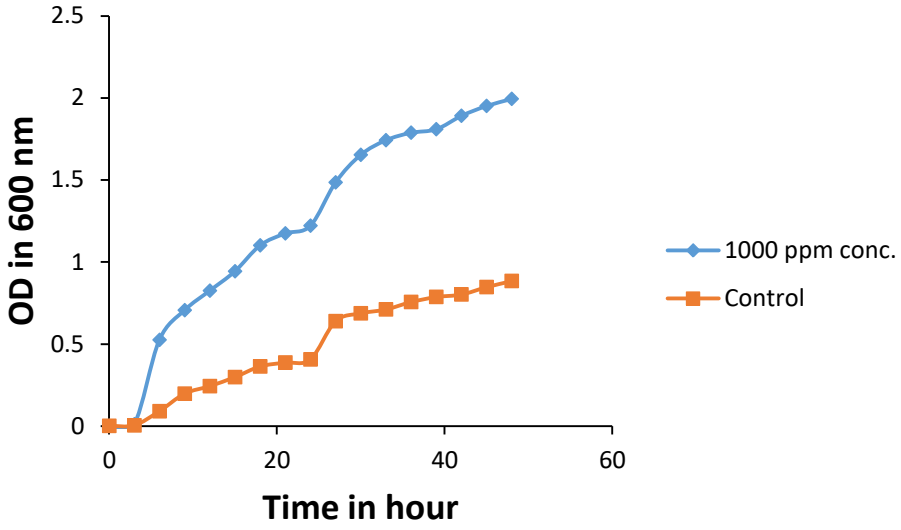
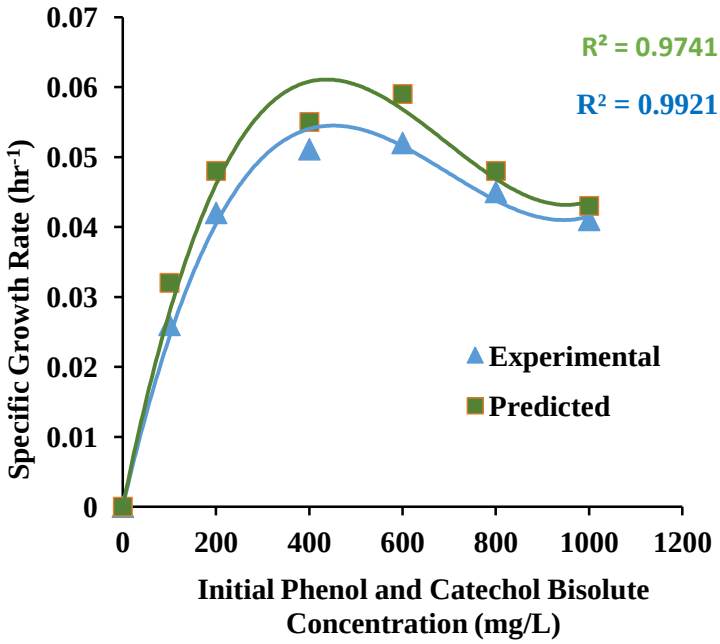


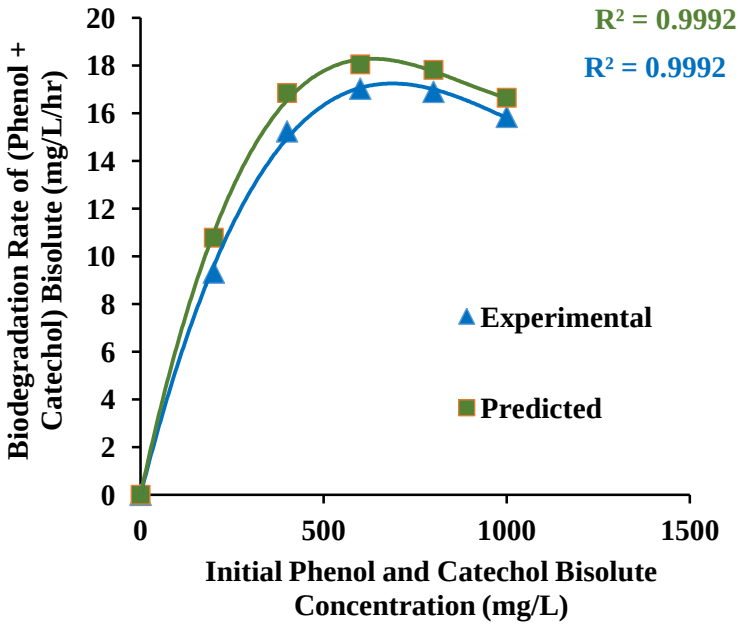
Figure VIIIC.3: The growth profile of the consortium in the highest conc. of the bi-solute mixture and control medium

VIIIC.2.2 Kinetics of degradation of the mixture and production of biomass:

The growth and degradation data might be explained by an integrated Haldane's substrate inhibition model with R^2 values more than 0.9, according to the progress curve analysis. The pattern of substrate inhibition was followed by the growth of the consortium and the biodegradation of the mixture of Phenol & Catechol. The growth rate and degradation of the mixture was initially increased when the concentration of the mixture increased. After that, both the rates decreased subsequently with the increasing conc. of the mixture. The Haldane model described overall growth of the consortium (R^2 = 0.9921) good at the S_0 range of 0 – 1000 mg/L. The kinetic parameters of the Haldane model have been displayed in Table VIII.1. Haldane model with R^2 values of 0.9992 for the consortium described the overall degradation rates very well.



(a)



(b)

Fig VIIIC.4: Experimental and predicted specific growth and degradation rate of the consortium during the biodegradation of bi- solute mixture

Sub chapter VI.I.D

Determination of growth kinetics of Phenol & 4- Chloro Phenol & Catechol (as a mixture) degrading consortium and inhibitory effect of this tri-solute mixture

VIIID.1 Materials and Methods:

VIIID.1.1 Materials:

Same as mentioned in chapter VI, section VID.1.1.

VIIID.1.2 Experimental set up:

The five operating parameters in case of the microbial consortium were as follows: pH: 8, Temperature: 45^{o}C, Media volume: 600 mL, Time: 48 hours & Inoculums size: 15percent (2.5percent inoculums of each of the six bacterial strains). All of these operating parameters were obtained at the centre point of RSM while optimizing the parameters in case of this consortium (chapter VII). The consortium was cultured at seven different initial conc. of the tri- solute mixture i.e. Phenol & 4- Chloro Phenol & Catechol. Individual initial conc. of Phenol & 4- Chloro Phenol & Catechol was kept equal in each case (in 1:1:1 ratio). Initial conc. of the tri- solute mixture in the seven mediums were maintained as 60 mg/L (20 mg/L Phenol, 20 mg/L 4- Chloro Phenol & 25 mg/L Catechol), 150 mg/L, 300 mg/L, 600 mg/L, 900 mg/L, 1200 mg/L and 1500 mg/L (500 mg/L Phenol, 500 mg/L 4- Chloro Phenol & 500 mg/L Catechol) along with a control. The highest initial conc. of the mixture was maintained as 1500 mg/L as this conc. was obtained at the centre point of the RSM (chapter VII). Periodically after 3 hours, all the samples were analyzed for biomass and degradation percentage of the tri- solute mixture (Phenol & 4- Chloro Phenol & Catechol).

Rest of the portion is same as described in the chapter V, section VA.1.2.

VIIID.1.3 Analytical Method:

The method of determination of residual tri- solute mixture in the media was same as mentioned in chapter VI, section VID.1.3. Cell mass was analyzed at 600 nm wavelengths in spectrophotometer.

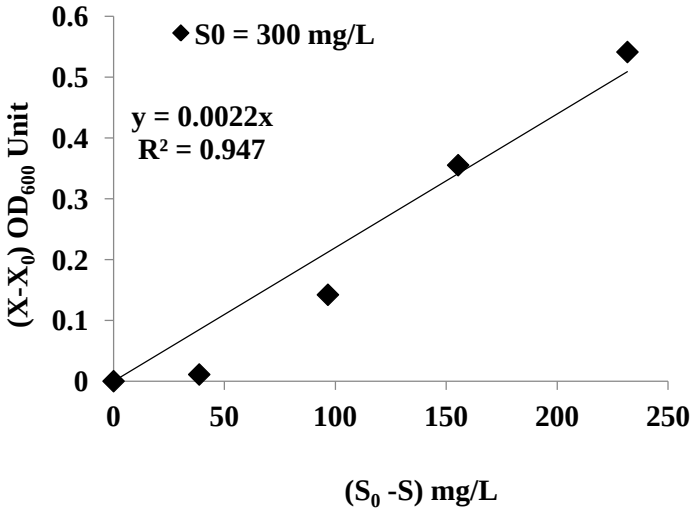
VIIID.1.4 Calculations:

Same as described the chapter V, section VA.1.4.

