Study of Industrial Dye Degradation Potential of a novel bacterial strain PSTD 06

A thesis submitted in fulfilment of the requirements for the degree of

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in

Biomedical Engineering

Submitted by

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It is certified that the work contained in this thesis entitled **"Study of Industrial Dye Degradation Potential of a novel bacterial strain PSTD 06"** by **"Tuhin Kahali"** has been carried out under my supervision and be accepted in fulfilment of the requirement for the Degree of Master of Engineering in Biomedical Engineering. The research results presented in the thesis has not been submitted elsewhere for a degree.

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I hereby declare that this thesis contains literature survey and original research work by the undersigned candidate, as part of his Master of Engineering in Biomedical Engineering studies during academic session 2018-2019.

All information in this document has been obtained and presented in accordance with academic rules and ethical conduct.

I also declare that, as required by this rules and conduct, I have fully cited and referred all material and results that are not original to this work.

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Abstract

In the present situation, dye utilizing industries play a significant role in causing immense pollution of water bodies by discharging colored effluents in them. Dyes significantly hamper the aquatic ecosystem by causing reduction in the photosynthetic activities within water bodies. Moreover a large section of industrial dyes are able to persist within the environment for extended time period while resisting degradation or transformation. Many of these dyes and their broken down end products are often toxic to the environment posing serious concern regarding its hazardous effects on human beings and nature. A previously isolated novel bacterial strain PSTD 06 was explored for its dye degradation potential. Industrial green dye (IGD) was most successfully degraded by PSTD 06. After confirming the proper degradation, toxicity of the degraded product (DP) of IGD was checked in comparison with that of IGD. Degradation pattern was studied under varying parameters and under the stress of heavy metals. Ultimately PVA-alginate beads of PSTD 06 whole cells were prepared to study the 19hrs IGD degradation by them. After the whole study, it can be concluded that PSTD 06 was efficient enough to be commercialized for the biodegradation purpose of IGD in presence of heavy metal stress provided by industrial effluents.

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Contents

List of Figures:

List of Tables:

List of Instruments:

Instruments used	**Company**
UV-Vis spectrophotometer	Agilent, Carry 60
Micro plate reader	BioRad
Optical microscope	Olympus, CH20i
Weighing Balance	Sartorious, BP 110 S
Centrifuge Machine	Biocoction
Autoclave	Ketan
Lyophilizer	Biobas

CHAPTER 1:

INTRODUCTION

1.1. Definition of Dye

1.2. History of Dyes

1.3. Types of Dyes

1.4. Classification of Dyes according to structure

1.5. Dye Pollution

1.6. Different Hazardous Dyes

1.7. Modern Pollution Prevention Techniques

1.8. Biological Treatments

1.9. Models for Comparative Study of dye toxicity and its treated product

1.10. Heavy metals, an important part of effluents from dye utilizing industries

1.11. Immobilization in bioremediation

1.1. Definition of Dyes:

Dyes are the substances which impart color to a variety of substrates, by binding to them when applied as aqueous solution. Substrates include leather, paper, plastics, hair, waxes, fur, cosmetics, drugs, greases and textile fibers.

Dye gets its utility for possessing four important properties:

a) Color
b) Water solubility
c) Ability of getting absorbed and retained by the substrate it is applied to.
d) Able to withstand light exposure, dry cleaning and washing.

Dyes are colored because they have

i. Capability of absorbing light within the 400-700 nm ranged visible spectrum
ii. Minimum one color bearing group, chromophore
iii. A conjugated system (structure bearing alternating single and double bonds)
iv. Stabilizing force due to resonance of electrons [1]

Along with choromophores, in most of the dyes, groups called auxochromes are found. They are polar groups which make the dyes water soluble and also helps in binding of the dye with fiber [2].

1.2. History of Dyes:

Previously, before mid 19th century almost all the colorants were derived from vegetable animal and mineral sources. Natural invertebrate dyes like crimson kermes and Tyrian purple were luxurious items in the past. Dyes derived from plants also played the role of important trade goods in Asian and European economies in the ancient and medieval ages [3]. Economic limitations of these coloring substances got very much prominent, around the end of the 18th century. This was due to the industrial revolution which emerged Europe by 1850 and this was the reason for the charging the world of cheap reliable and easily available synthetic dyes. Major challenge was offered to the die chemists, by two of the most important natural dyes of vegetable origins indigo and madder [4]. Mauve was the first dye of synthetic origin discovered by Perkin in 1856 [4, 5].

Natural dyes:

Natural dyes are the color important substances, obtained from invertebrates, minerals and plants. Majority of the natural dyes from plant sources like berries, leaves, roots, barks and wood. Some natural dyes also come from lichens and fungi.

Synthetic dyes:

Synthetic dyes are the color imparting substances, which are produced by utilizing synthetic resources such as petroleum by products, earth Minerals and Chemicals.

1.3. Types of Dyes:

1.3.1. Acid Dyes

Acid dyes are generally the sodium salts of the acidic color, consisting of phenolic or sulphonic acid groups. Being anionic dye, they possess the color in their negative ions. They are mainly applied to materials like nylon, wool and silk along with acid solutions. Synthetic food colors mostly fall in this class. Examples- Methyl orange, Methyl red, Martius yellow etc.

1.3.2. Basic Dyes

Basic dyes are generally the salts of the basic dye as zinc chloride and hydrochloride complexes. Being cationic they possess the color in their positive ions. They were the first among the synthetic dyes to get isolated from derivatives of coal tar. They are usually used for coloring leather, wool, silk, linen, hemp etc. Recently their successful application having exceptional fastness with brilliant color was seen for acrylic fibers. Triphenylmethane and azo dyes can be given as examples.

1.3.3. Direct Dyes

Direct dyes are acid color salts, which can dye the fabric by forming hydrogen bonds when the fabric in directly placed within aqueous hot solution of these dyes. Direct Black EW and congo red are typical direct dyes. These dyes got popular, as mordant was not required for the binding. They are used for coloring wool, rayon, silk, linen, cotton and nylon. Example- Direct orange 26.

1.3.4. Mordant Dyes

Mordant dyes require application of a mordant or a binding agent which makes the dye bind to the fabric. Type of mordant depends on the nature of the mordant dye. Acidic dye dyes require basic mordant (salts of Fe, Sn, Cr, Al), whereas basic dyes require acidic mordants (tanning being the most common one). Color imparted by the mordant dye also changes, depending on the mordant applied. Example- Alizarin.

1.3.5. Ingrain Dyes

These dyes are synthesized within the fibers. First one half of the dye is applied to produce the dye in the fabric. Then the other half of the dye is applied on the fabric. The combination

leads to the final finished color. This type of dyeing is specifically done with dyes. An important example of ingrain dye is Para red.

1.3.6. Vat Dyes

Vat dyes are soluble in their reduced form which is obtained with the help of some reducing agents like alkaline sodium hyposulphite. Anthraquinone and indigo are some examples of vat dyes.

1.3.7. Reactive Dyes

These dyes form covalent bonds with cellulosic fibers giving fast and long lasting color to the fiber. Reactive group reacts with the amino or hydroxyl group of the fiber. Wool, cotton and silk can be colored by this dyeing. Example: Reactive blue 5.

1.3.8. Disperse Dyes

These dyes being relatively insoluble in water are prepared for dyeing as fine powder along with dispersing agents. They are used to color nylon, polyester, and cellulose acetate fibers. Example: Disperse yellow 3.

1.4. Classification of Dyes according to structure

Azo dyes: They form the most important and the largest section of organic dyes. Azo group forms the chromophore part of these dyes. Examples: Methyl orange, Congo red etc.

Nitro dyes: They are the poly nitrophenols. Example: Picric acid.

Triphenylmethane dyes: They claim to be the oldest of the synthetic dyes, having triphenylmethane backbones with intense color. Examples: Malachite green, Crystal violet, Rosaniline, Cotton blue etc.

Phthalein dyes: They are mainly used for pH indication tests. Examples: Phenolphthalein, eosin, erythrosine, mercurochrome etc.

Anthraquinone dyes: Example: Alizarin.

Indigo dyes: Example: Indigo [6].

1.5. Dye Pollution:

Dye industries, being an important sector for the economical advancement consumes a large volume of chemicals and water. Different types of dyes, consisting of varying amount of chemical compounds get exploited during production depending on the textile type or the material being dyed. Over 3600 types of dyes are utilized by the textile industries alone [7]. Over 100,000 dyes are available commercially and above $7x10^5$ tones are produced annually

[8, 9]. Though large amount of water is consumed for manufacturing, application (mostly by textile, paper and leather) and dye disposal [10], effective cleaning of the used water before releasing it to the environment is not done in many cases. Hence, making the effluents from dye utilizing industries, play the role of a major pollutant. Some dyes do not degrade in water and some degrade to form toxic end products. More than 1mg/l concentration of dye is normally noticeable. But, average concentration of dyes in textile industry effluents is around 300 mg/l [11].

- **Hazards of Dye Pollution:**

- Dyes being colored are able to absorb and reflect the sunlight entering the water. And this is the main concern regarding the dye pollution. Hence in presence of dye, photosynthetic activity of the algae present in the water gets reduced, causing serious problem to the food chain.
- Many of the industrial dyes and the breakdown products of many are mutagenic, carcinogenic and/or toxic for the environment to which the effluents are released into.
- Liver, kidney and urinary bladder cancer of the dye industry workers have been reported.
- Textile dyes are often responsible for causing allergic reactions like skin and eye irritation, irritation in mucous layer and in upper part of the respiratory tract causing respiratory diseases, contact dermatitis etc.
- Respiratory troubles were reported for the workers, who got regular exposure to the reactive dyes for occupational purpose.

1.6. Different Hazardous Dyes:

1.6.1. Crystal Violet

This dye has many uses, but is recalcitrant, posing toxic effects to the environment due to long time persistence in the environment. It is a significant carcinogen, a potent tumor growth promoting factor in some fishes. Hence the dye is a biohazard substance [12, 13].

1.6.2. Methylene Blue

Stomach upset, vomiting, nausea, diarrhea, bladder irritation, are some of the prominant side effects due to its exposure. Excessive exposure may lead to fainting, irregular heartbeat, dizziness, high fever, unusual tiredness, chest pain, skin irritation, breathing trouble etc [14].

1.6.3. Methyle Orange

Methyle Orange can cause diarrhea and vomiting due to significant irritation to the digestive tract. Heavy exposure to this dye can even cause death of the victim [15].

1.6.4. Congo Red

It is a synthetic, reactive dye. Its exposure can cause symptoms like heart failures, irregular heartbeat, respiratory tract disease, enlarged spleen, skin lesions, macroglossia, problems in motor functions. Moreover gastrointestinal tract can also get affected causing vomiting, diarrhea [16].

1.6.5. Safranin

It is a commonly used dye, which is cationic in nature but is toxic to human beings. It causes severe eye irritation, conjunctiva irritations in respiratory tract and skin. It is also able to permanently damage human and rabbit cornea and conjunctiva [17, 18].

1.6.6. Remazol Brilliant Blue

An important textile dye which is often used as the starting material for many polymeric dyes, and hence acts as a source of organopollutants, have recalcitrant and xenobiotic properties. It may cause irritation to eye, skin, digestive and respiratory tract. It is an experimental carcinogen.

1.6.7. Cotton Blue

Cotton blue is cytotoxic. It is suspected of causing genetic defects. Prolonged repeated exposure causes damage to organs. Direct exposure can even cause severe skin burn and serious eye damage.

1.6.8. Cibacron Brilliant Yellow

Reactive dyes like Cibacron brilliant yellow get widely used for dyeing. Even presence of a very small amount of the dye causes undesirable, highly visible color affecting photosynthetic activity harming aquatic ecosystem. Kidney, brain and liver dysfunctions are the caused by the exposure [19].

1.6.9. Malachite Green

Malachite green, being a member of the tryphenylmethane family, shows animal carcinogenicity. Malachite green has been nominated as a priority chemical for carcinogenicity testing by the National Toxicology Program 1993 [20]. Malachite green and its reduced form tends to persist within tissues of edible fishes for extended time period, posing serious concern regarding the hazardous effects of its bioaccumulation in aquatic and terrestrial ecosystems. Though the dye has been banned in several countries, it is still being

used in many regions for treating fungal infections, dyeing in textile, leather and food industries, etc [21].

1.7. Modern Pollution Prevention Techniques:

1.7.1. Chemical Precipitation

Chemical precipitation involves addition of a reagent to the wastewater, which forms an insoluble compound with the dissolved dye to be removed and hence precipitation removes the dye.

1.7.2. Adsorption

Adsorption is used as a industrial waste water purification technique, mainly to remove a variety of non-degradable organic compounds present in lower concentrations. In the process molecules to be removed from the liquid sample, binds to the solid absorbent surface. Absorbents provide a huge amount of internal surface area to allow adsorption of the targeted molecules.

1.7.3. Electro-coagulation

Electrocoagulation can be used for treating waste water, as it involves flocculation of the contaminants to be removed by directly using electric current. Hence no coagulants are required. It was stated by Shammas et al. that with current being applied coagulation of the small particles occurs as motion is imparted into them by the current [22].

1.7.4. Ozonation

Ozonation involves passing of ozone by either pulling the gas by creating a vacuum or by providing bubbles of ozone through the water. Being reactive with the metals ozone forms insoluble metals oxides with the metals present in the dyes and hence by filtration they can ultimately be removed [23].

1.7.5. Photo oxidation

Photo Oxidation is mainly involved in producing highly reactive hydroxyl radicals. Due to its high oxidation potential, these radicals in presence of organic matter cause a series of chemical reactions to take place. Thus the organic compounds get mineralized along with production of carbon dioxide and water. Photo-oxidation has two variants: Photolysis and photocatalysis.

In photolysis the effluent gets irradiated by ultraviolet light, making the chemical compounds to absorb it forming free radicals. Free radical source for non-selective oxidation of the

oraganic compounds is provided by the combination of oxidizing agents like hydrogen peroxide and ozone with ultraviolet radiation.

In photocatalytic oxidation, catalysts are used to increase formation of hydroxyl ions for oxidizing the contaminants.

1.7.6. Ion exchange

Ion exchange is used for removing unwanted cations and anions from the waste water. While passing the waste water through ion exchange resins the cations and anions get exchanged with that provided by these resins.

Disadvantages of modern techniques for tackling dye pollution:

The modern chemical and physical techniques used for tackling dye pollution are not cost effective. Moreover high amount of energy and chemicals are required for most of them. In many cases, toxic end products form secondary pollutants and again further treatment is needed.

1.8. Biological Treatments:

Bioremediation can be defined as the process for degrading and reducing or for detoxifying the pollutants and waste products by utilizing living organisms mainly microorganisms and plants. Presently bioremediation is commonly practiced for restoring natural useful values of polluted sites by involving microorganisms which are able to transform, degrade or chelate the toxic compounds [24].

Microorganisms break down organic pollutants while utilizing them as carbon source and energy source, or by cometabolism. Heavy metals get transformed from one oraganic complex or oxidative state to another reducing its solubility and toxic effects [24, 25].

Bioremediation is a non-invasive, cheap, eco-friendly and permanent solution causing degradation or transformation of pollutants to their less toxic or harmless forms [24, 25, 26, 27].

Bacterial biodegradation (Kolekar et al. 2013), biosorption (Guendouz et al. 2016), biodegradation by fungi(Taha et al. 2014), and phytoremediation using Portulacaceae, Araceae, and Verbenaceae have been reported by Kagalkar et al. (2010), Khandare et al. (2011a) and Kabra et al. (2012; 2011), respectively.

Positive aspects of biological treatment:

Biological treatments for dye containing waste water are far more advantageous over the conventional chemical and physical ways. They are so far the cheapest solution with high efficacy for getting rid of dye pollution. They provide permanent settlement to the pollution by forming non hazardous or less toxic end products without requiring much maintenance, expense of energy or chemicals.

1.9. Models for comparative study of dye toxicity and its treated product:

Bacillus pumilus & Bacillus subtilis

They are considered as the beneficial soil bacteria having important contribution towards the soil ecosystem. Thus something showing toxic effect on them will surely conclude its hazard on the ecosystem.

Oryza sativa

Being the source of staple food in many regions of the world, this plant obviously clams to be an ideal model for the toxicity of the pollution causing industrial dyes and their degraded products.

Eisenia fetida

Earthworm (*Eisenia fetida*) is considered as farmer's friend as it hugely contributes in improving soil attributes. They do show prominent contribution towards the fertility and enrichment of the soil. Dye containing effluents from industries while contaminating water bodies, indirectly also enters the soil causing hazardous effects. So earthworms can also be dealt as an ideal model for comparative study of toxicity of industrial dyes before and after treatment.

1.10. Heavy metals, an important part of effluents from dye utilizing industries:

The heavy metals of major concern from dye utilizing sectors include: Cadmium, mercury, copper, selenium, nickel, cobalt, lead, chromium, chromium, arsenic, antimony.

In some reactive dyes, heavy metals remains bonded within the chromophore as metal complexes. But often free metals can stay as impurity due to incomplete reaction.

Some heavy metals relatable to such occurrence include:

- Copper
- Cobalt
- Nickel
- Chromium

For metal-free chromophore containing dyes metallic impurities from the raw materials can also be present.

Another source for heavy metals in dyes can be from the catalysts used.

Heavy metals on microorganisms:

Indications of specific inhibitory action in microbes, by heavy metals have been reported previously. Heavy metals selectively target specific pathways and enzymatic systems resulting certain members of the microbial community to be more sensitive to heavy metal exposure than others, depending on the sensitivity of their critical metabolic pathways [28, 29, 30].

1.11. Immobilization in bioremediation:

Enhanced efficiency in biodegradation observed while using immobilized cells in comparison to free cells lead to increase in interest of implementing immobilization for biodegradation [31, 32].

It is assumed carriers protects the cells and hinders spreading of pollutants causing reduction in the concentration of surface contaminants on these immobilized microbial cells.

Recently, immobilization methods are very often used for the purpose of bioremediation. Immobilization means limiting of the microbial cells' mobility or that of their enzymes along with preservation of the cell viability and catalalytic fuctions respectively [33-37].

Five main techniques of immobilization:

Adsorption, entrapment, binding on a surface (electrostatic or covalent), encapsulation and flocculation (natural or artificial) [38]

Advantages of Immobilization [39, 40]:

- Immobilization significantly improves the pollution degradation efficiency and reduces the cost of bioremediation.
- Cell filtration stage is not required.
- Stable microenvironment is ensured for the cells or enzymes.

- The risk of genetic mutation gets reduced.
- Resistance to the shear forces in bioreactors is ensured.
- Resistance of biocatalysts against heavy metals and environmental hazards is enhanced.
- Biocatalyst survival during storage gets increased,
- Tolerance against high pollutant concentrations increases.

❖ **Entrapment**

Entrapment is an immobilization technique in which microbes are only able to move within the carrier. Hence it prevents leaking out of the cells. It is a inexpensive, non-toxic, rapid and versatile method, widely used for bioremediation [37, 41].

Support materials (carriers) for immobilization:

A suitable carrier should be non-toxic for both environment and the immobilized materials, insoluble, inexpensive, easily accessible and stable. Carriers also get selected based on the immobilization being applied or the nature of the bioremediation. Carriers can be classified into natural and synthetic or as organic and inorganic.

Organic carriers which are natural by origin possess many functional groups which help in stabilizing the biocatalysts. This class include: chitosan, carrageenan, alginate, charcoal, plant fibers etc [42-45].

These supports are biocompatible and non toxic to environment, hydrophilic, biodegradable and generally inexpensive. But their application in bioremediation gets limited due to the sensitivity to organic solvents, low resistance to organic solvents and due to stability in narrow pH range [46, 47].

Organic carriers of synthetic origin possess a number of diverse functional groups. Polyvinyl chloride, polystyrene, polypropylene, polyacrylonitrile and polyvinyl alcohol fall in this group [48-51].

A major advantage provided by this class is that, their structure at macromolecular level can be altered.

Inorganic carriers (volcanic rocks, porous glass, ceramics, etc.), both natural and synthetic provide high resistance to physical, chemical and biological resistance. But significant disadvantage is there due to lack of functional groups preventing sufficient bonding with the biocatalysts. So they are generally used to develop hybrid carriers.

CHAPTER 2:

REVIEW OF LITERATURE

2. Review of Literature:

Environmental pollution has become a major issue over the last century. With increasing population and increasing demands, industrialization has flourished to a great extent and has severely invaded nature, causing destruction of various ecosystems by its immense contribution to the environmental pollution. In case of water bodies, pollution is mainly caused by the discharge of the inadequately treated waste water from these industries. Among the different pollution causing attributes of the industrial effluents, dye is one of the significant pollutants present in the wastewaters discharged from industries like leather, textile, paper, cosmetics, food, pharmaceuticals etc. [52]. These industries produce huge amount of colored water. Colors when present in water cause hindrance to entry of the sunlight into water, as portions of the light gets absorbed by the dyes. This ultimately hampers the photosynthetic activities within the water bodies, ultimate harming the aquatic system to great extent. Moreover many dyes and their breakdown products are persistent in environment for long periods of time without getting degraded easily and are toxic, mutagenic, carcinogenic and growth inhibitor of plants, leading to potential hazard for human race and environment [53]. There are over 100,000 commercial dyes and annually, over 7 x 10^5 metric tons of dyes are produced [54]. The amount of dye which doesn't bind with the fiber gets released into the water bodies. Thus in recent years pollution created by these dyes, has gained great concern. The physical and chemical water treatment methods for combating dye pollution like froth floatation, electro coagulation, advanced oxidation or photo oxidation, ion exchange etc are not always feasible due to their high cost, high consumption of chemicals and energy etc. [54, 55]. On the other hand biological methods for treating the waste water are cheaper, less energy intensive, safe and result in bioconversion of organic pollutants to their non toxic or less toxic stable end products. There are many fungal, bacterial and algal species having the ability to adsorb or degrade dyes [56]. Bacterial colorization is normally faster than that by fung [57]. Decolorization takes place under conventional and facultative anaerobic and aerobic conditions by different bacterial groups. Several research works have been done to explore the potential of the wide variety of bacteria having the capability of decolorizing dye containing waste waters, by adsorbtion or degradation of the dyes present. Dye containing effluents serve as a rich niche for isolating these bacteria having dye degrading potential.

Dye decolorization potential of *Escherichia coli [58], Phanerochaete chrysosporium [59], Pseudomonas aeruginosa [60], Aspergillus terricola [59], Chlorella vulgaris [61]* have been reported of showing dye degradation potential.

Microbial degradation of organic synthetic colorants has also been reported by Meyer in 1981, Omar in 2013, [62, 63]. Several *Streptomyces* species, decolorizing dyes have been reported by Ball, Zimmermann and Don Crawford [64, 65, 66]. Moreover several species of Pseudomonas sp. have been reported by Zimmermann et al. 1982, TL Hu in 1998, Oranusi et al. 2005, Kalyani et al. 2009 [67, 68, 69, 70]. In 1996, decolorization of industrial effluents by bacteria has been reported by Poonam nigam and Guthrie et al 2003 [71]. Alhassani et al. reported dye degradation by *Brevibacillus. Sp.* [72].

CHAPTER 3:

MATERIALS AND METHODOLOGY

3.1. Morphological and Biochemical Characterization of the noble bacteria PSTD 06

3.2. Preparation of inocula

3.3. Growth Curve of PSTD 06

3.4. Various Dye Degradation by PSTD 06

3.5. Industrial Green Dye Degradation by PSTD 06

3.6. Analysis of the Treated Product

3.7. Toxicity Studies

3.8. Degradation of IGD by whole cell, lysed cell and cell free extract of PSTD 06

3.9. Degradation study of IGD using PSTD 06 under different conditions

3.10. Study of the effect of heavy metals on IGD Degradation by PSTD 06

3.11. IGD Degradation study by PVA-Alginate beads of PSTD 06

3.1. Morphological and Biochemical Characterization of the noble bacteria PSTD 06:

3.1.1. Morphological Characterization

- **Gram Staining:**

Materials: Glass slide, crystal violet, gram's iodine, safranin, PSTD 06 culture.

Method:

1. Bacterial specimen was taken and heat fixed on grease free, clean glass slides.
2. Slide was flooded with the primary stain, crystal violet and was kept for 40 seconds.
3. Gentle rinsing of the slide was done with distilled water.
4. Iodine solution was applied as the mordant and was kept for 1min.
5. Rinsing was again done with distilled water and the smear appeared on the slide as a purple circle.
6. The slide was then flooded with 95% ethanol as decolorizer for five to ten seconds, till the alcohol runs almost clear.
7. Immediately the slide was again rinsed with distilled water.
8. The slide was then flooded with safranin as the counter stain and was kept for 45 seconds.
9. Blot drying was done with filter paper.
10. Observation of the smear was done using light-microscope under oil immersion.

- **Endospore Staining:**

Materials: Glass slide, blotting paper, IGD, safranin, PSTD culture.

Method:

1. Bacterial smear was made on the grease free clean glass slide.
2. The slide was air died and heat fixing was done.
3. Blotting paper was placed on the slide, IGD solution was poured.
4. The slide was heated using spirit lamp, until the solution evaporated.
5. The slide was heated, removed and reheated for about five minutes. Throughout this step, the blotting paper was kept moist by adding drops of the IGD solution. Thus the slide got steamed, and wasn't overheated.
6. The slide was cooled and then gently washed with distilled water.
7. Safrannin was applied as the counter stain.
8. After rinsing the slide, it was air dried and observation was done under light-microscope.

3.1.2. Biochemical Characterization

- **Catalase Production Test:**

Materials: Glass slide, H_2O_2, PSTD 06 culture.

Method:

1. Small inoculum of the bacteria was taken on clean glass slide.
2. Drop of H_2O_2 was added for the observation of the test.

- **Amylase Production Test:**

Materials: Petri plates, nutrient broth, agar agar, starch, PSTD 06, iodine solution.

Method:

1. Plate of nutrient agar along with 0.4% starch was prepared.
2. The plate was spotted with the PSTD 06
3. Incubation was done for 48hrs at 37°C
4. Plates were then flooded with iodine solution

- **Lipase Production Test:**

Materials: Nutrient Agar plates with added 1% Tween 80, PSTD 06

Method:

1. 1% Tween containing Nutrient Agar plate was prepared.
2. Plate was spotted with PSTD 06.
3. Incubation was done for 96hrs at 37°C.

- **Protease Production Test:**

Materials: 1% Casein containing nutrient agar plate, 10% HCL, PSTD 06 culture.

Method:

1. 1% Casein containing nutrient agar plate was prepared.
2. The plate was spotted with PSTD 06.
3. Incubation was done for 72 hrs at 37°C.
4. Then 10% HCL was added.

- **Indole Production Test:**

Materials: Tryptophan broth medium, Kovac's reagent, PSTD 06 culture.

Method:

1. PSTD 06 was grown in one tryptophan broth tube for 48 hrs at 37°C. The other tube was kept as control.
2. Kovac's reagent (0.5ml) was added to each of the tubes.

- **Nitrate Reductase Test:**

Materials: Nitrate broth [KNO_3- 1g/l, Beef Extract- 3g;l, Peptone, pH- 7.0], Nitrate reagent, PSTD 06 culture.

Method:

1. Nitrate broth was prepared in two test tubes.
2. One was inoculated with 100 µL of PSTD 06 culture and the other one was kept as control.
3. Incubation was done at 37°C for 48hrs.
4. 1ml of nitrate reagent was added to both the tubes.

- **Citrate Utilization Test:**

Materials: Simmon's citrate medium, PSTD 06 culture.

Method:

1. Simmon's citrate plate was prepared.
2. The plate was spotted with PSTD 06.
3. Incubation was for 48hrs at 37°C.

- **Gelatinase Production Test:**

Materials: Nutrient broth with 12% gelatin, ice, PSTD 06 culture.

Method:

1. Two nutrient broth with 12% gelatin media were prepared
2. One was inoculated with 100µl of PSTD 06 culture and another was kept as control.
3. Incubation was done at 37°C.

After 48-72 hrs incubation

3.2. Preparation of inocula:

MSM was prepared following Khalid et al. [73] with pH set to 7. In 250ml conical flsk, 100ml broth was placed and inoculated. After 24hr incubation at 37°C, PSTD 06 culture with O.D 1 at 600nm was obtained. For inoculating the 100ml MSM, 5ml of inocula was added.

3.3. Growth curve of PSTD 06:

Materials: 250ml conical flask, Nutrient broth, PSTD 06 culture, UV-Vis spectrophotometer

Method:

1. PSTD 06 was allowed to grow in nutrient broth.
2. At regular intervals O.D at 600nm was measured with UV-Vis spectrophotometer (Cary 60, Agilent).
3. Graph was plotted against time.