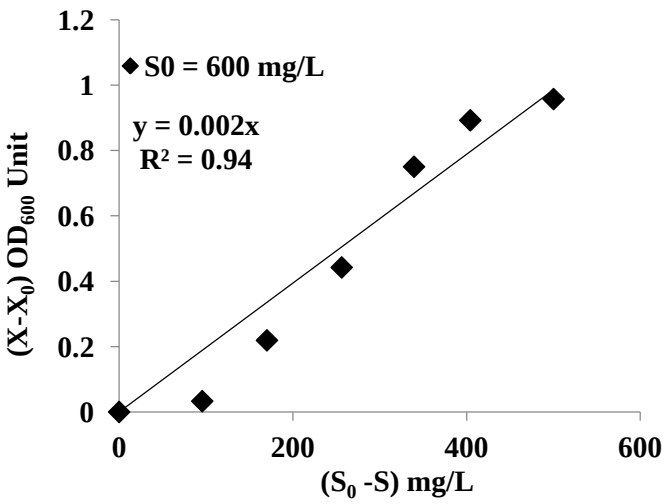
VIIID.2 Results and Discussions:

VIIID.2.1 Degradation of tri- solute mixture (Phenol & 4- Chloro Phenol & Catechol) & biomass production:

A bacterial consortium was utilized in this study. Incubation time varied with respect to the initial conc. of tri- solute mixture. 48 hours of residence time was taken by this consortium for complete degradation of 1500 mg/L of the tri- solute mixture. When the initial conc. of the mixture was low, it was removed within very short time span. The consortium completely removed 60 mg/L and 150 mg/L of the tri- solute mixture within just 3 hours and 9 hours respectively. And thereby, 300 mg/L, 600 mg/L, 900 mg/L and 1200 mg/L of the mixture was degraded by this consortium within 15 hours, 21 hours, 39 hours and 42 hours respectively. The effects of initial conc. of the mixture on the growth of the consortium with respect to that of the biomass yield have been shown in Figure: VIIID.1.The yield of biomass per unit mass of consumed mixture i.e. Phenol & 4- Chloro Phenol & Catechol ($Y_{x/s}$) have been displayed in the Table VIIID.1. Maximum yield of 0.0022 OD units. L/mg was achieved by the consortium when initial conc. was 300 mg/L.



(a)



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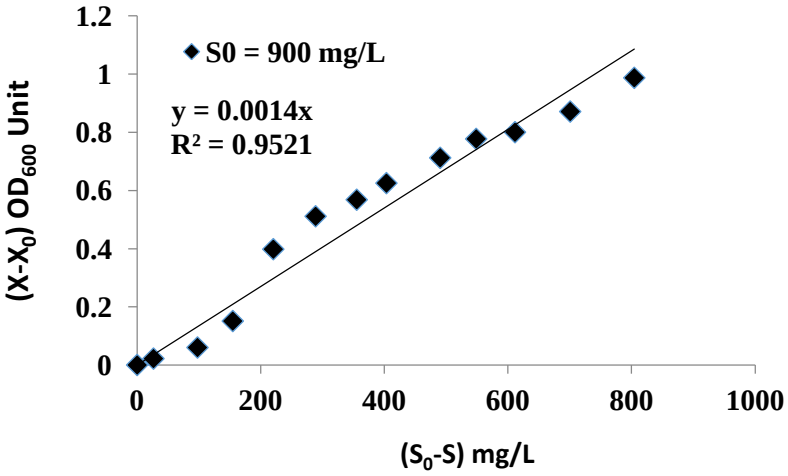

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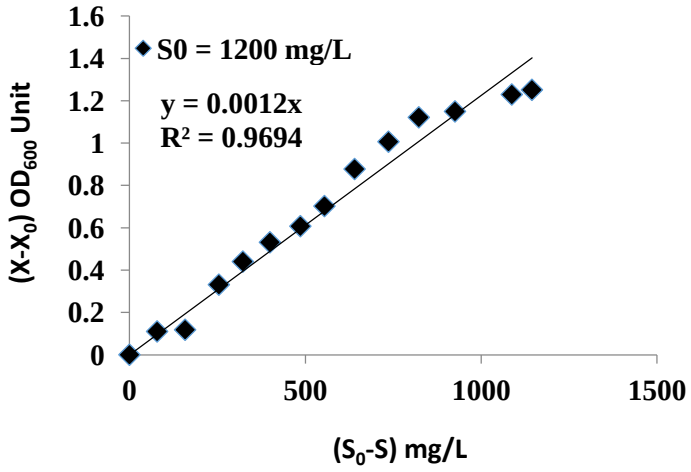
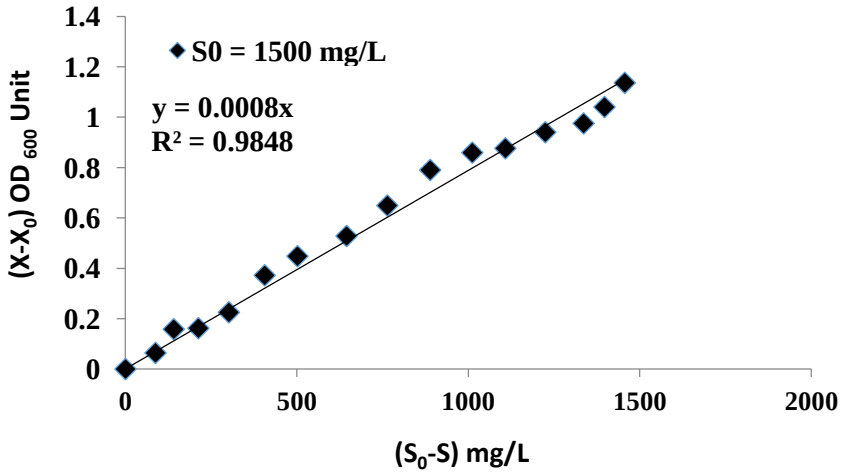


(d)



(e)

Figure VIIID.1: Yield coefficients for the consortium; growth on the mixture of Phenol & 4- Chloro Phenol & Catechol

Table VIIID.1: Yield coefficients for the for the growth of consortium on the tri- solute mixture determined from the plots

S_0 (mg/L)	Yield coefficients ($Y_{x/s}$) [l_{600} units. L/mg]
300	0.0022
600	0.002
900	0.0014
1200	0.0012
1500	0.0008

Here in this study, yield coefficients were not deducted for 60 mg/L and 150 mg/L initial conc. of the mixture. The time duration of the microbial growth and degradation of the mixture for 1200 mg/L and 1500 mg/L have been shown in Figure VIIID.2. The growth profile of the consortium both in the highest conc. of the tri- solute mixture and control medium, has been displayed in Figure VIIID.3. The highest OD value and the incubation time to reach the stationary phase depend upon the initial conc. of the mixture. The highest optical density (λ_{600}) of 1.404 was attained by the consortium when the conc. of the mixture was 1200 mg/L. The corresponding highest OD value of 1.315 was obtained by the consortium at the same initial conc. of the tri- solute mixture. The growths had exponential and stationary phase with increasing lag phase. Duration of the lag phase increased with the increasing initial conc. of the mixture and in turn, increasing the incubation time to attain the complete degradation of the mixture.

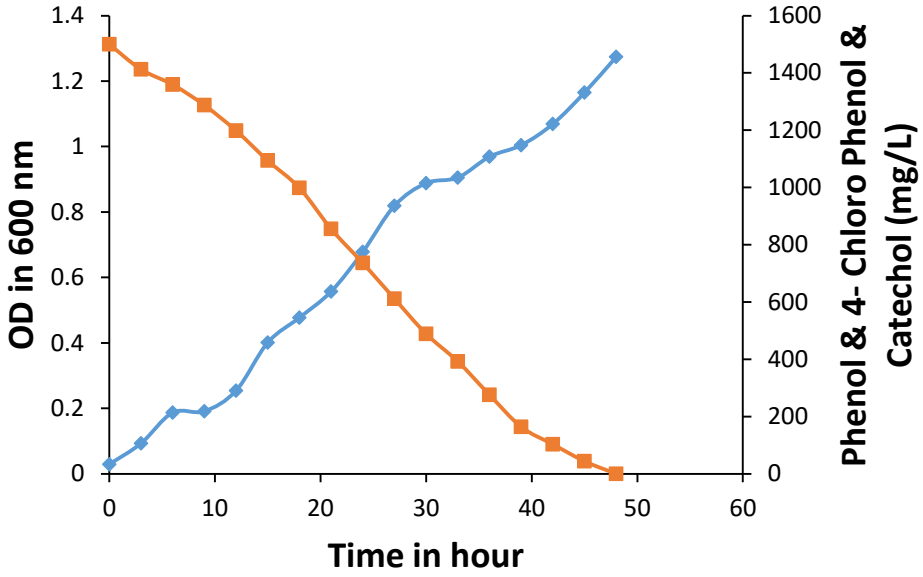


Figure VIIID.2: Time duration of the microbial growth and degradation of the tri- solute mixture (Phenol & 4- Chloro Phenol & Catechol)

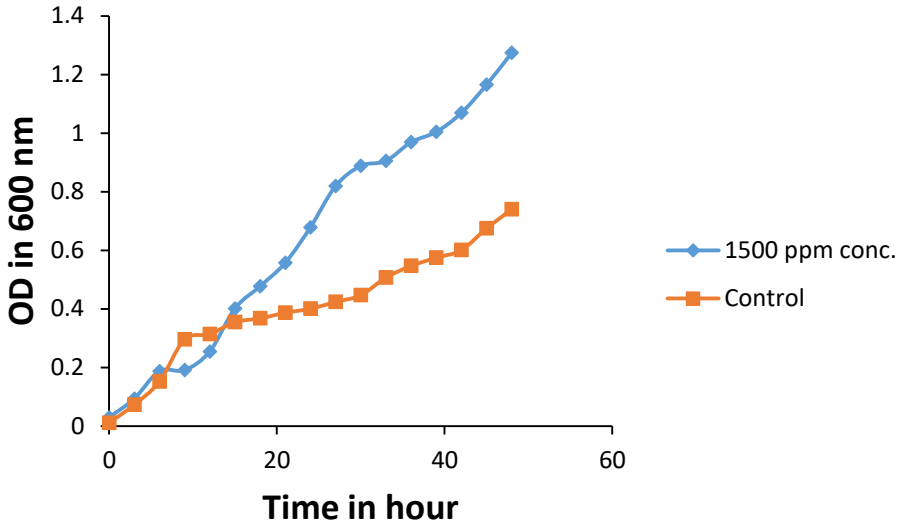
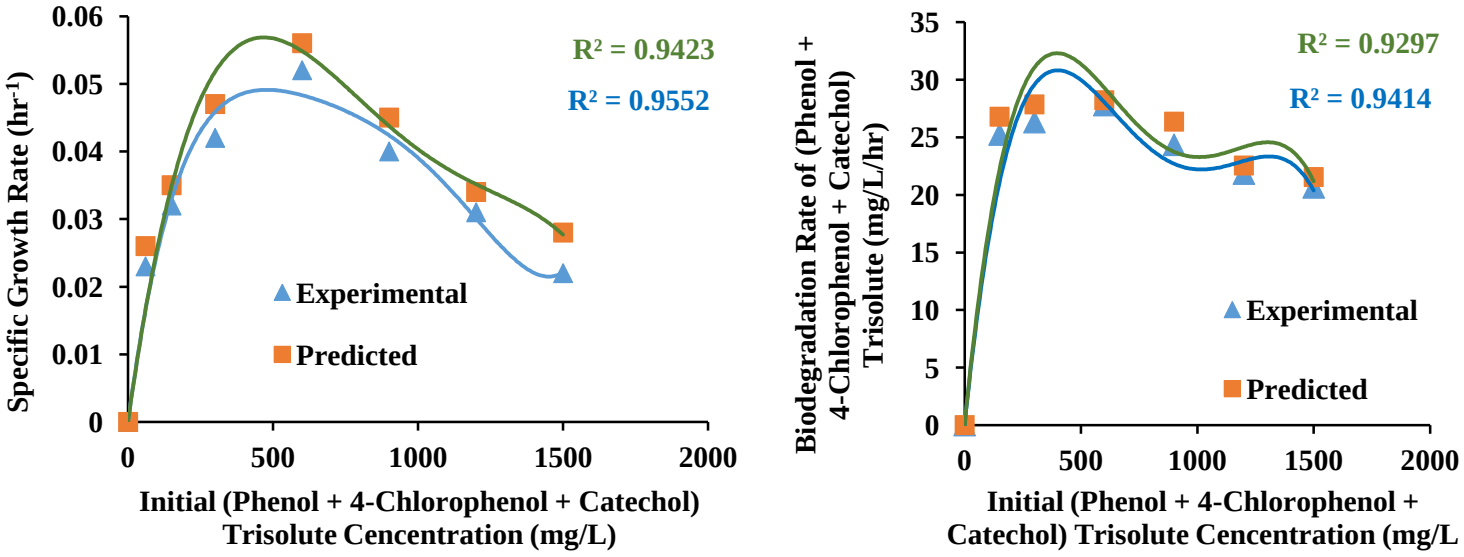


Figure VIIID.3: The growth profile of the consortium in the highest conc. of the tri-solute mixture and control medium

VIIID.2.2 Kinetics of degradation of the tri- solute mixture and production of biomass:

The growth and degradation data might be explained by an integrated Haldane's substrate inhibition model with R^2 values more than 0.9, according to the progress curve analysis. The pattern of substrate inhibition was followed by the growth of the consortium and the biodegradation of the mixture of Phenol & 4- Chloro Phenol & Catechol. The growth rate and degradation of the mixture was initially increased when the concentration of the mixture increased. After that, both the rates decreased subsequently with the increasing conc. of the mixture. The Haldane model described overall growth of the consortium (R^2 = 0.9552) good at the S_0 range of 0 – 1500 mg/L. The kinetic parameters of the Haldane model have been displayed in Table VIII.1. Haldane model with R^2 values of 0.9414 for the consortium described the overall degradation rates very well.



(a) (b)

Fig VIIID.4: Experimental and predicted specific growth and degradation rate of the consortium during the biodegradation of tri- solute mixture

Conclusions:

In this review, coordinated Haldane model with factors of most extreme specific growth rate, half immersion, biomass yield and inhibition constants were used to assess the progress of cell development during the degradation of the bi- solute and tri-solute mixtures of the phenol, 4- Chloro Phenol and Catechol, respectively at various initial concentrations. In such manner, non-linear least squares mechanized curve fitting system of Table Curve 2D was utilized to gauge the kinetic parameters. The values of μ_m, K_s, K_i & $Y_{x/s}$ are displayed in Table VIII.1. For the most part, the Haldane model gave great description of the experimental information where $R^2 > 0.9$. From the Table no VIII.1, it can be displayed that the values of the kinetic parameters, obtained in the current study, are almost similar to that of the previous studies. So, it can be concluded that the biomass production data were successfully fitted into the integrated Haldane model. Moreover, the obtained data implied that inhibitory effects of the bi- or tri- solute mixtures of the Phenol, 4- Chloro Phenol or Catechol arose at higher initial concentrations.

Table VIII.1: Haldane kinetic parameters for the biodegradation of Phenol and mixed Phenolic compounds in the batch reactor of respective consortiums

Bacterial strains/ consortiums	Initial conc. of Phenol (mg/L)	Initial conc. of bi- solute mixture (mg/L)	Initial conc. of tri- solute mixture (mg/L)	μ_m (h^{-1})	K_s (mg/L)	K_i (mg/L)	Q_s (mg/l/h)	References
Pseudomonas sp. DAF1	0 - 1000	Nil	Nil	0.799	25.276	84.591	74.075	Nweke and Okpokwasili., 2014
Brevibacillus formosus *Pseudomonas otitidis* *Bacillus timonensis* *Bacillus cereus*	Nil	0 – 1000 (Phenol & 4- Chloro Phenol)	Nil	0.034	34.25	146.36	26.11	Current study
Bacillus timonensis *Bacillus cereus* *Bacillus Pseudomycoides* *Bacillus Paramycoides*	Nil	0 – 1000 (4- Chloro Phenol & Catechol)	Nil	0.098	35.62	136.52	22.13	
Brevibacillus formosus *Pseudomonas otitidis* *Bacillus Pseudomycoides* *Bacillus Paramycoides*	Nil	0 – 1000 (Phenol & Catechol)	Nil	0.061	38.65	138.24	18.05	
Brevibacillus formosus *Pseudomonas otitidis* *Bacillus timonensis* *Bacillus cereus* *Bacillus Pseudomycoides* *Bacillus Paramycoides*	Nil	Nil	0 – 1500 (Phenol & 4- Chloro Phenol & Catechol	0.057	40.25	156.24	32.57	

Chapter IX

Fabrication of a bioreactor for the degradation of tri- solute mixture (Phenol & 4-Chloro Phenol & Catechol)

IX.1 Introduction:

The prime objective of the study was to design and fabricate a bioreactor for the degradation of Phenolic mixtures from the pharmaceutical waste water. Normally, bioreactor refers to a kind of reactor where biological processes are performed in pilot scale. As a large amount of water, containing Phenol & Phenolic derivatives, discharged from the pharmaceutical industries, bioreactor is required here to treat this large amount of water. A number of Phenolic compounds may be occurred in the pharmaceutical waste water. Biodegradation of those Phenolic compounds was to be carried out in bioreactor.