3.4. Various Dye Degradation by PSTD 06:

Materials: Cyrstal Violet, IGD, Cotton Blue, Methyle Orange, Cibacron Brilliant Yellow, Methylene Blue, Congo Red, Safrannin, Remazol Brilliant Blue, PSTD 06 culture (O.D. 1 at 600nm), 50ml Falcons, UV-Vis spectrophotometer, centrifuge, Minimal salt media(MSM), eppendorf tubes.

Method:

1. MSM with different dyes (20IGD/l concentration) were prepared.
2. 5ml of PSTD 06 culture was added to each falcon containing 45ml of the solutions, and were kept at room temperature.
3. Each time for observation, 3ml of the mixture was centrifuged at 6000rpm for 2mins and O.D. (614 nm) of the supernatant was noted.
4. Degradation percentages were calculated to plot against time.

Degradation efficiency was measured by the following formula :-

$$\text{Percentage degradation} = \frac{(I-F)}{I} \times 100$$

Where, I= initial absorbance, F= final absorbance

3.5. Industrial Green Dye Degradation by PSTD 06:

Materials: IGD dye (IGD), PSTD 06 culture (O.D 1 at 614nm), 50ml falcon, Minimal salt media (MSM), eppendorf tubes.

Method:

1. MSM with IGD (40g/l concentration) was prepared.
2. PSTD 06 culture was added to the media and was kept at room temperature.
3. 3ml culture media was taken out at regular intervals.
4. Centrifugation was done at 6000rpm for 2 mins.

5. O.D (614 nm) of the supernatant was noted till the degradation took place.
6. Degradation percentage was calculated using the previously given formula, and graph was plotted against time.

3.6. Analysis of the Treated Product:

3.6.1. UV-Vis spectroscopy

The IGD and DP were scanned from 300 to 800 nm in the UV-VIS spectroscopy (Carry 60).

3.6.2. Thin Layer Chromatography

Materials: TLC plate, glass beakers, Solvent-I (Propanol), Solvent-II (Butanol : acetic acid : water = 4:1:2), IGD solution (40mg/l), degraded product (DP) of IGD by PSTD 06 (DP), Sublimed Iodine.

Method:

1. Line was drawn with pencil, 1cm above the base of the TLC plate.
2. Small spots with IGD and degraded product were made on the drawn line.
3. Two such plates were prepared for the two solvents or mobile phase.
4. Two beakers were separately filled with two different solvents, such that when TLC plate is immersed, solvent level remains below the drawn line.
5. Beakers were covered and the setup was kept undisturbed, till the solvent front ran almost to the top the plate.
6. The plates were then carefully taken out, and kept in presence of iodine to check the separated out spots, and the Rf values were calculated.

3.6.3. Fourier Transform Infrared (FTIR) Spectroscopy

Both IGD and DP were examined using FTIR spectroscopy, to check if the IGD got properly degraded by PSTD 06.

3.7. Toxicity Studies:

3.7.1. Beneficial bacteria growth inhibition assay:

Materials: Nutrient agar medium, cultures (O.D 1 at 600nm) of *Bacillus subtilis* and *Bacillus pumilus*, IGD solution (40mg/l), DP, petri plates, conical flasks.

Method:

1. Two conical flasks, each containing 25ml of Nutrient broth with agar, were autoclaved.
2. After autoclaving, the flasks were allowed to come closer to the normal room temperature, under aseptic conditions.
3. While the nutrient agar was still liquid and mild warm, 1ml *Bacillus subtilis* culture was added to one flask and 1ml *Bacillus pumilus* culture was added to another flask aseptically. Mixing was done with the help of micropipette.
4. Then from the flasks, the mixtures of nutrient agar and cultures were separately plated in two petri plates.
5. Three wells were formed in each petri dish.
6. One well was loaded with distilled water as control. One was loaded with IGD solution and remaining one was loaded with degraded product.
7. Incubation was done for 24hrs at 37°C.
8. Observation was done.

- **Growth inhibition curve of beneficial bacteria:**

Materials: Nutrient broth, cultures (O.D 1 at 600nm) of *Bacillus subtilis* and *Bacillus pumilus*, IGD solution (40IGD/l), DP, Conical flasks.

Method:

1. Two separate setups were made for the two beneficial bacteria.
2. Each setup consisted of three 250ml conical flask.
3. In each of the three flasks, 250 ml nutrient broth was taken.
4. One flask was kept as control, in which 10ml distilled water was added. In one flask 10ml of IGD solution and in the remaining one 10 ml of the degraded product was added.
5. Each flask was inoculated with bacterial cultures.
6. Incubation was done at 37°C.
7. At 30mins interval O.D of the growth media was measured using UV-Vis spectrophotometer at 600nm.

Same procedure was followed for both the bacterial strains.

3.7.2. Phytotoxicity Study using *Oryza sativa*

Materials: Petri plates, Whatman filter paper, IGD solution (40mg/l), DP, distilled water, *Oryza sativa* (High yield variety, Satabdi) seeds supplied by Rice Research Institute, Chinsurah.

Method:

1. Surface sterilization of the seeds was done according to the following steps:
    a) Washing with tap water.
    b) Washing with distilled water.
    c) Washing with 70% Ethanol.
    d) Washing with distilled water, twice.
2. Three replicates of the setup were prepared.
3. Each setup consisted of three petri dishes with filter papers aseptically placed in them.
4. 22 seeds were placed on the filter paper of each petri dish.
5. One Petri plate was kept as the control in which filter paper was kept moistened with distilled water. Similarly, among other two petri dishes, one was provided with IGD solution and another was provided with DP.
6. Germination and shoot lengths were observed.

3.7.3. Toxicity Study on *Eisenia fetida*

Contact toxicity test and survival test (based on artificial soil test) were conducted on *Eisenia fetida* by referring to Gopinathan et al., 2015 [74, 75].

- **Contact Toxicity Test:**

Materials: *Eisenia fetida* (Earthworm) supplied by Aastha Earthworms, Flat bottomed vials (8cm length, 3cm diameter), IGD. dye in six different amounts (3.25mg, 6.5 mg, 13mg, 26mg, 39mg) , Whatman filter paper, distilled water.

Method:

1. 40 flat bottomed vials were divided into 8 sets, thus each containing 5 vials.
2. One set of five vials was kept as control, six sets of vials were taken for the six different amounts of IGD and one set was taken for DP.
3. Internal surface of all the vials were covered with filter paper cut to suitable size.
4. Filter papers of the control vials were moistened with 1ml distilled water. Filter papers within the vials specific for IGD were moistened with 1ml solutions of 6 different amounts of IGD. Vials of the set specific for DP were provided with 1ml of DP.

5. Then they were air dried.
6. Each vial's filter paper was moistened with 1ml of distilled water and was provided with an adult earthworm.
7. Caps of the vials were kept open, and all the sets were kept in a cool dark place.
8. Observation was noted after 72 hrs.

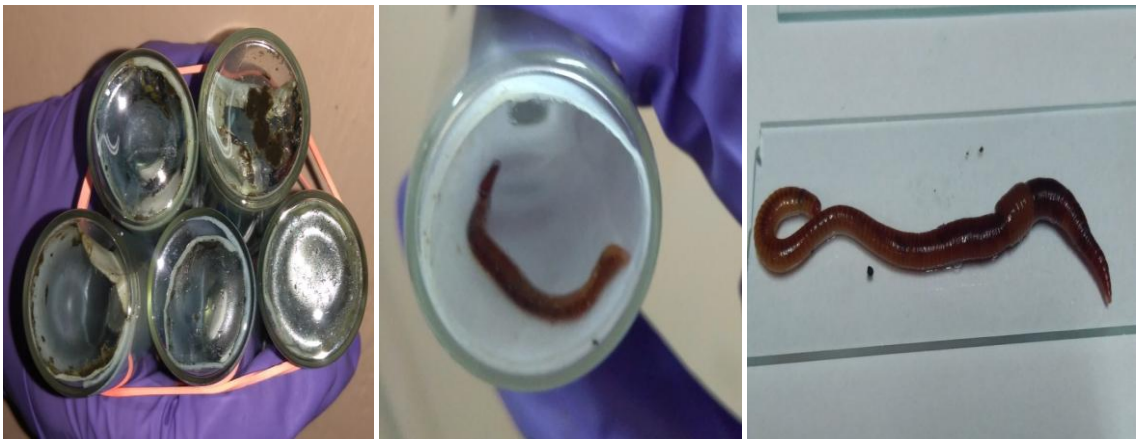

Fig. 1: Experimental setup for contact toxicity test on *Eisenia fetida*

- **Survival Test:**

Materials: *Eisenia fetida* (Earthworm) and breeding soil of the earthworms were supplied by Aastha Earthworms, seven 250ml conical flasks, IGD in five different amounts (1mg, 2mg, 4mg, 6mg, 8mg), distilled water, PSDT-DP

Method:

1. Each of the seven conical flasks was filled with 5 gm of soil (supplied by breeder).
2. One was taken as the control. Five flasks were taken for five different amounts of IGD and one was for DP.
3. Soil in the control flask was sprayed and mixed with 10ml of distilled water. Flasks for IGD were sprayed and mixed with 10ml solutions of the fixed five amounts of IGD accordingly.
4. Soil of the DP flask was sprayed and mixed with 10 ml of DP.
5. Each flask was then provided with five adult earthworms.
6. Mouths of the conical flasks were covered with aluminium foil with holes for air passage and were kept in dark place at room temperature for observation.

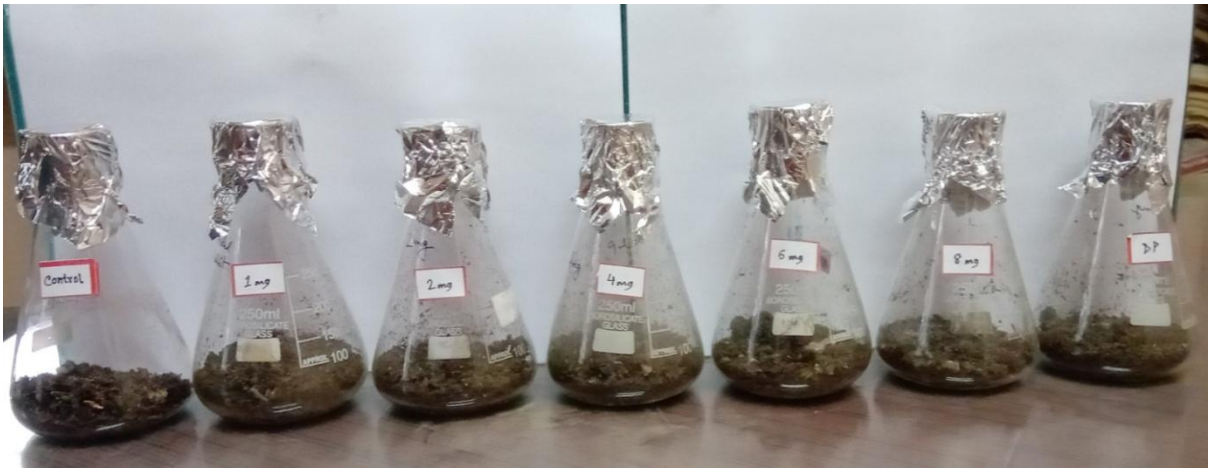

Fig. 2: Experimental setup for survival test on *Eisenia fetida*

3.7.4. Cytotoxicity assay on PBMC

MTT assay was done on PBMCs, to check the cytotoxicity of DP into healthy human cells, in comparison with that of IGD.

Materials: Cis-platin (CIPLA 10mg/20ml), IGD, DP, 96 wellplate, periferal blood mononuclear cells (PBMCs) were isolated, cultured and supplied by School of Bioscience and engineering, Jadavpur University.

Method:

1. PBMCs were seeded in 24 well with a seeding density of $0.3x10^4$ cells per well containing 500µL complete media.
2. Media was replaced after reaching 70% confluence.
3. IGD (sample 1) and DP were added.
4. Standard drug Cis-Platin (working concentration 16.66µg/ml) was added to the standard marked wells.
5. Incubation was done for 24hrs and then MTT (Thiazol Blue Tetrazolium Bromide) to read absorbance at 590nm using Agilent Technologies Cary 60 Uv-Vis Version 2.00 Spectrophotometer (Riss et al. 2013).
6. Cell viability percentage was ultimately calculated against blank with no treatment.

3.8. Degradation of IGD by whole cell, lysed cell and cell free extract of PSTD 06

Materials: MSM with IGD (40IGD/l), PSTD 06 (O.D 1 at 614nm).

Method:

1. PSTD 06 cells were lysed using probe sonicator (40 amp output, giving 7 strokes, each of 1 sec with 1min interval, temperature below 4°C**)** [76]
2. Sonicated culture was centrifuged at 12000rpm for 10min, to get the cell free extract.
3. 5ml of PSTD culture, 5 ml of sonicated and hence lysed cells, and 5ml of cell free extract was added separately to 50 ml of MSM with IGD.
4. At regular intervals of 15mins, 3ml of these inoculated media were centrifuged at 6000rpm for 2mins, and O.D of the supernatants was noted at 614 nm.
5. Simultaneously data for all three setups were collected.
6. Degradation percentages were calculated.
7. Triplicate of this study was performed.

3.9. Degradation study of IGD using PSTD 06 under different conditions

3.9.1. Inoculum Variation

Materials: PSTD 06 culture (O.D 1 at 600nm), MSM with IGD (40mg/l)

Method:

1. Six different amounts of inoculums of PSTD 06, 1ml, 3ml, 5ml, 7ml, 9ml and 11ml were separately added to 50ml of MSM with IGD. Thus 2%(v/v), 6%(v/v), 10%(v/v), 14%(v/v) and 18%(v/v) inoculum doses were used.
2. Incubation was done at room temperature.
3. At regular intervals of 15mins 3ml of the growth media was centrifuged at 6000rpm for 2mins.
4. O.D of the supernatant was checked by UV-Vis spectrophotometer at 614nm.
5. The study was done thrice, and averages of the obtained readings were used to calculate the percentage degradation.
6. Percentage degradations calculated were plotted against time.

3.9.2. Initial Dye Concentration Variation

Materials: PSTD 06 culture (O.D 1 at 600nm), MSM, IGD.

Method:

1. MSM with five different concentrations of IGD (20 mg/l , 40 mg/l, 60 mg/l, 80 mg/l, 100 mg/l) were prepared.
2. 5ml of PSTD 06 culture was added to 50 ml of each MSM with IGD separately.
3. Incubation was done at 37°C.
4. At 15mins regular interval 3ml of the growth media was centrifuged at 6000rpm for 2mins.
5. O.D of the supernatant was noted at 614nm using UV-Vis spectrophotometer.
6. The study was done thrice, and averages of the obtained readings were used to calculate the percentage degradation.
7. Percentage degradations calculated were plotted against time.

3.9.3. Temperature Variation

Materials: PSTD 06 culture (O.D 1 at 600nm), MSM with IGD (40IGD/l),

Method:

1. 5ml of PSTD 06 was added to 50ml of MSM with IGD. Five such setups were made.
2. Incubation of the five setups were done at five different temperatures (20°C, 30°C, 40°C, 50°C, 60°C, 70°C).
3. After regular interval of 15mins, 3ml of the growth media was centrifuged at 6000rpm for 2min.
4. O.D of the supernatant was checked at 614nm by using UV-Vis spectrophotometer.
5. The study was done thrice, and averages of the obtained readings were used to calculate the percentage degradation.
6. Percentage degradations calculated were plotted against time.

3.9.4. pH Variation

Materials: PSTD 06 culture (O.D 1 at 600nm), MSM with IGD (40IGD/l).

Method:

1. MSM with IGD was prepared with different pH values (1.5, 2.5, 3.5, 4.5, 5.5, 6.5, 7.5, 8.5, 9.5, and 10.5)
2. 5ml of PSTD 06 cultures were added to 50ml of each MSM prepared separately.
3. Incubation was done at 37°C.
4. After regular interval of 15mins, 3ml of the growth media was centrifuged at 6000rpm for 2min.

5. O.D of the supernatant was checked at 614nm by using UV-Vis spectrophotometer.
6. The study was done thrice, and averages of the obtained readings were used to calculate the percentage degradation.
7. Percentage degradations calculated were plotted against time.

3.9.5. Different Carbon Sources

Materials: PSTD 06 culture (O.D 1 at 600nm), modified MSM (sucrose substituted with dextrose, fructose, lactose, ribose, maltose, molasses and yeast extract) with IGD (40IGD/l),

Method:

1. Five modified MSM with IGD were prepared by substituting the standard carbon source sucrose with dextrose, fructose, lactose, ribose, maltose, molasses and yeast extract separately.
2. 5ml of PSTD 06 culture was added to 50ml of each of these media.
3. Incubation was done at 37°C. pH: 7
4. After regular interval of 15mins, 3ml of the growth media was centrifuged at 6000rpm for 2min.
5. O.D of the supernatant was checked at 614nm by using UV-Vis spectrophotometer.
6. The study was done thrice, and averages of the obtained readings were used to calculate the percentage degradation.
7. Percentage degradations calculated were plotted against time.

3.9.6. Different Nitrogen Sources

- **Organic Nitrogen Sources**

Materials: PSTD 06 culture (O.D 1 at 600nm), modified MSM with IGD (40IGD/l), soybean meal, tryptone, thiourea, urea, beef extract, peptone, yeast extract.

Method:

1. Seven modified MSM with IGD were prepared (pH 7), where nitrogen source diammonium hydrogen phosphate was substituted with soybean meal, tryptone, thiourea, urea, beef extract, peptone and yeast extract separately.
2. 50ml of each of these medias was inoculated with 5ml PSTD 06 culture.
3. Incubation was done at 37°C.
4. After regular interval of 15mins, 3ml of the growth media was centrifuged at 6000rpm for 2min.
5. O.D of the supernatant was checked at 614nm by using UV-Vis spectrophotometer.

6. The study was done thrice, and averages of the obtained readings were used to calculate the percentage degradation.
7. Calculated percentage degradations were plotted against time.

- **Inorganic Nitrogen Sources**

Materials: PSTD 06 culture (O.D 1 at 600nm), modified MSM with IGD (40mg/l), ammonia, sodium azide, calcium nitrate, sodium nitrite.

Method:

1. Four modified MSM with IGD were prepared, where nitrogen source diammonium hydrogen phosphate was substituted with ammonia, sodium azide, calcium nitrate and sodium nitrite separately.
2. 50ml of each of these media was inoculated with 5ml PSTD 06 culture.
3. Incubation was done at 37°C.
4. After regular interval of 15mins, 3ml of the growth media was centrifuged at 6000rpm for 2min.
5. O.D of the supernatant was checked at 614nm by using UV-Vis spectrophotometer.
6. The study was done thrice, and averages of the obtained readings were used to calculate the percentage degradation.
7. Calculated percentage degradations were plotted against time.

3.9.7. Salinity Variation

Materials: PSTD 06 culture (O.D 1 at 600nm), MSM with IGD (40mg/l), Sodium chloride (NaCl).

Method:

1. Ten sets of 50ml MSM with IGD were prepared with ten varying concentrations of sodium chloride (NaCl): 20g/l, 40g/l, 60g/l, 80g/l, 100g/l, 120 g/l, 140g/l, 160g/l, 180g/l, 200g/l .
2. 5ml of PSTD 06 was added to each of the prepared medias.
3. Incubation was done at 37°C.
4. After regular time interval, 3ml of the growth media was centrifuged at 6000rpm for 2min.
5. O.D of the supernatant was checked at 614nm by using UV-Vis spectrophotometer.
6. The study was done thrice, and averages of the obtained readings were used to calculate the percentage degradation.
7. Calculated percentage degradations were plotted against time.

3.10. Study of the effect of heavy metals on IGD Degradation by PSTD 06:

Materials: PSTD 06 culture (O.D 1 at 600nm), MSM with IGD (40mg/l), salts of arsenic (As), lead (Pb), mercury (Hg), chromium (Cr), cadmium (Cd).

Method:

1. Sets of 50ml MSM with IGD were prepared.
2. Varying concentrations of heavy metals were added to the medias separately and degradation study was done individually for each heavy metal.
3. 5ml of PSTD 06 was added to each of the prepared media.
4. Incubation was done at 37°C.
5. After regular time intervals, 3ml of the growth media was centrifuged at 6000rpm for 2min.
6. O.D of the supernatant was checked at 614nm by using UV-Vis spectrophotometer.
7. The study for each heavy metal, was done three times, and averages of the obtained readings were used to calculate the percentage degradation.
8. Calculated percentage degradations were plotted against time.

3.11. IGD Degradation study by PVA-Alginate beads of PSTD 06:

- **Preparation of PVA-Alginate beads [77]**

Materials: PSTD 06 culture (O.D 1 at 600nm), polyvinyl alcohol (PVA), sodium alginate, 5% calcium chloride solution, distilled water.

Method:

1. 1gm of PVA and 1gm of Sodium alginate were mixed in 70ml of boiling distilled water.
2. Solution was autoclaved and the solution was cooled.
3. 20ml of PSTD 06 culture was mixed with the solution.
4. Cold, sterile 5% Calcium chloride solution was prepared separately in two glass beakers. Each beaker contained 100ml of the solution.
5. Drop wise extrusion of the PVA, alginate and PSTD 06 mixture was done in cold sterile calcium chloride solution to obtain bio beads of diameter, approximately 2mm.
6. Hardening of beads was done by keeping the beads into another beaker containing fresh calcium chloride solution at 4°C for 24hrs.
7. The hardened beads were finally washed with distilled water to remove the excess calcium chloride and untrapped cells of PSTD 06 [66].

- **IGD Degradation study with PVA-Alginate beads**

Materials: Bio-beads of PSTD 06, MSM with IGD(40 mg/l), UV-Vis spectrophotometer.

The total weight of the beads obtained was equivalent to 20ml of PSTD 06 culture. Accordingly, weights of beads equivalent to 1ml, 3ml, 5ml, 7ml and 9ml were calculated and measured.

Method:

1. Five batches of MSM with IGD were prepared.
2. Beads equivalent to 2%(v/v), 6%(v/v), 10%(v/v), 14%(v/v) and 18%(v/v) inoculum doses, were separately added to each of the prepared media.
3. The setups were kept at room temperature.
4. At regular time intervals, O.D of the media was measured at 614 nm with the help of UV-Vis spectrophotometer for 19hrs.
5. The study was done thrice, and averages of the obtained readings were used to calculate the percentage degradation.
6. Calculated percentage degradations were plotted against time.

CHAPTER 4:

EXPERIMENTS AND RESULTS

4.1. Morphological and Biochemical Characterization of the noble bacteria PSTD 06

4.2. Growth Curve of PSTD 06

4.3. Various Dye Degradation by PSTD 06

4.4. Industrial Green Dye Degradation by PSTD 06

4.5. Analysis of the Treated Product

4.6. Toxicity Studies

4.7. Degradation of IGD by whole cell, lysed cell and cell free extract of PSTD 06

4.8. Degradation study of IGD using PSTD 06 under different conditions

4.9. Study of the effect of heavy metals on IGD Degradation by PSTD 06

4.10. IGD Degradation study by PVA-Alginate beads PSTD 06

4.1. Morphological and Biochemical Characterization of the noble bacteria PSTD 06:

4.1.1. Morphological Characterization

- **Gram Staining:**

Gram staining showed PSTD 06 is a gram positive, small rod shaped bacteria.

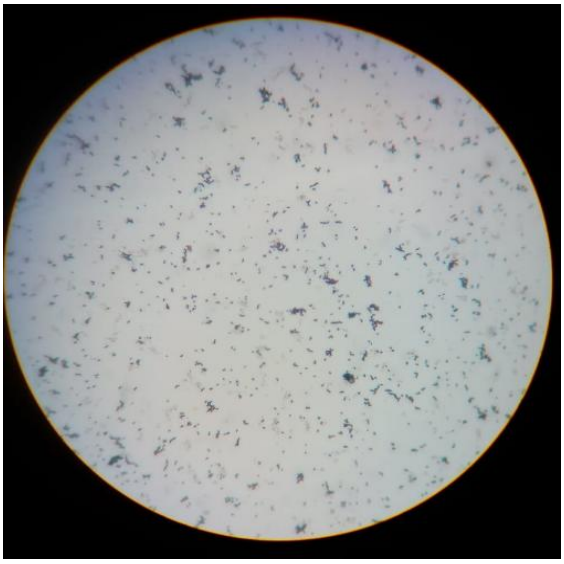

Fig. 3: Gram's staining of PSTD 06.

- **Endospore Staining:**

Endospore formation by PSTD 06 was not observed.

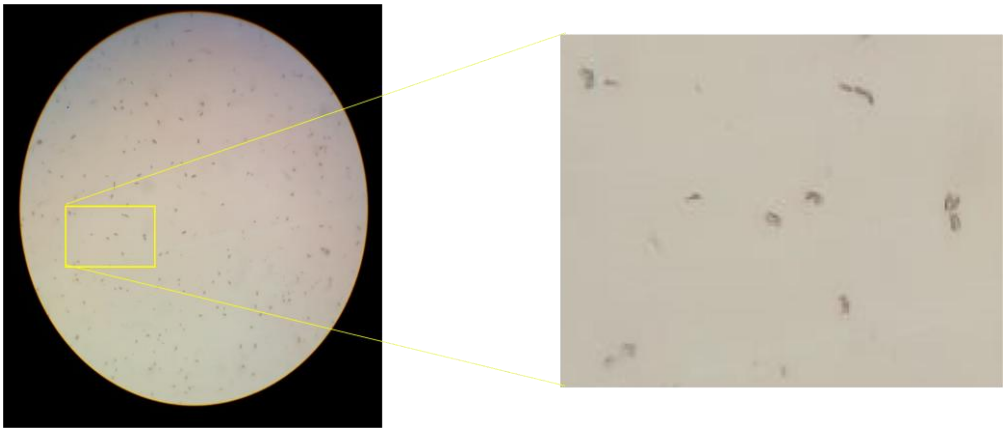

Fig. 4: Endospore staining of PSTD 06.

4.1.2. Biochemical Characterization

- **Catalase Production Test:**

Strong effervescence by PSTD 06 confirmed catalase production by PSTD 06.

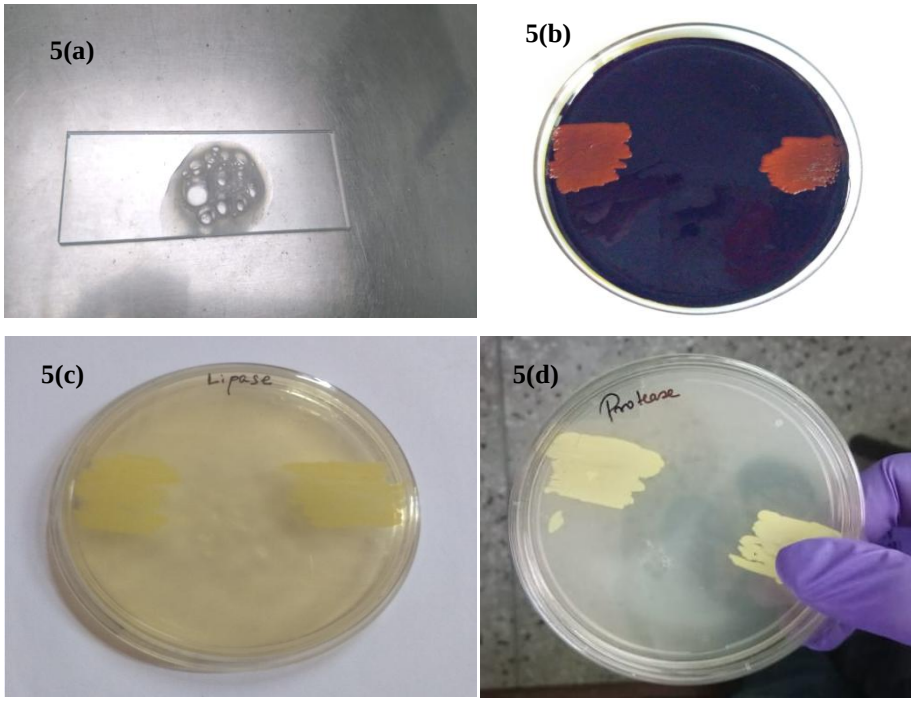

Fig. 5(a,b,c,d): Result of Catalase production test, Amylase production test, Lipase production Test, Protease production test.

- **Amylase Production Test:**

No clear zones were observed in nutrient agar plate (containing starch) around the growth of PSTD 06. Thus negative result was observed, proving amylase wasn't produced by PSTD 06.

- **Lipase Production Test:**

Opaque zones were not seen around the growth of PSTD 06 in the plate, which meant lipase wasn't produced by PSTD 06.

- **Protease Production Test:**

Halo formation was observed around the growth of PSTD 06, showing protease production by PSTD 06.

- **Indole Production Test:**

Absence of prominent blood red ring gave negative result.

- **Nitrate Reductase Test:**

Color change confirmed that PSTD 06 can produce nitrate reductase.

- **Citrate Utilization Test:**

No color change was observed in the plate, giving negative result for the test.