In the current study, Phenol & 4- Chloro Phenol & Catechol, these three compounds were considered to be treated in bioreactor. Six favourable conditions which were optimized in earlier chapters, was to be deployed in the bioreactor. Those were: Temperature, pH, Incubation time (or residence time), Inoculums size, Media volume and Initial concentration of the Phenolic substances.

IX.2 Materials and Methods:

IX.2.1 Materials:

Six different strains were utilized to make a microbial consortium. Those strains were: *Brevibacillus formosus* strain NRRL NRS- 863, *Pseudomonas otitidis* strain MCC10330, *Bacillus timonensis* strain 10403023, *Bacillus cereus* strain K1, *Bacillus pseudomycoides* strain NBRC 101232 and *Bacillus paramycoides* strain MCCC 1A04098. Isolation and identification process of these strains has been described in chapter II.

Preparation of pharmaceutical waste water was performed by mixing various amounts of Phenol & 4- Chloro Phenol & Catechol which were prepared in laboratory. Conc. of the stock solutions of these compounds was maintained as 10 g/L.

Mineral Salt Medium (MSM) was utilized for the culture media of the consortium.

1L and 2L Erlenmeyer flasks were used as reactor.

IX.2.2 Experimental set up:

In case of the percent of inoculums, total percent of inoculums of all the six bacterial strains was taken into account where percent of each of the six strains was maintained equally (1:1:1:1:1:1 ratio). Where outputs were not satisfactory, ratio of some strains were increased and some decreased as per requirement keeping the total percentage as constant. In case of initial conc. of the tri- solute mixture to be removed i.e. Phenol & 4- Chloro Phenol & Catechol, total initial conc. (mg/L) of the three compounds was used where individual initial conc. of the compounds was kept unequal (i.e. either 1:2:1 ratio or 1:1:2 ratio or 2:1:1 ratio and so on) but keeping the total conc. constant.

Rest of the portion is same as mentioned in the chapter III, section IIIA.1.2.

IX.2.3 Analytical Method:

Same as described in chapter VI, section VID.1.3

IX.2.4 Favourable parameters for the reactor:

Three phases were performed there in this study. In the very 1st phase, all the parameters were maintained as the centre point of RSM, obtained during the study of tri- solute mixture (chapter VII, subchapter VIID). Six (6) parameters maintained as: pH 8; Temperature: 45^{o}C; Incubation time: 48 hours; Media volume: 600 mL; and Inoculums size of the consortium: 15percent. Also, the total initial conc. of the tri- solute mixture maintained as 1500 mg/L but here, individual concentrations of the three Phenolic substances were not equal like that of the previous studies. Individual concentrations of those compounds were varied in a number of ways. Total sixty three (63) experiments were performed in this phase (Table IX.1, IX.2, IX.3 & IX.4).

In the next phase, some of the experiments, which did not obtain fruitful outcomes, were repeated. Here, all the five parameters were same as the previous experiments except the inoculums size. Individual inoculums size of the six microbial strains were increased and

decreased here as per requirements maintaining the total inoculums size of the consortium constant i.e. 15percent. Ten (10) experiments were repeated in this phase (Table IX.5).

After successful completion of the above two phases, some selected experiments were performed finally where some optimized parameters were same while inoculums size, initial conc. of the mixture and media volume were changed. 2L media volume was utilized here for the culture of the consortium instead of 600 mL and, initial conc. of the tri- solute mixture maintained same and increased also to verify the reliability of the bioreactor. Inoculums size was also increased up to 18percent when the total initial conc. of the mixture was increased up to 1800 & 2100 mg/L. Seven (7) tests were performed in this phase. In the first experiment, 15% consortium (2.5% of each of the six microbial strains) was deployed to deal with the 1500 mg/L initial conc. of the mixture. In the second experiment same initial conc. was maintained while inoculums size of the consortium was increased up to 18% (3% of each of the strains). After that, in all the experiments, 18% inoculums were maintained even after increasing the initial conc. up to 2100 mg/L. At this conc. the individual initial concentrations of the three compounds were varied also (Table IX.6). Degradation percentages at this phase were checked after 24 hours, 48 hours & 72 hours respectively instead of checking only after 48 hours.

IX.2.5 HPLC analysis:

HPLC analysis was performed in order to detect the individual degradation of the three compounds i.e. Phenol & 4- Chloro Phenol & Catechol. It was done only in case of one sample, experimented at pilot scale (i.e. in 2L volume) at 48 hours, where initial conc. of the tri- solute mixture was 2100 mg/L (i.e. 1200 mg/L Phenol & 500 mg/L 4- Chloro Phenol & 400 mg/L Catechol) and inoculums size of the consortium was 15% as a whole (2.5% inoculums of each of the six strains). The chromatogram image (IX.8) depicted satisfactory degradation of the three Phenolic compounds individually.

Rest of the portion is same as described in the chapter VII, section VIIA.1.5.

IX.3 Results and Discussions:

IX.3.1 Experiments in bioreactor:

In this phase, the experiments were subdivided into four stages. Those were: increase in Phenol conc. and subsequent decrease in the conc. of 4- Chloro Phenol & Catechol (Table IX.1); increase in the conc. of 4- Chloro phenol and subsequent decrease in the conc. of Phenol & Catechol (Table IX.2); increase in the conc. of Catechol and subsequent decrease in the conc. of Phenol & 4- Chloro Phenol (Table IX.3); initial conc. of all the three compounds varied together miscellaneously (Table IX.4). In all the four tables, the highlighted sets of the mixtures represented those combinations of initial concentrations where satisfactory outcomes were not achieved.

Table IX.1: Effects on degradation percentage when initial conc. of Phenol was increased

SI No.	Phenol (mg/L)	4-Chloro phenol (mg/L)	Catechol (mg/L)	Percentage of degradation
1	600	450	450	87.17
2	**700**	**400**	**400**	**82.91**
3	750	375	375	86.51
4	800	350	350	89.73
5	900	300	300	93.47
6	1000	250	250	92.73
7	1100	200	200	91.13
8	1200	150	150	91.11

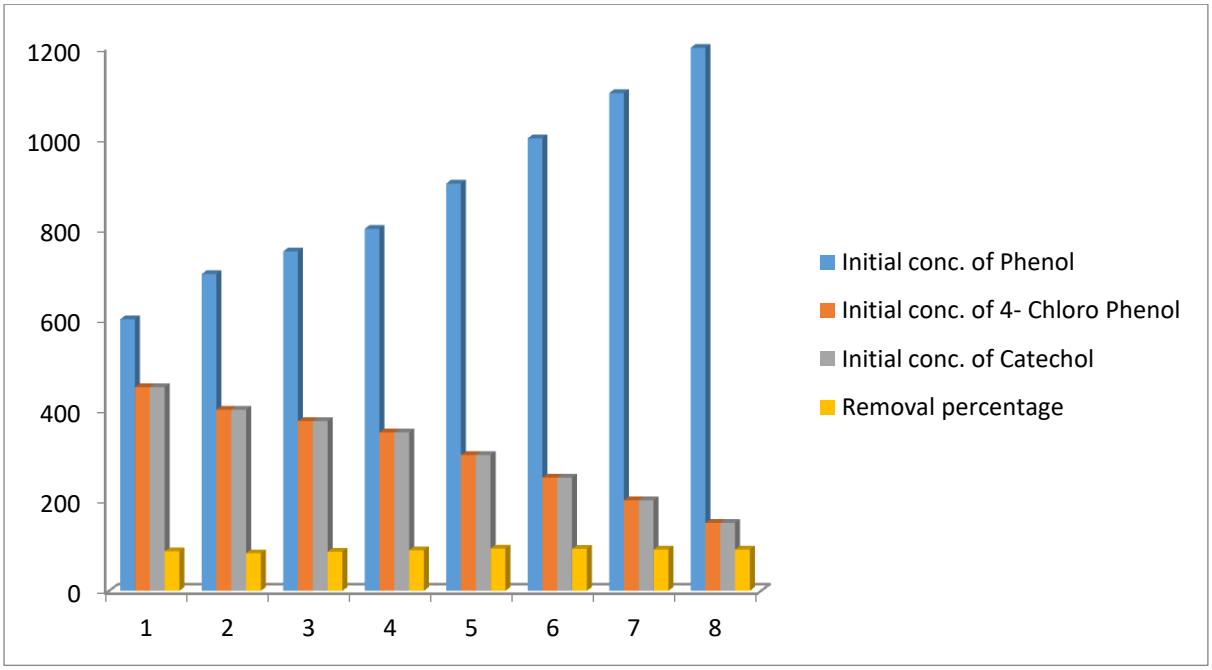


Figure IX.1: Bar diagram regarding the effects on degradation percentage when initial conc. of Phenol was increased