- **Gelatinase Production Test:**

Under cold condition, media was observed to be solid for both the control and the inoculated tubes. Thus proving that gelatin was not degraded by PSTD 06, as it cannot produce gelatinase enzyme.

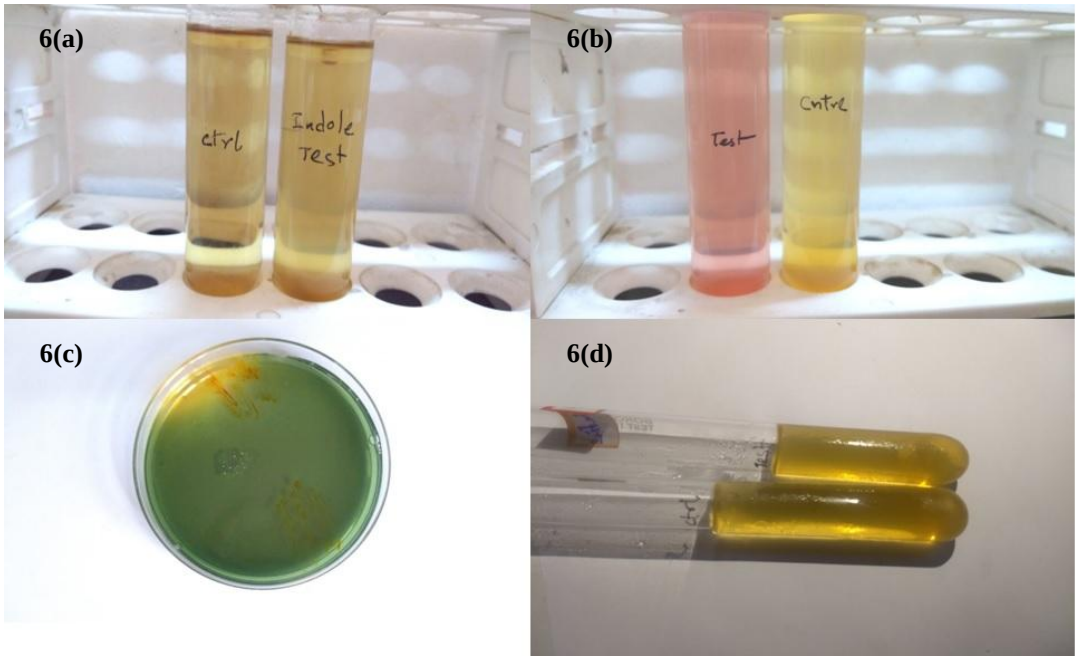

Fig. 6(a,b,c,d): Indole production test, Nitrate reductase test, Citrate Utilization Test, Gelatinase Production Test.

4.2. Growth Curve of PSTD 06:

Fig. 7 shows the growth curve of PSTD 06, which was obtained over a time period of 30hrs (1800 min).

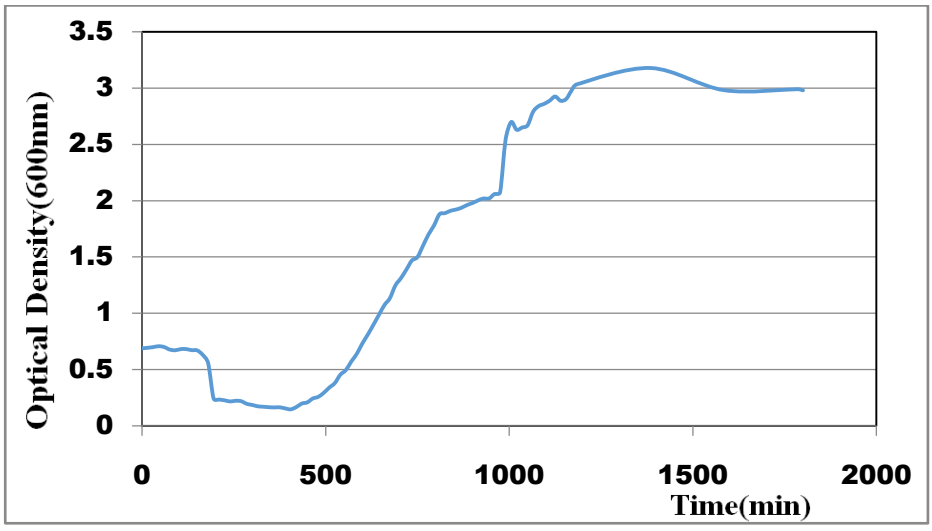

Fig. 7: Growth curve of PSTD 06 in nutrient broth.

4.3. Various Dye Degradation by PSTD 06:

Fig. 8(b) shows the degradation study of the nine selected industrial dyes by PSTD 06. Three dyes, industrial red dye I, industrial yellow dye and industrial blue dye II were degrade less than 50%. Industrial green dye (IGD) and industrial orange dye got almost 100% degraded, by PSTD 06. Most rapid degradation was observed for IGD. IGD reached its maximum degradation percentage within 8 hrs of the study. Whereas maximum degradation of the industrial orange dye was observed in the reading obtained at 240 hrs. So IGD was selected for further study. For getting a more precise degradation pattern of IGD, a degradation study at 30min interval was performed.

Fig. 8 (a): Exploring Dye Degradation capability by PSTD 06.

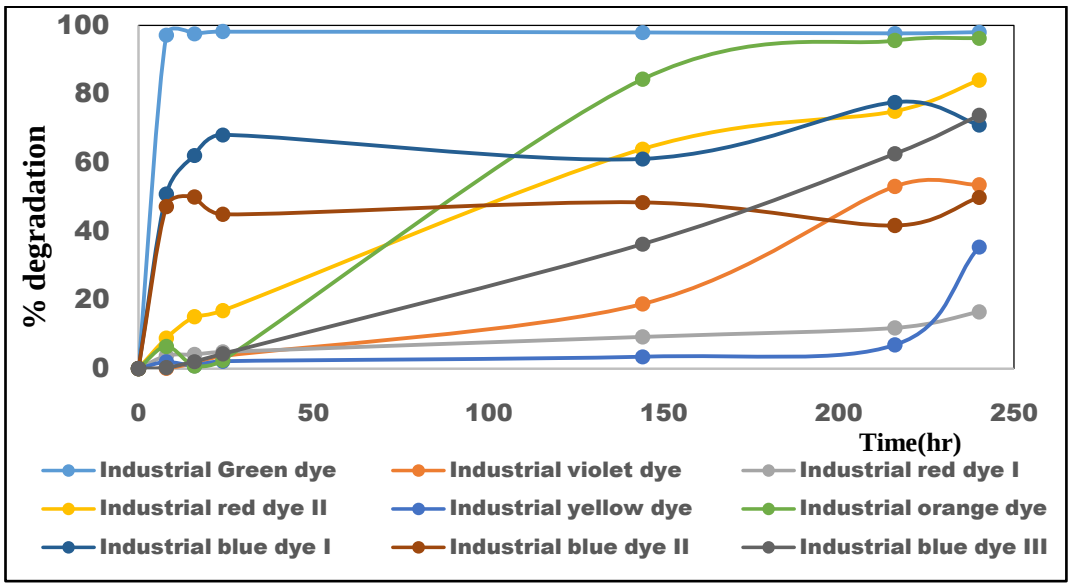

Fig. 8 (b): Graphical plot representing degradation pattern of the selected dyes by PSTD 06.

4.4. Industrial Green Dye Degradation by PSTD 06:

Fig. 9 shows degradation study of IGD by PSTD 06. In this study maximum degradation percentage of IGD by PSTD 06, was obtained after 4hr 50min.

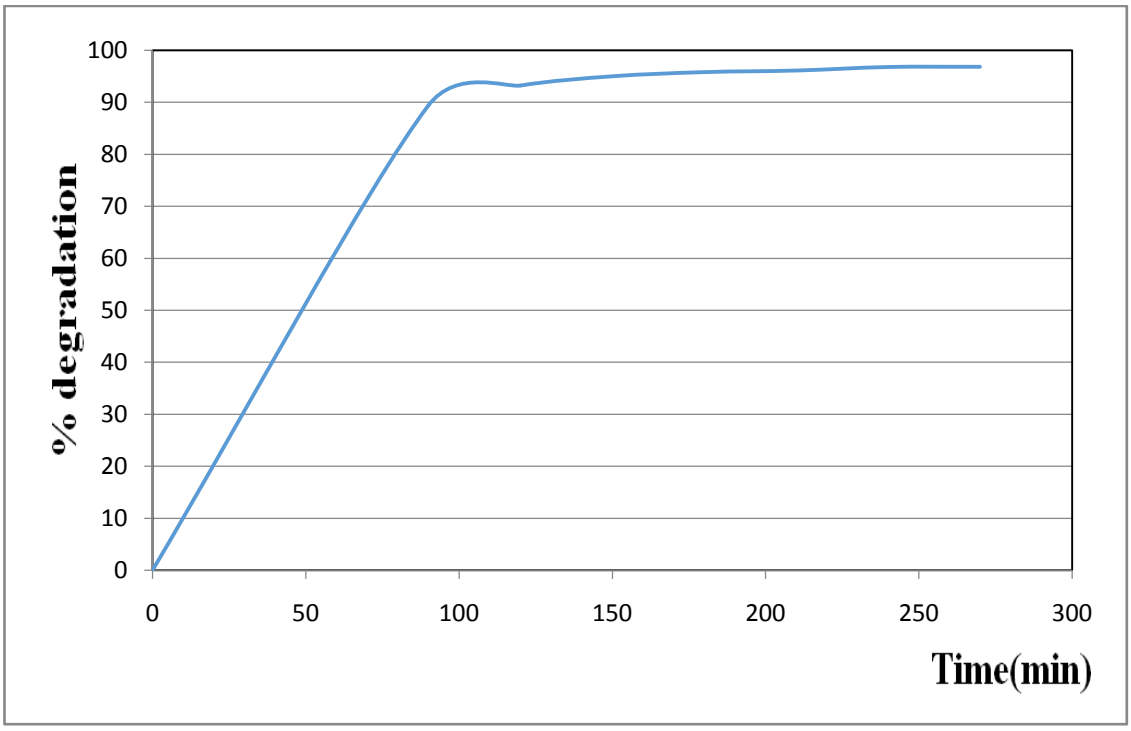

Fig. 9: IGD degradation in MSM by PSTD 06.

4.5. Analysis of the Treated Product:

4.5.1. UV-Vis spectroscopy analysis

Previously it has been reported that if bacterial decolorization of dye is by biosorption, then characteristic absorption peaks of the dye decrease proportionately with decolorization. But, if the decolorization is due to degradation, then either the characteristic absorption peak of the dye decreases completely or a new peak appears (Ayed et al. 2009).Fig 10 shows UV-Vis spectroscopy of IGD and the degraded product (DP) obtained. It is observed that the characteristic absorbance peak of IGD at 614nm got fully decreased and absorbance peak of DP came at 220nm. Hence it was proved that IGD was getting degraded by PSTD 06.

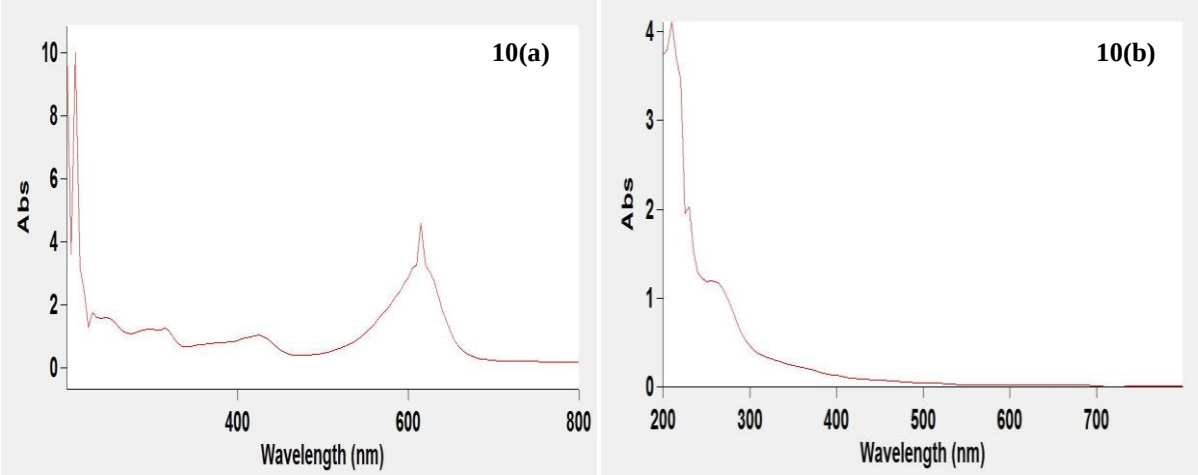

Fig. 10(a, b): UV-Vis spectroscopy of IGD and DP respectively.

4.5.2. Thin Layer Chromatography

Degradation of DP by PSTD 06, was furhter supported by TLC, which was performed by utilizing two diferent solvents.

Solvent I (Propanal):

On observing the dye chromatogram (Figure11.a.) after keeping in iodine chamber, DP spot with R_f values 0.571 and 0.714 were found. No similar bands were observed for spot of IGD and uninoculated medium. Hence indicating the decolorization due to degradation.

Solvent II (Butanol:Acetic Acid:Water=4:1:2,v/v):

Similarly for solvent II dye chromatogram (Figure 11.b.), DP spot showed R_f values 0.488, 0.627 and 0.744, but IGD with uninoculated medium spot showed R_f value of 0.651 claiming the degradation of DP.