Table IX.2: Effects on degradation percentage when initial conc. of 4- Chloro Phenol was increased

SI No.	Phenol (mg/L)	4-chloro phenol (mg/L)	Catechol (mg/L)	Percentage of degradation
9	450	600	450	93.07
10	400	700	400	90.57
11	375	750	375	89.81
12	350	800	350	94.11
13	300	900	300	93.71
14	250	1000	250	95.23
15	200	1100	200	99.92
16	150	1200	150	96.73

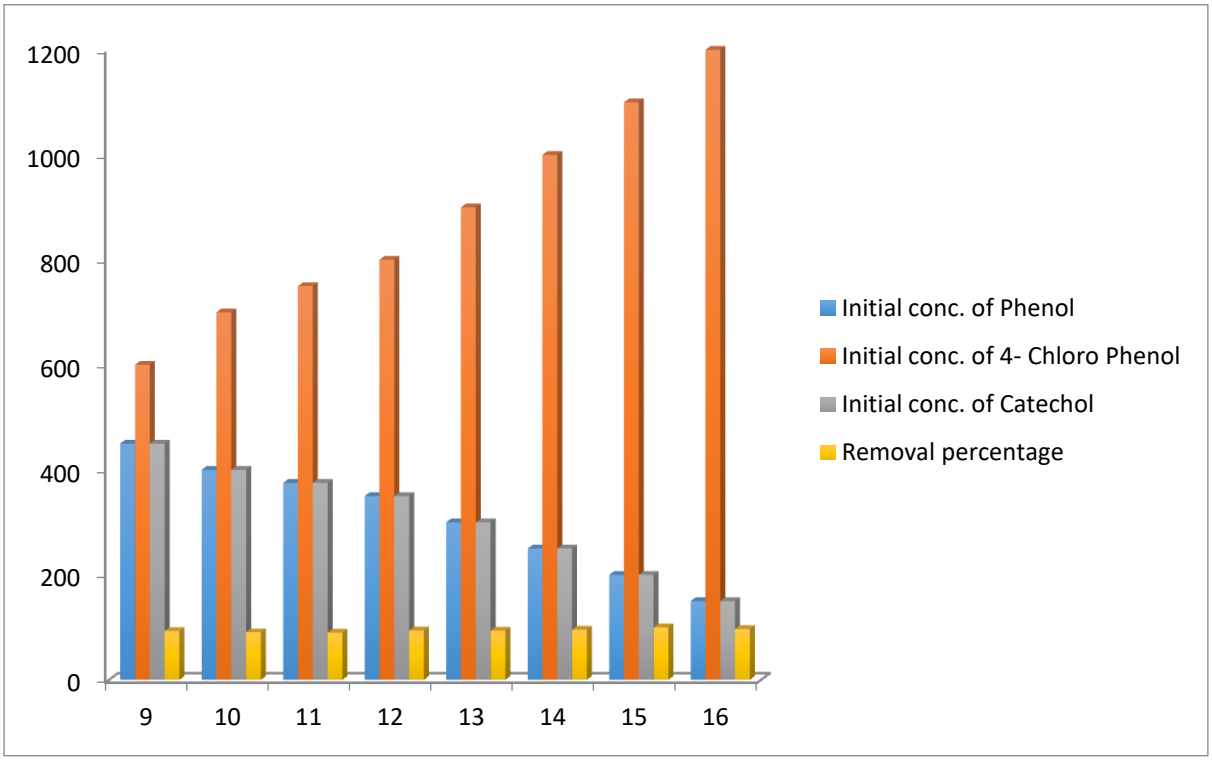


Figure IX.2: Bar diagram regarding the effects on degradation percentage when initial conc. of 4- Chloro Phenol was increased

Table IX.3: Effects on degradation percentage when initial conc. of Catechol was increased

SI No.	Phenol (mg/L)	4-chloro phenol (mg/L)	Catechol (mg/L)	Percentage of degradation
17	450	450	600	95.77
18	**400**	**400**	**700**	**75.63**
19	375	375	750	87.07
20	350	350	800	88.92
21	300	300	900	88.63
22	250	250	1000	89.71
23	200	200	1100	88.92
24	**150**	**150**	**1200**	**80.57**

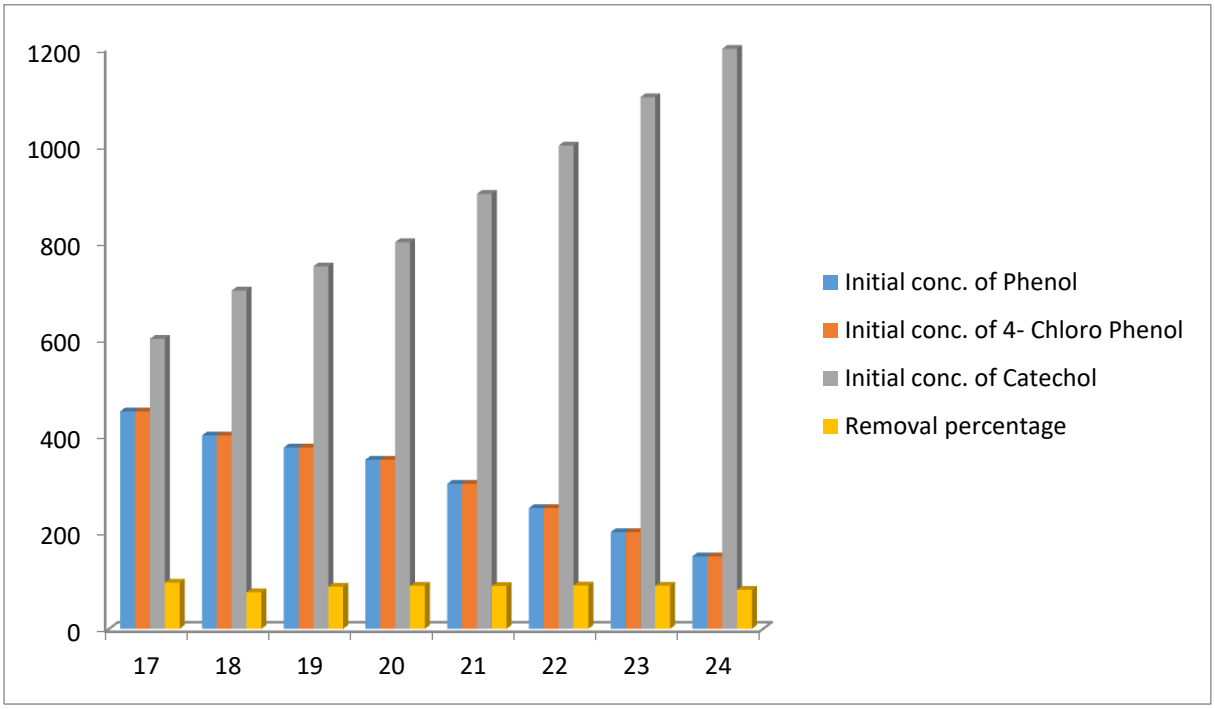


Figure IX.3: Bar diagram regarding the effects on degradation percentage when initial conc. of Catechol was increased

Table IX.4: Effects on degradation percentage when initial conc. of all three compounds were varied together

SI No.	Phenol (mg/L)	4-chloro phenol (mg/L)	Catechol (mg/L)	Percentage of degradation
25	600	600	300	98.63
26	**600**	**300**	**600**	**85.83**
27	**300**	**600**	**600**	**84.33**
28	700	600	200	96.77
29	600	200	700	94.77
30	200	700	600	93.97
31	600	500	400	96.27
32	**600**	**400**	**500**	**84.83**
33	500	600	400	89.32
34	400	600	500	99.27

35	500	400	600	89.07
36	400	500	600	88.63
37	525	525	450	93.63
38	450	525	525	89.63
39	525	450	525	95.41
40	700	500	300	95.53
41	700	300	500	89.77
42	500	700	300	95.21
43	500	300	700	87.23
44	300	500	700	93.13
45	300	700	500	96.63
46	800	400	300	99.26
47	**800**	**300**	**400**	**82.57**
48	400	800	300	90.94
49	**400**	**300**	**800**	**81.27**
50	**300**	**400**	**800**	**81.21**
51	**300**	**800**	**400**	**81.13**
52	900	400	200	93.87
53	900	200	400	96.81
54	400	900	200	98.79
55	400	200	900	92.97
56	200	900	400	98.95
57	200	400	900	91.36
58	1000	300	200	99.93
59	1000	200	300	93.86
60	200	1000	300	99.99
61	200	300	1000	94.33
62	300	1000	200	99.97
63	300	200	1000	93.88

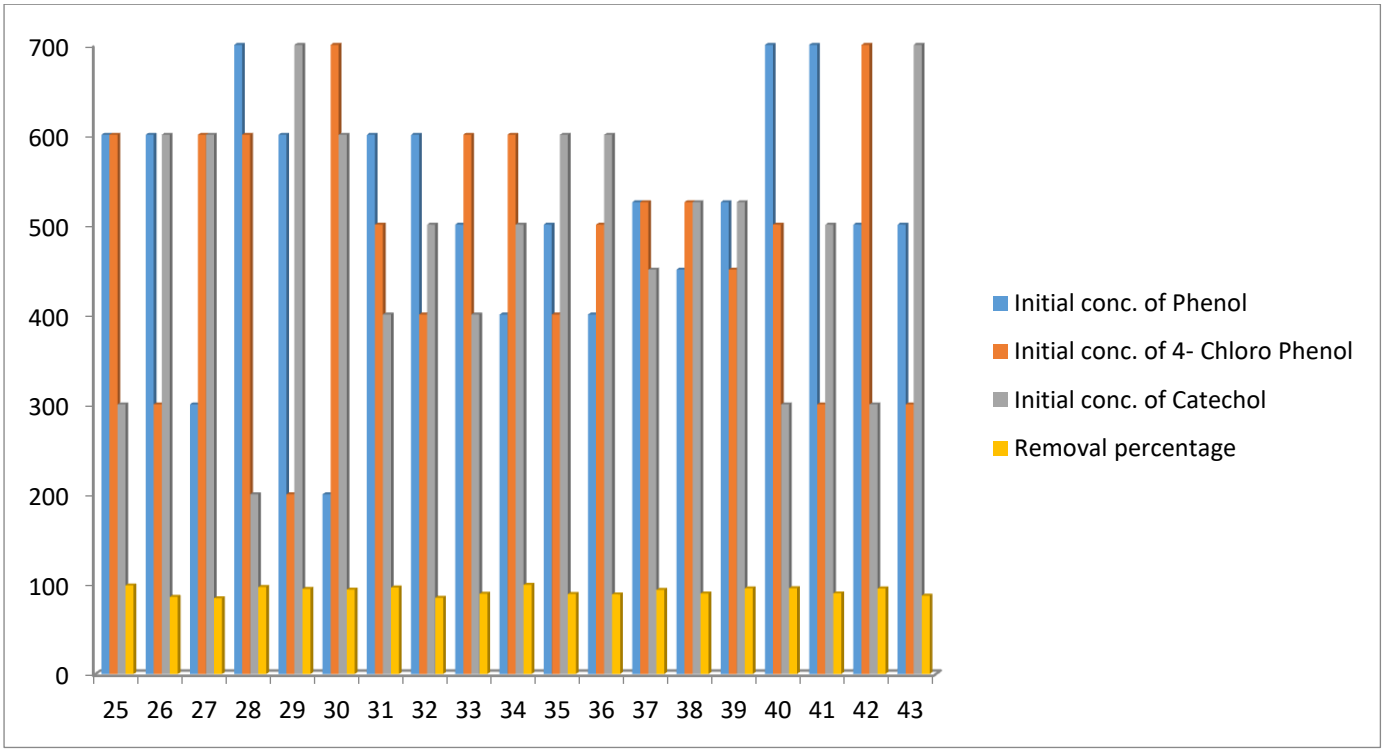


Figure IX.4: Bar diagram regarding the effects on degradation percentage when initial conc. of all three compounds were varied together (from SI no 25 to 43)

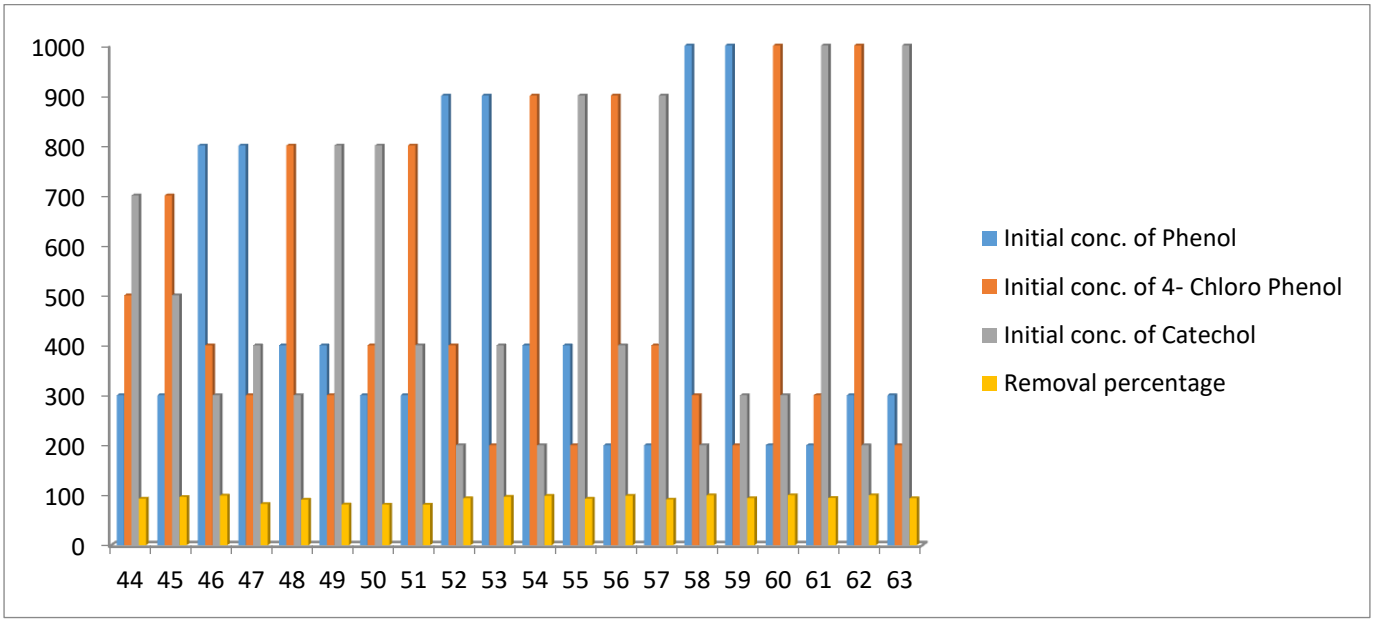


Figure IX.5: Bar diagram regarding the effects on degradation percentage when initial conc. of all three compounds were varied together (from SI no 44 to 63)

From the Table no IX.1, only the test at SI No. 2 found to be dissatisfactory. Thus, from the Table no IX.3 & IX.4, respectively two and seven experiments were not found to be fruitful. In the second stage of the study, these ten (10) experiments were repeated where only the individual inoculums sizes of the strains were adjusted keeping the total inoculums size constant. Furthermore, rest of the five parameters were also unchanged. In this phase, satisfactory outputs were obtained from those experiments (Table IX.5).