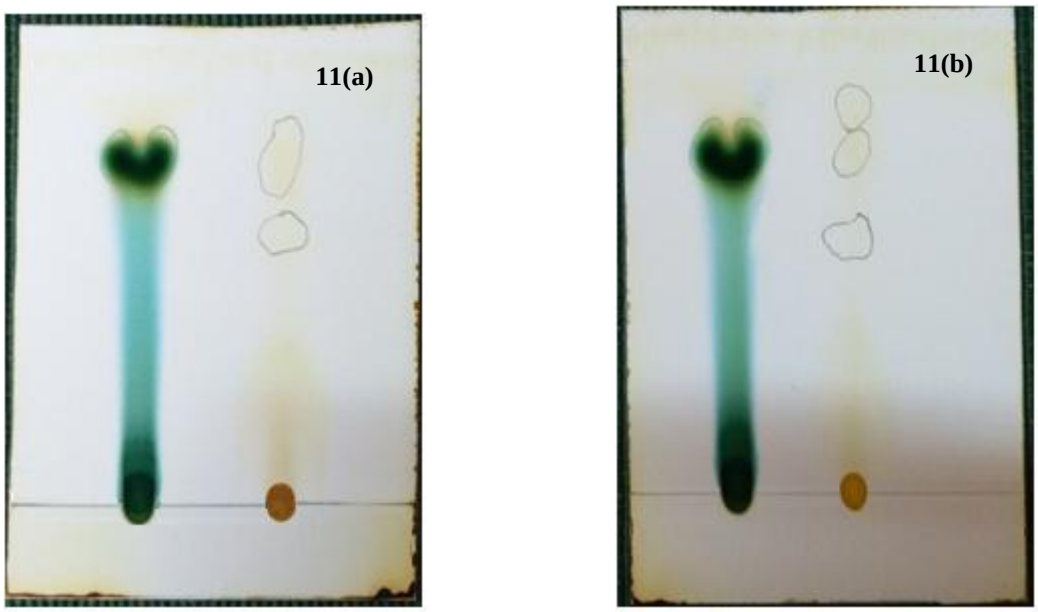

Fig. 11(a,b): TLC plates with mobile phase solvent I and solvent II respectively.

Propanol

Solvent front = 4.2 cm

Industrial green dye = 3.1 cm

Treated sample= 2.4 cm and 3cm

Rf (Malachite green dye) = 3.1cm/4.2cm=0.7380

Rf 1(Treated Sample) = 2.4cm/4.2cm=0.571

Rf 2 (Treated sample) = 3cm/4.2cm=0.714

Butanol :acetic acid : water(4:1:2)

Solvent front = 4.3 cm

Industrial green dye = 2.8cm

Treated sample= 2.1cm, 2.7cm, 3.2cm

R_f (Malachite green dye) = 2.8cm/4.3cm=0.651

R_{f1} (Treated sample)=2.1cm/4.3cm=0.488

$R_{f\,2}$ (Treated sample)=2.7cm/4.3cm=0.627

$R_{f\,3}$ (Treated sample)=3.2cm/4.3cm=0.744

4.5.3. Fourier Transform Infrared Spectroscopy (FTIR) analysis

Fig 12 (a) and Fig 12 (b) are showing the FTIR spectrum of IGD and DP respectively. The peaks observed in these two spectra are different from each other. Thus, degradation of IGD by PSTD 06 was again confirmed by the FTIR spectroscopy.

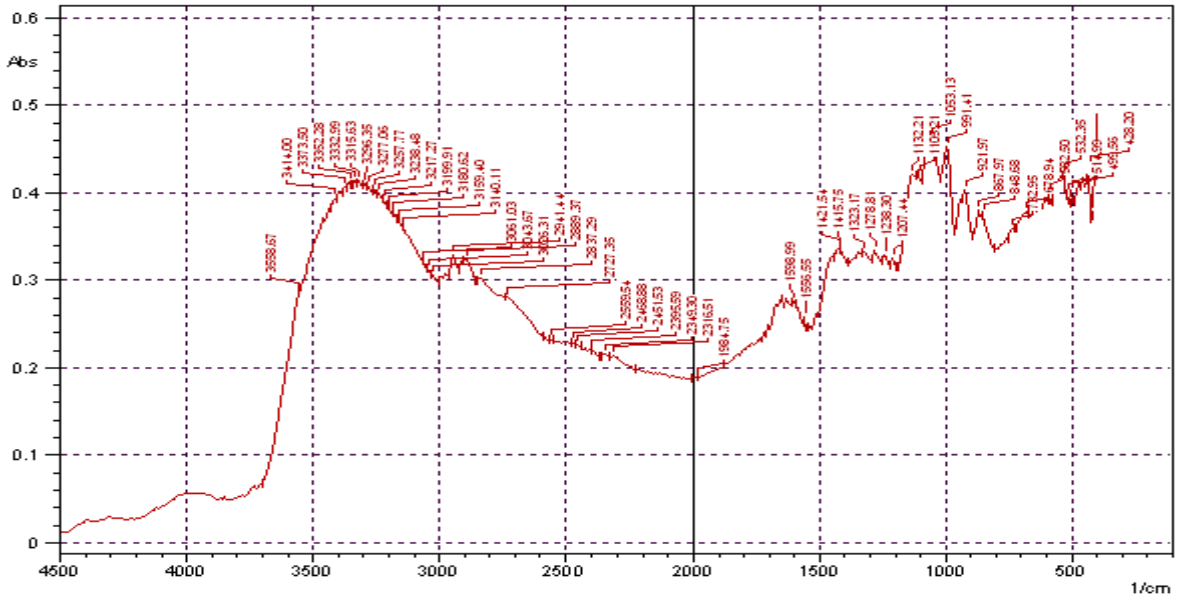

Fig. 12 (a): FTIR spectrum of IGD.

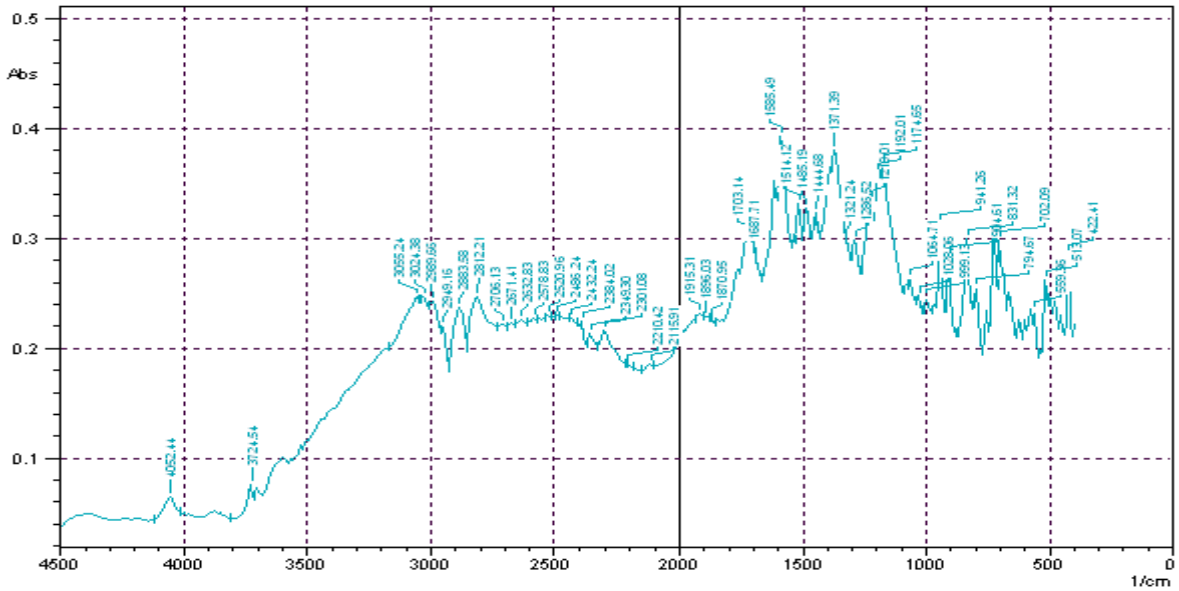

Fig. 12 (b): FTIR spectrum of DP.

4.6. Toxicity Studies:

After confirming the proper degradation of IGD, checking the toxicity of DP in comparison with that of IGD was essential.

4.6.1. Toxicity study using beneficial bacteria

Growth inhibition assay of both the beneficial bacteria showed no zone of inhibition incase of PATD-DP similar to that of the control, though IGD gave zone of inhibition of 2.29cm and 2.045cm for *Bacillus pumilus* and *Bacillus subtilis* respectively. Hence confirming that for these bacteria DP wasn't toxic.

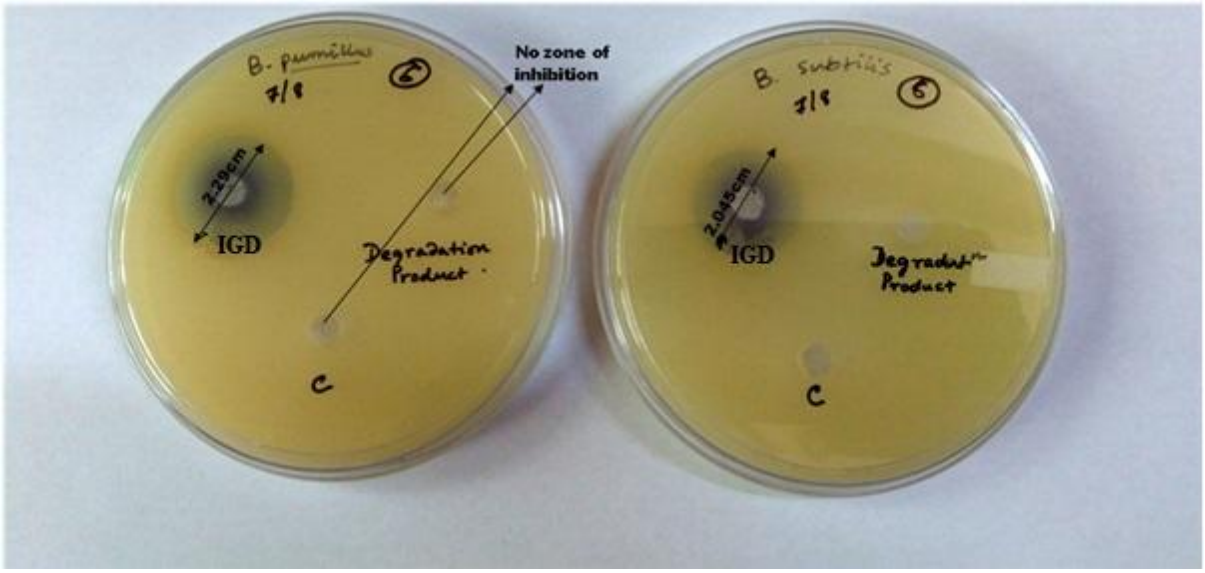

Fig. 13: Growth inhibition assay on *Bacillus pumilus* and *Bacillus subtilis*.

Growth inhibition curve of *Bacillus pumilus* and *Bacillus subtilis* are shown below. Fig 14 and Fig 15 are the growth inhibition curves of *Bacillus pumilus* and *Bacillus subtilis* respectively. For both the bacteria it was observed, that the growth curve obtained in presence of DP was very much similar to that obtained in presence of the control distilled water.

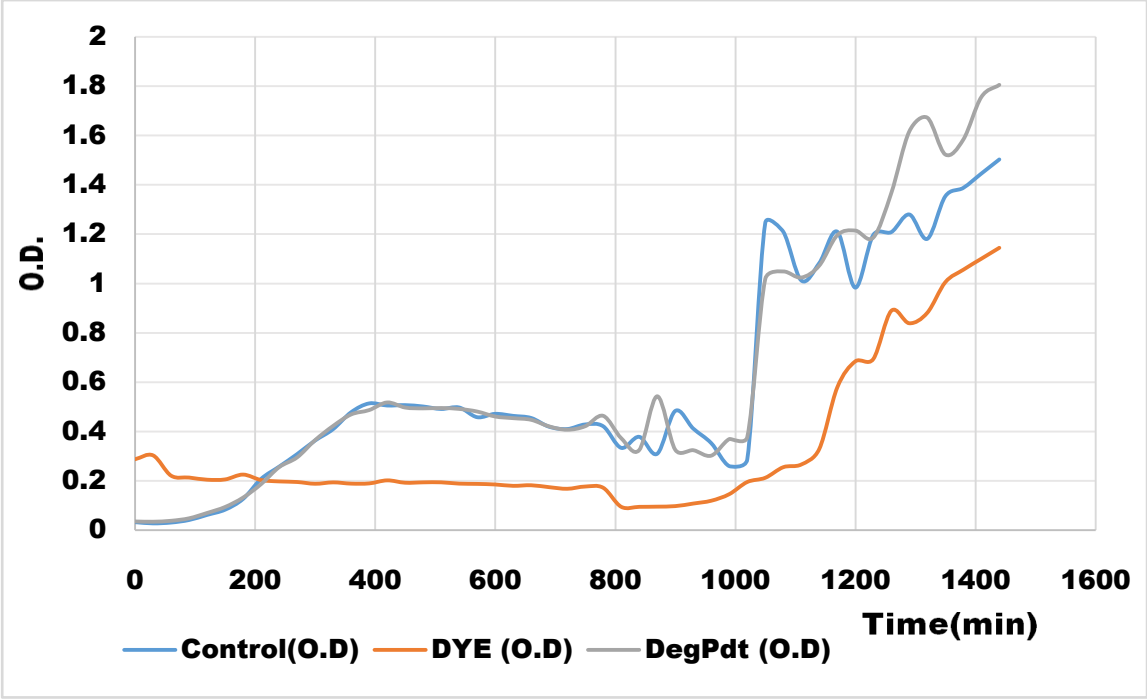

Fig. 14: Growth inhibition curve of *Bacillus pumilus*.

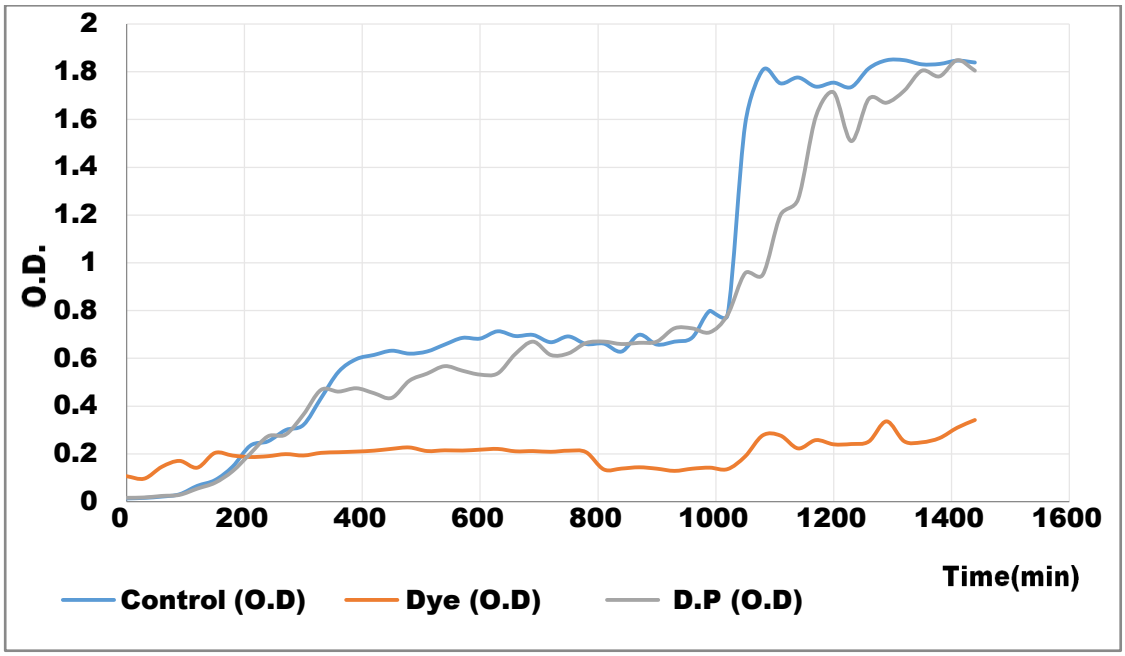

Fig. 15: Growth inhibition curve of *Bacillus subtilis*.