Table IX.5: Repetition of the experiments where individual inoculums sizes were adjusted

	Inoculums size of the six microbial strains individually						
SI No.	**Percent of inoculums of *Brevibacillus formosus***	**Percent of inoculums of *Pseudomonas otitidis***	**Percent of inoculums of *Bacillus timonensis***	**Percent of inoculums of *Bacillus cereus***	**Percent of inoculums of *Bacillus pseudomycoides***	**Percent of inoculums of *Bacillus paramycoides***	**Percent of degradation**
2	4	4	1.75	1.75	1.75	1.75	98.86
18	2	2	2	2	3.5	3.5	89.57
24	2	2	2	2	3.5	3.5	76.53
26	3	3	1.5	1.5	3	3	85.33
27	1.5	1.5	3	3	3	3	85.77
32	3.5	3.5	1.5	1.5	2.5	2.5	96.51
47	3.5	3.5	1.5	1.5	2.5	2.5	94.11
49	1.5	1.5	2	2	4	4	88.17
50	1.5	1.5	2.5	2.5	3.5	3.5	80.47
51	1.5	1.5	3.5	3.5	2.5	2.5	88.13

In the above table (Table IX.5), the inoculums sizes of the six microbial strains utilized to make the consortium in case of the repetition of some experiments, have been displayed. Moreover, the SI Nos. of the experiments, which were not satisfactory, have been mentioned

in the table. When some experiments were found not to be satisfactory, inoculums sizes were adjusted for each strain. It can easily be deduced that individual sizes of the inoculums of the six bacterial strains have been adjusted in a way that total inoculums size i.e. 15percent would remain constant (Table IX.5). In the earlier stages, inoculums size of each strain was 2.5percent. In case of the repetition of some experiments, the inoculums sizes were adjusted according to the initial conc. of the Phenolic substances. Where initial conc. of Phenol was higher than that of the 4- Chloro Phenol & Catechol, inoculums size of the two Phenol degrading strains (*Brevibacillus formosus* & *Pseudomonas otitidis*) were increased and rest were decreased keeping the total inoculums size constant. Similar adjustment was performed when the initial conc. of 4- Chloro Phenol or Catechol was increased. Thus, each experiment was found to be successful after adjusting the inoculums size as in each experiment, degradation efficacy was increased than the former (table IX.5).

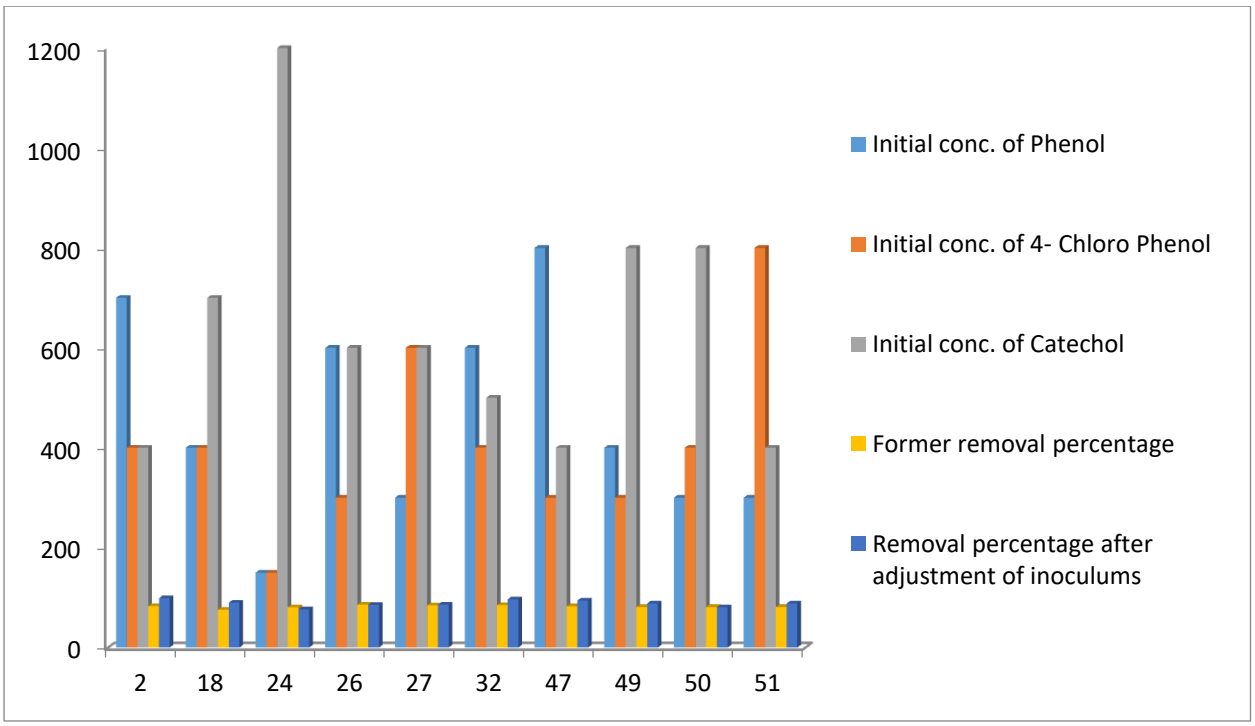


Figure IX.6: Bar diagram regarding the experiments which were again performed after adjusting the individual sizes of the inoculums of six microbial strains; both the earlier and later degradation parentages have been depicted

In the final stage, some experiments were selected and further performed in a large volume of media i.e. 2L to verify the efficacy of the bioreactor in pilot scale. In this phase, temperature and pH were unchanged. Three time periods were considered here (24 hrs, 48 hrs & 72 hrs) instead of only 48 hrs. Total seven (7) experiments were performed in this phase. Out of these, in four experiments, the initial conc. of the Phenolic compounds was increased gradually keeping the ratio 1:1:1. And in case of rest of the three experiments, the initial conc. of the compounds was varied. 15% inoculums (2.5% of each of the six microbial strains) of the consortium was used where initial conc. of the compounds was 1500 mg/L. After that, in each experiment, 18% inoculums (3% of each of the six microbial strains) were used as the initial conc. of the mixture was further increased up to 1800 mg/L and even up to 2100 mg/L. In all cases, overall satisfactory degradation was achieved (Table IX.6).

Table IX.6: Study in a large volume

SI No.	Initial conc. of Phenol	Initial conc. of 4- Chloro phenol	Initial conc. of Catechol	Overall inoculums size of the consortium	percent of degradation		
					24 hr	48 hr	72 hr
1	500	500	500	15	77.53	93.67	91.51
2	500	500	500	18	81.71	93.87	92.77
3	600	600	600	18	81.22	93.47	90.78
4	700	700	700	18	79.98	94.91	94.74
5	1000	600	500	18	75.54	91.22	89.66
6	1100	500	500	18	75.76	96.92	91.29
7	1200	500	400	18	74.88	95.67	90.47

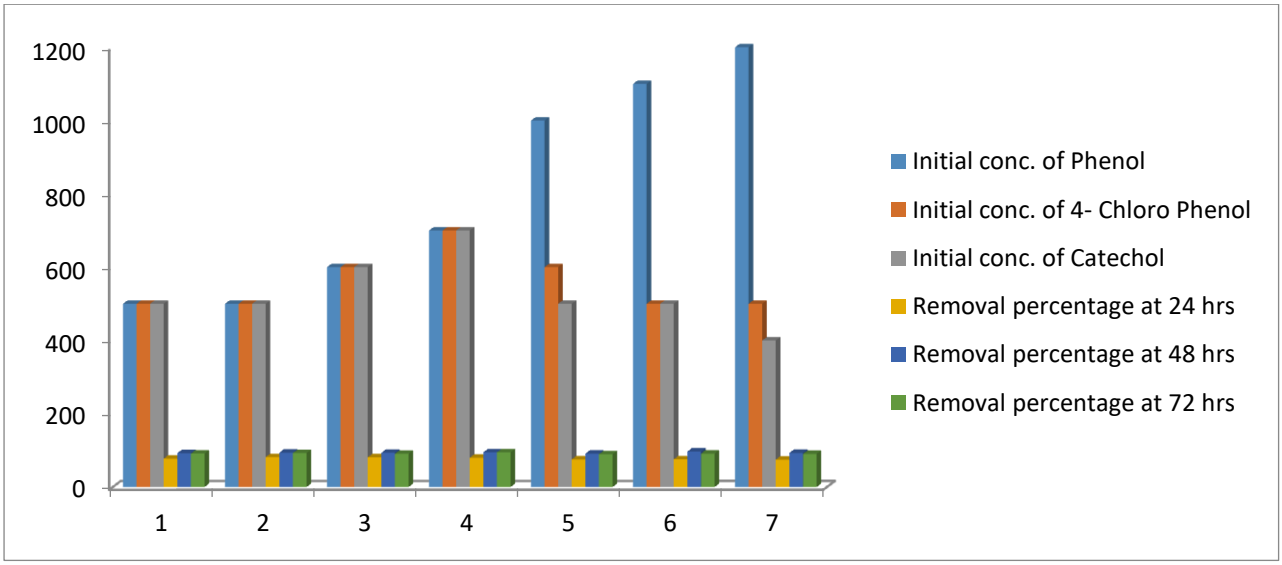


Figure IX.7: Bar diagram regarding the experiments at 2L media volume

From the above data, it can be deducted that 48 hours is the perfect incubation time for the consortium regarding this bioreactor as maximum degradation was gained at that point. Beyond that, degradation efficacy slightly decreased.

IX.3.2 HPLC analysis:

From the standard chromatogram of known conc. of Phenol, 4- Chloro Phenol and Catechol, it can be deduced that the peak of 6.619 AU represents the residual conc. of Catechol, 6.787 AU represents the residual conc. of Phenol and 7.069 AU represents 4- Chloro Phenol respectively (Figure IX.8). Calculating the area of the chromatograms (both the standard and the sample), it was found that individually 99.72% of Phenol, 99.77% of 4- Chloro Phenol and 98.58% of Catechol were removed from the tri- solute mixture, while cultured at a high volume of media (2L), 45^{o}C temperature, 8 pH for 48 hours and where initial conc. of tri solute mixture was 1500 mg/L. The HPLC analysis almost supported the experimental result, obtained at Table IX.6, SI No. 1.

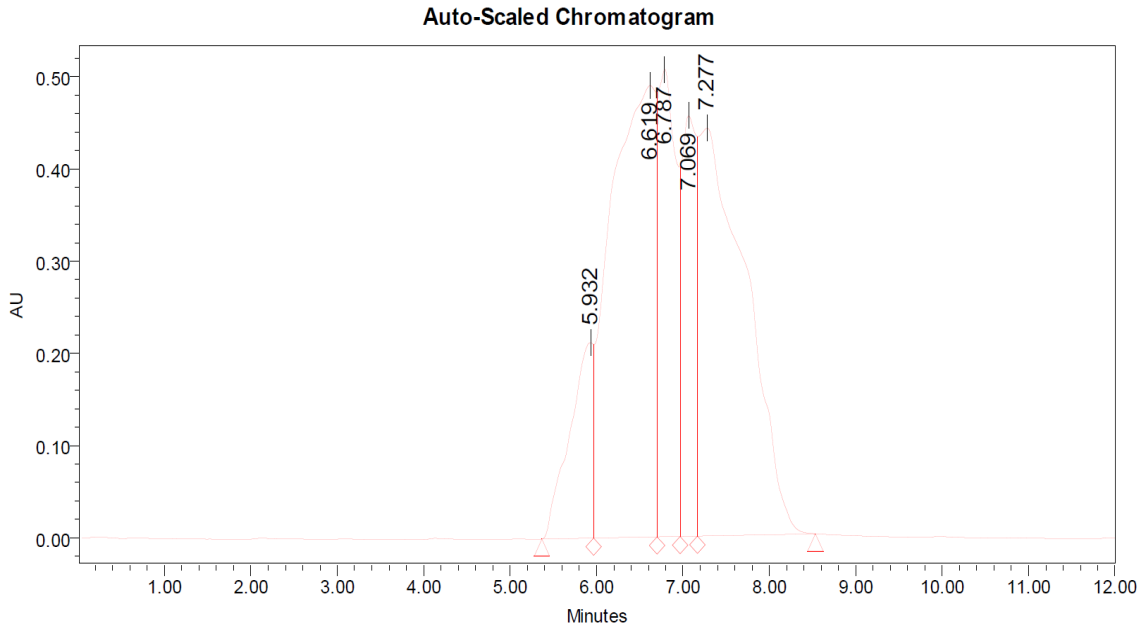


Figure IX.8: Chromatogram for the of the sample regarding the Table IX.6, SI no. 7

IX.4 Conclusions:

The ultimate target of this study was to design and fabricate a bioreactor, to be used in the treatment of pharmaceutical waste to remove the mixture of Phenol & 4- Chloro Phenol & Catechol. To do this, it was necessarily required to check whether the bioreactor was effective in pilot scale. Also it was required to check whether it was effective in the variation of initial concentrations of the three Phenolic substances keeping their total conc. unchanged. In both cases, satisfactory outcomes were gained. Some experiments did not obtain fruitful outputs but after adjustment of inoculums, satisfactory results were obtained from those also. While studying in pilot scale, 15% inoculums of the consortium was deployed to degrade 1500 mg/L of tri- solute mixture only. Later, for the same as well as increased initial concentrations, 18% inoculums were involved. In all cases, satisfactory outcomes were achieved at 48 hours of incubation. Beyond that time period, degradation efficacy slightly decreased. Analyzing the data obtained while studying bioreactor (Table IX.6 & Figure IX.7), it may be concluded that the bioreactor can be used not only in case of pharmaceutical wastewater but also in case of petrochemical industries and oil refineries because of its efficacy. Normally, in case of pharmaceutical wastewater, around 1000 mg/L of Phenolic

waste can be found. But from the experiments, it was found that ~93% degradation can be achieved even when the initial conc. was 2100 mg/L (Table IX.6). So in case of future studies, similar experiments with higher concentrations are recommended. To degrade higher concentrations of Phenolic waste, further rearrangements in the parameters may be required.

Chapter X

Conclusions & References

Conclusions:

Now- a- days, human civilization and thereby, industrialization is growing very rapidly. Consequently, their adverse impacts on the environment cannot be denied. Pollution of different environmental factors has become a burning question to the present world. One of them is water pollution. There are different points and non- point sources of the water pollution. Human induced organic pollutants are one of the most dangerous causative agents of the water pollution. These organic compounds are very harmful to the human beings as well as other aquatic organisms. Phenolic compounds are very common organic pollutants found to be hazardous in case of aquatic ecosystem. Some of the hazardous phenolic substances are Catechol, Cresol, Chlorophenols and Phenol itself. There are several physical and chemical methods to remove phenolic compounds from the water bodies. But these traditional treatment methods possess some demerits as they are cost effective and also produce some toxic intermediate compounds. On the other hand, biological treatment methods have easily overcome these drawbacks. Moreover, complete degradation can be achieved via biological methods. That's why biological methods of degradation of Phenolic substances are widely accepted throughout the world. A number of native microbes are involved in the biological treatment methods by the researchers. Many studies were previously conducted regarding the bioremediation of the Phenolic substances. However, there were some gaps in those studies. First of all, most of the previous studies dealt with two or three parameters. Besides, the studies were conducted in small scale rather than field scale. Time span to remove the Phenolic substances were also high. Keeping those gaps in mind, the framework of the current study was designed.

In this thesis, some native bacterial strains were isolated from the local hospital area to remove some Phenolic wastes found in the pharmaceutical waste water. Later those were characterized biochemically and identified via 16s rDNA assay. Some parameters were selected to find out the optimized values of those parameters to reveal the best performances of the isolated strains in batch reactor. Besides, those parameters were optimized by deploying Response Surface Methodology (RSM) also and, kinetic study was performed in case of those strains to obtain the yield coefficients and Haldane model of kinetics validated the substrate inhibition model regarding those strains. Parameter optimization study and kinetic study was deployed both in case of single Phenolic compound as well as mixed compounds. At last, some studies were justified in pilot scale by deploying the optimized

parameters in case of the tri- solute mixture of Phenolic compounds. The major findings of the study have been summarised below:

1. More than two hundred of naïve bacterial strains were isolated via enrichment technique which was able to degrade Phenol or 4- Chloro Phenol or Catechol.
2. Six bacterial strains were selected possessing the maximum capability of degradation. Those strains were characterized and identified for further studies. *Brevibacillus formosus* strain NRRL NRS-863 and *Pseudomonas otitidis* strain MCC10330 were selected to degrade Phenol, *Bacillus timonensis* strain 10403023 and *Bacillus cereus* strain K1 were isolated for the degradation of 4- Chloro Phenol, *Bacillus pseudomycoides* strain NBRC 101232 & *Bacillus paramycoides* strain MCCC 1A04098 were selected to remove Catechol respectively.
3. Six parameters were selected as well as optimized for each of the six strains respectively. Those are: Temperature, pH, Incubation time, Media volume, Inoculums size & Initial conc. of the Phenol or its compounds.
4. Those parameters were optimized statistically also by involving Response Surface Methodology (RSM).
5. Kinetic study was performed for each of the six strains while degrading Phenol, 4-Chloro Phenol & Catechol respectively and yield coefficients were deducted for each microbial strain. Moreover, the substrate inhibition models of the each strain were validated by Haldane kinetic model.
6. Three bi- solute mixtures and one tri- solute mixture of the Phenol and Phenolic compounds were prepared to be removed. Here microbial consortiums were prepared by mixing those isolated strains together and deployed to remove those mixtures. Parameter optimization study was also conducted here separately for all the four mixtures.
7. Kinetic studies were performed for the bi- and tri- solute mixtures also.
8. A bioreactor study was performed where some of the previous studies were re-conducted in pilot scale i.e. in large volume of media (2L). The bioreactor was found to be suitable for dispose of Phenol & 4- Chloro Phenol & Catechol from the pharmaceutical waste water within 48 hours when the initial conc. of the mixture was 1500 mg/L.

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