4.6.2. Phytotoxicity study using *Oryza Sativa*

Germination observation of *Oryza sativa*:

From Table 1, it is evident that number of seeds germinated in presence of DP was same with that germinated in control. Thus showing that, DP didn't cause any inhibition to the germination of the seeds. But only three seeds germinated in presence of toxic IGD.

Fig. 16: Experimental setup of phytotoxicity study on *Oryza sativa*, showing its germination in presence of IGD, DP and water.

Experimental condition	No. of seeds germinated
Control(water)	**21**
DP	**21**
IGD	**3**

Table 1: Observation of *Oryza sativa* germination.

Shoot length observation of *Oryza sativa* w for 21 days:

From table 2 and fig 17, 18; it is observed that growth of shoot in presence of DP was much closer to that obtained in the control. But growth of shoot got stunned in presence of IGD.

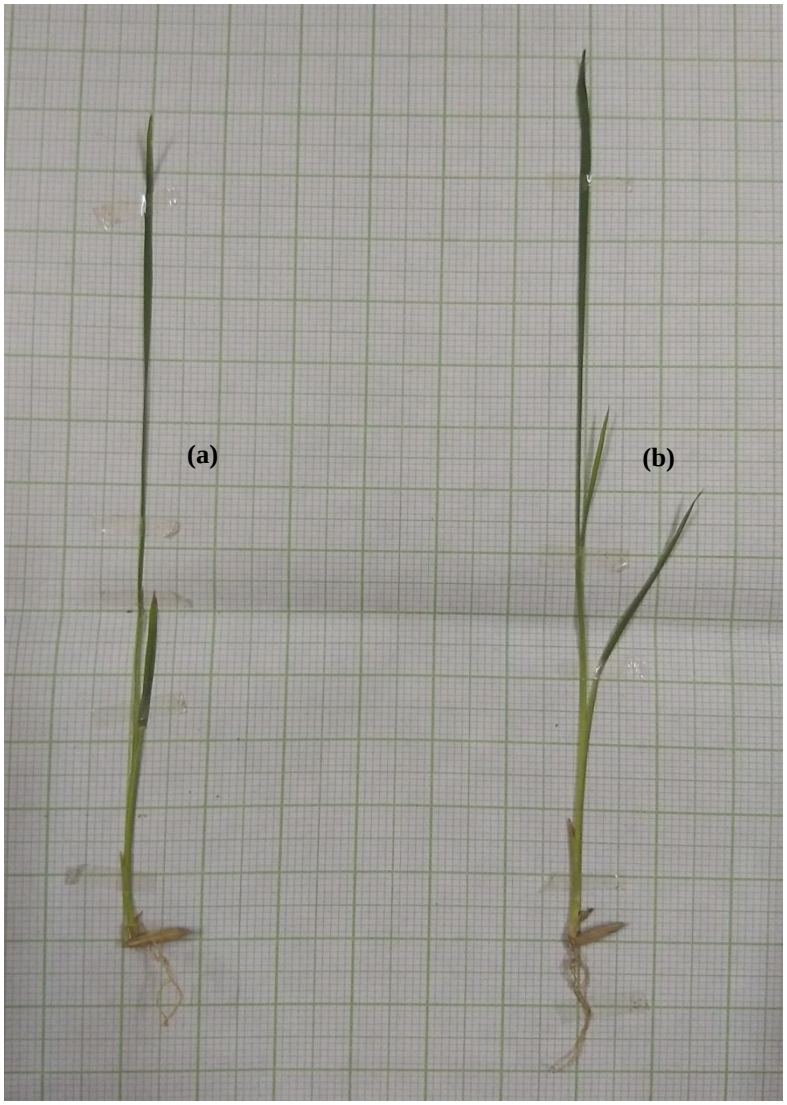

Fig. 17(a, b): *Oryza sativa* shoot development on 21st day of observation in presence of DP and Water (Standard).

Day	Standard (avg growth in cm)	DP (avg growth in cm)	IGD (avg growth in cm)
0	**0**	**0**	**0**
11	**8.53**	**7.59**	**0.33**
13	**9.15**	**8.02**	**0.33**
15	**9.56**	**8.40**	**0.33**
17	**10.23**	**9.51**	**0.33**
19	**11.49**	**10.33**	**0.33**
21	**12.83**	**11.90**	**0.33**

Table 2: Shoot length observation of *Oryza sativa*

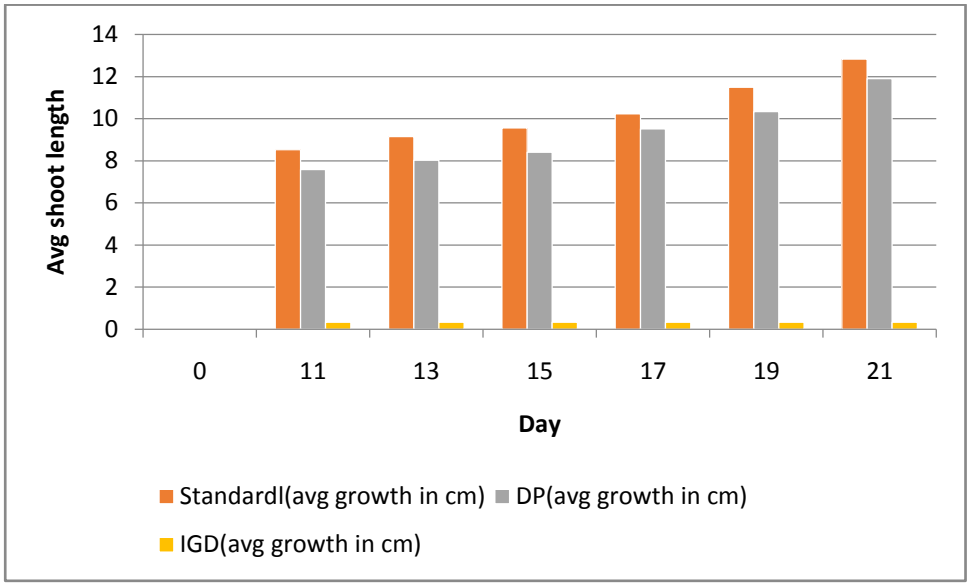

Fig. 18: Graphical representation of shoot length observation of *Oryza sativa.*

4.6.3. Toxicity study on *Eisenia fetida*

Observation of contact toxicity test after 72 hrs:

The study showed (as illustrated in table 3) that DP wasn't toxic when in contact with the body of earthworm, so all the five adult earthworms survived 72 hrs of contact treatment by DP as was seen for the control. In all the contact treatments involving varying amounts of IGD, more than one death of earthworms were observed.

Contact treatment done with	**No. of living earthworms at 0 hr**	**No. of living earthworms at 72 hrs**
Control (Distilled water)	5	5
3.25 mg of IGD	5	4
6.5 mg of IGD	5	2
13 mg of IGD	5	2
19.5 mg of IGD	5	1
26 mg of IGD	5	1
39 mg of IGD	5	1
DP	5	5

Table 3: Observation of contact toxicity test on *Eisenia fetida*

Observation of Survival test after 14 days:

The survival test showed that with the increasing amount of IGD, number of surviving earthworms decreased. Out of five adult earthworms, only one died in presence of DP. Thus again confirming that DP is less toxic than IGD for earthworms (*Eisenia fetida*).

Survival test done with	No. of living earthworms on Day 1	No. of living earthworms on Day 14
Control (Distilled water)	5	5
1 mg of IGD	5	3
2 mg of IGD	5	2
4 mg of IGD	5	2
6 mg of IGD	5	1
8 mg of IGD	5	0
DP	5	4

Table 4: Observation of Survival test on *Eisenia fetida*

4.6.4. Cytotoxicity Assay on PBMC

Percentage of cell viability was found to be similar in case of both the Cis-Platin (control) and IGD, but that was more in case of DP. So it can be concluded that DP is comparatively less toxic to mammalian cells than IGD.

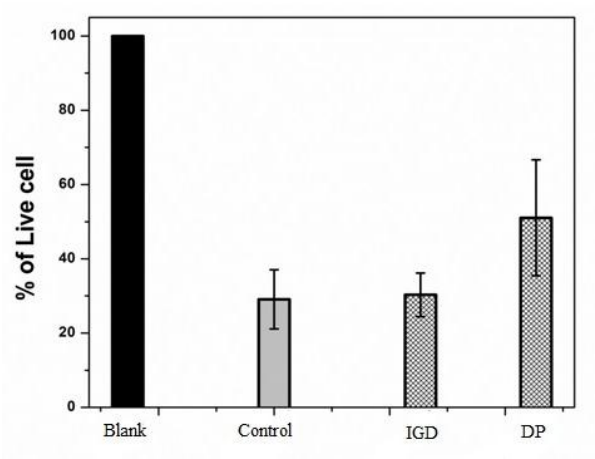

Fig. 19: MTT Assay on PBMC

4.7. Degradation of IGD by whole cell, lysed cell and cell free extract of PSTD 06

Observation of the Fig 20, leads to the conclusion that PSTD-06 whole cells were found to be having the best potential for degradation of IGD, followed by lysed cells and cell free extracts of PSTD 06.

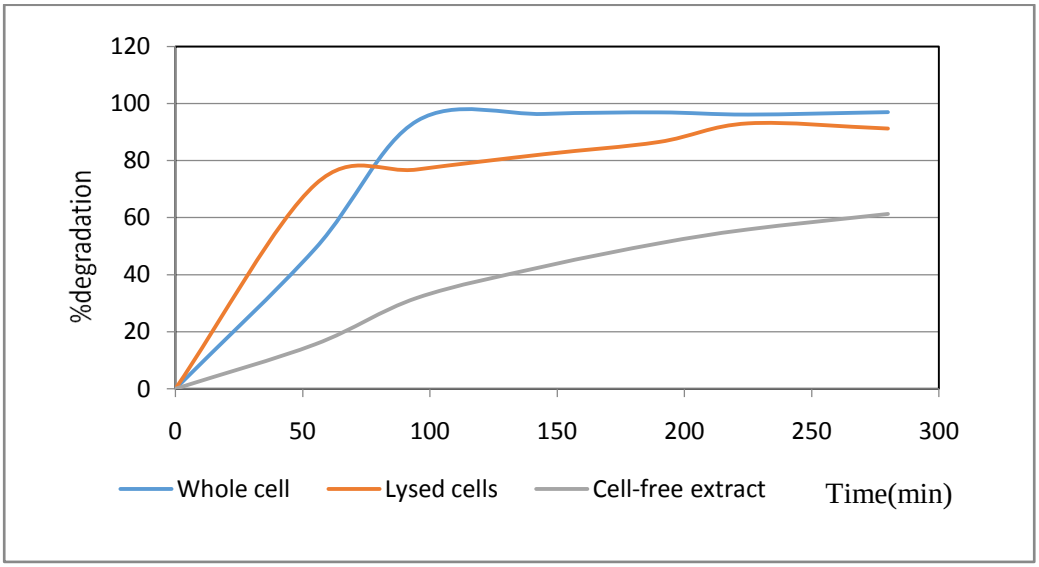

Fig. 20: Degradation of IGD by whole cell, lysed cell and cell free extract of PSTD 06 in MSM.

4.8. Degradation study of IGD using PSTD 06 under different conditions:

4.8.1. Inoculum Variation

Figure 21 represents the degradation pattern of IGD by PSTD 06 depending on the varying dose of inoculums. With the increasing inoculum dose from 2%(v/v) till 22%(v/v), initially the degradation percentage of IGD increased showing the best result for 10%(v/v) and

14%(v/v). But then it started decreasing and showed around 80% degradation for inoculums dose 22% (v/v).

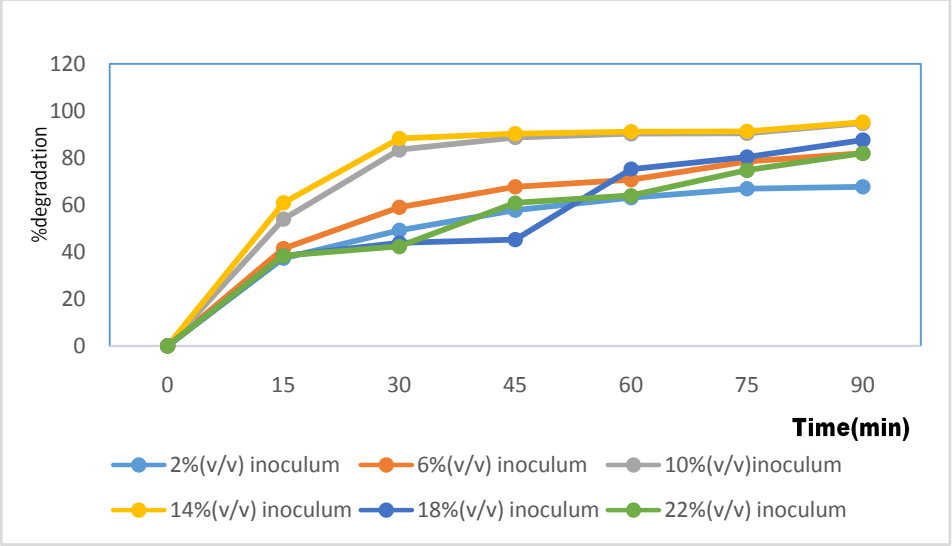

Fig. 21: Degradation study of IGD using PSTD 06 under varying inoculum dose.

4.8.2. Initial Dye Concentration Variation

Initial dye concentration affects the degradation of IGD significantly. When initial IGD concentration was from 20mg/l till 60mg/l, there was nearly 100% degradation of IGD. For concentrations of 80mg/l and 100mg/l, degradation was below 70% and below 50% respectively.

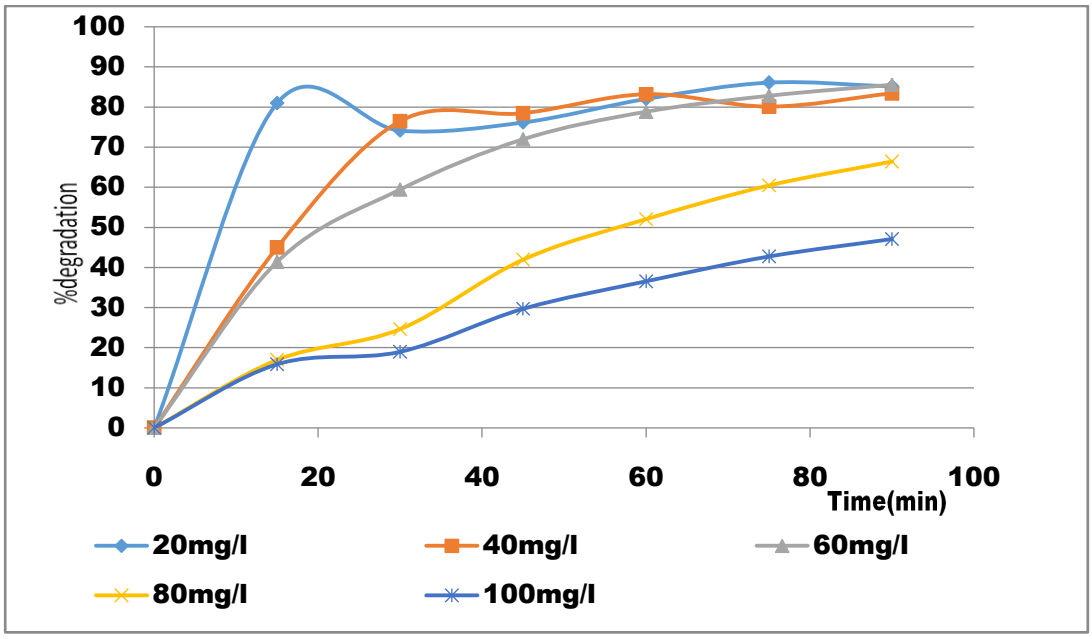

Fig. 22: Degradation study of IGD using PSTD 06 under varying initial dye concentration.

4.8.3. Temperature Variation

At temperature of 20°C, degradation of IGD was just above 50%. Percentage of degradation sharply reached nearly 100% when the temperature applied was increased to 30°C and 40°C. At 50°C, percentage degradation was just above 80% and got decreased to around 60% at temperatures 60°C and 70°C.

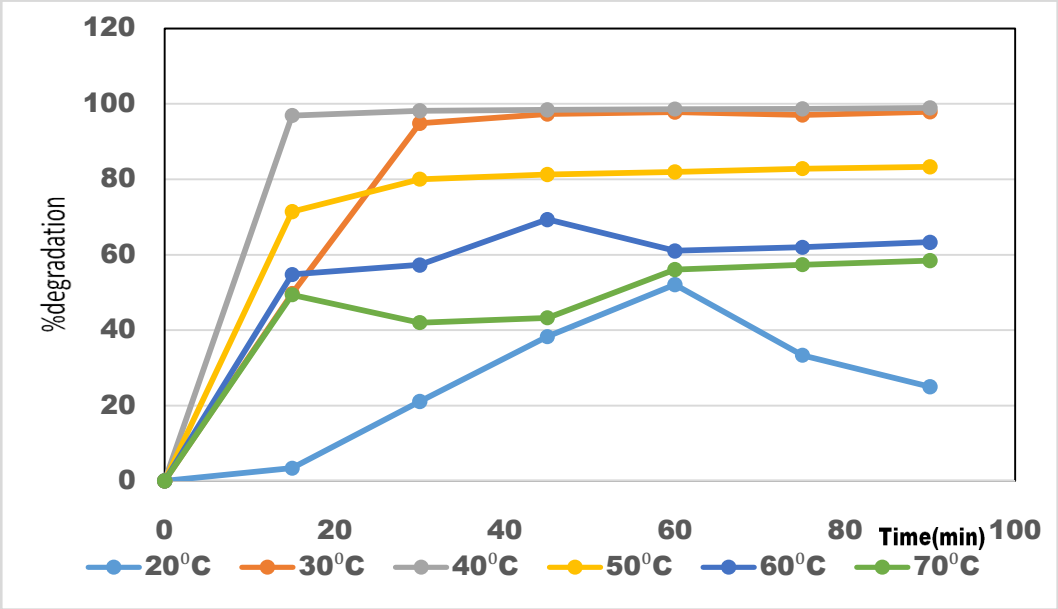

Fig. 23: Degradation study of IGD using PSTD 06
under varying temperatures.

4.8.4. pH Variation

PSTD 06 was able to perform above 90% degradation of IGD when implemented pH of the media was from 4.5 to 10.5. Within this range, efficiency of degradation gradually increased reaching the maximum at pH 6.5 and again there was slight decrement in efficiency with the increasing pH. For pH 3.5 degradation percentage was just above 20%. Less than 5% degradation was obtained at highly acidic pH values of 1.5 and 2.5.

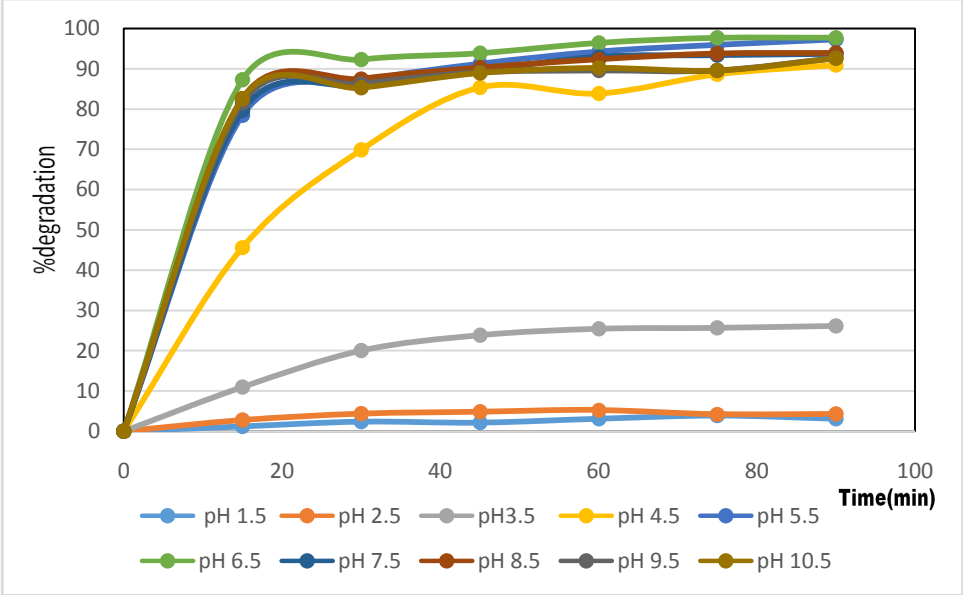

Fig. 24: Degradation study of IGD using PSTD 06 under varying pH.

4.8.5. Different Carbon Sources

Among the seven different carbon sources utilized for the study, degradation was found to be most efficient, above 90% for dextrose and ribose. Degradation percentage reached just above 80% for lactose. For media substituted with molasses, fructose, maltose and yeast extract as carbon source, degradation percentage remained within 70-80%.

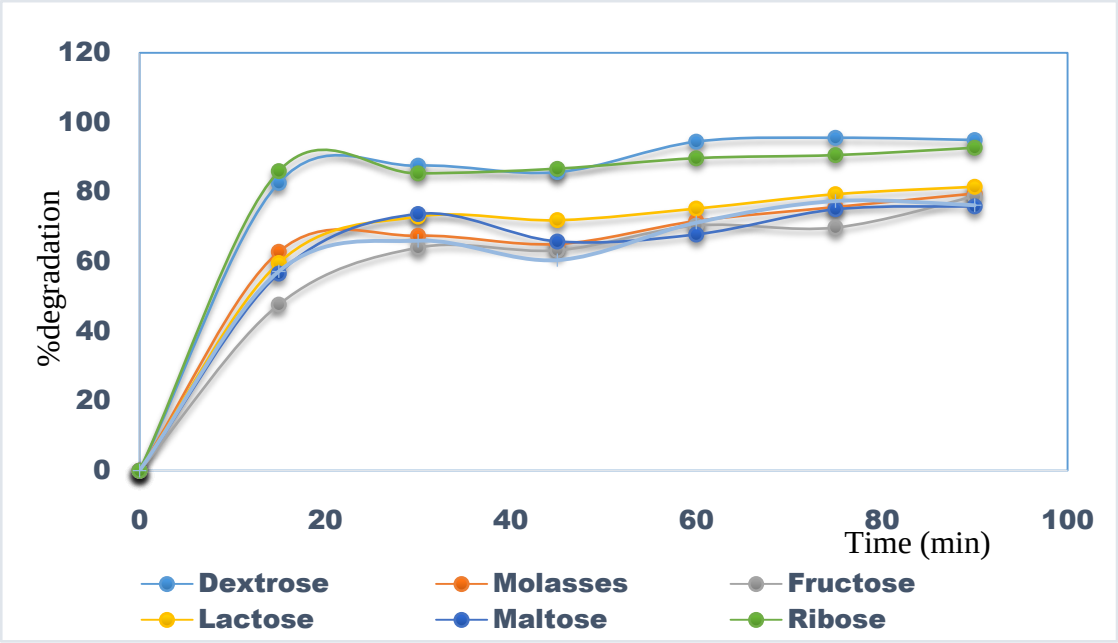

Fig. 25: Degradation study of IGD using PSTD 06
in different carbon source substituted MSM.

4.8.6. Different Nitrogen Sources

Organic Nitrogen Sources

For media substituted with organic nitrogen sources soyabean meal, tryptone, urea, beef extract and yeast extract, PSTD 06 showed above 90% degradation of IGD. Among these soyabean meal showed the best result. Almost 90% degradation was obtained in case of thiourea and almost 70% was for peptone.

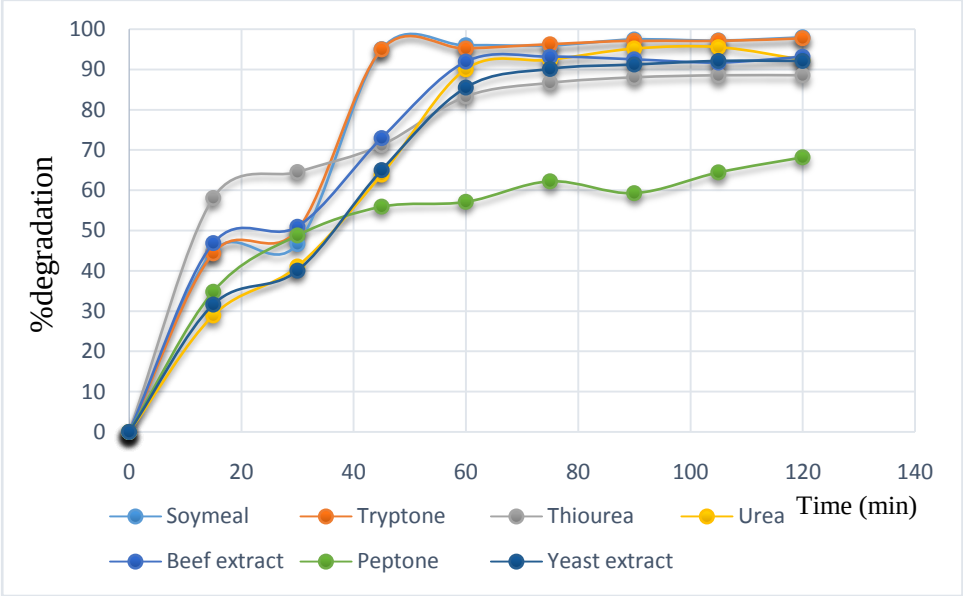

Fig. 26: Degradation study of IGD using PSTD 06
in different nitrogen source (organic) substituted MSM.

Inorganic Nitrogen Sources

Among the four media prepared with substituted inorganic nitrogen sources, 97-96% degradation was observed for those containing sodium azide, calcium nitrate and sodium nitrite. 89% degradation of IGD was achieved by PSTD 06 in presence of ammonium hydrogen phosphate.

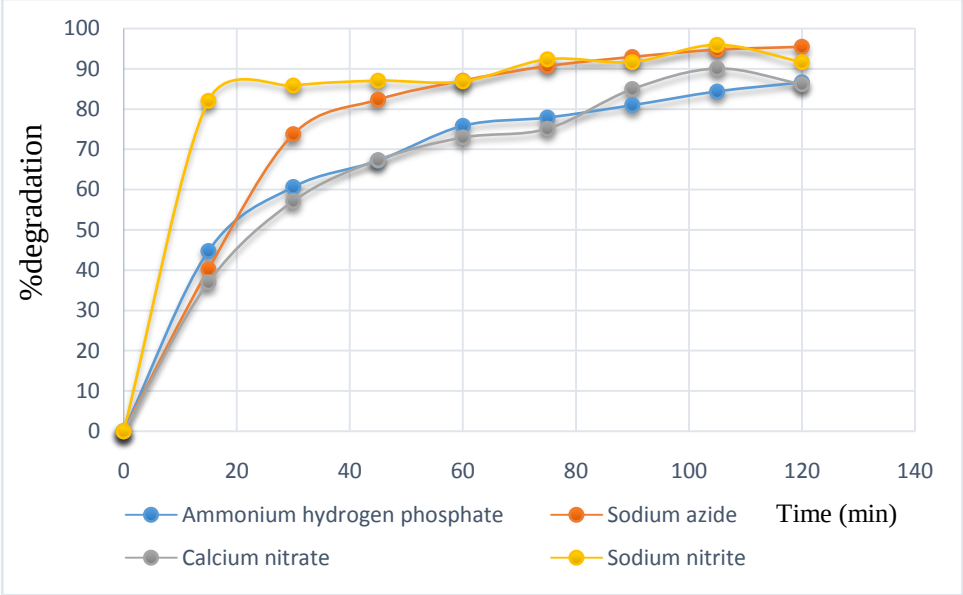

Fig. 27: Degradation study of IGD using PSTD 06
in different nitrogen source (inorganic) substituted MSM.

4.8.7. Salinity Variation

Figure 28 illustrates the result obtained from the study of IGD degradation by PSTD 06 under varying salinity. PSTD 06 showed decrease in efficiency of IGD degradation with the increasing salinity, while achieving over 90% degradation in 40g/l and lower salinity concentrations. PSTD 06 was capable of over 50% degradation till 140g/l salt concentration.

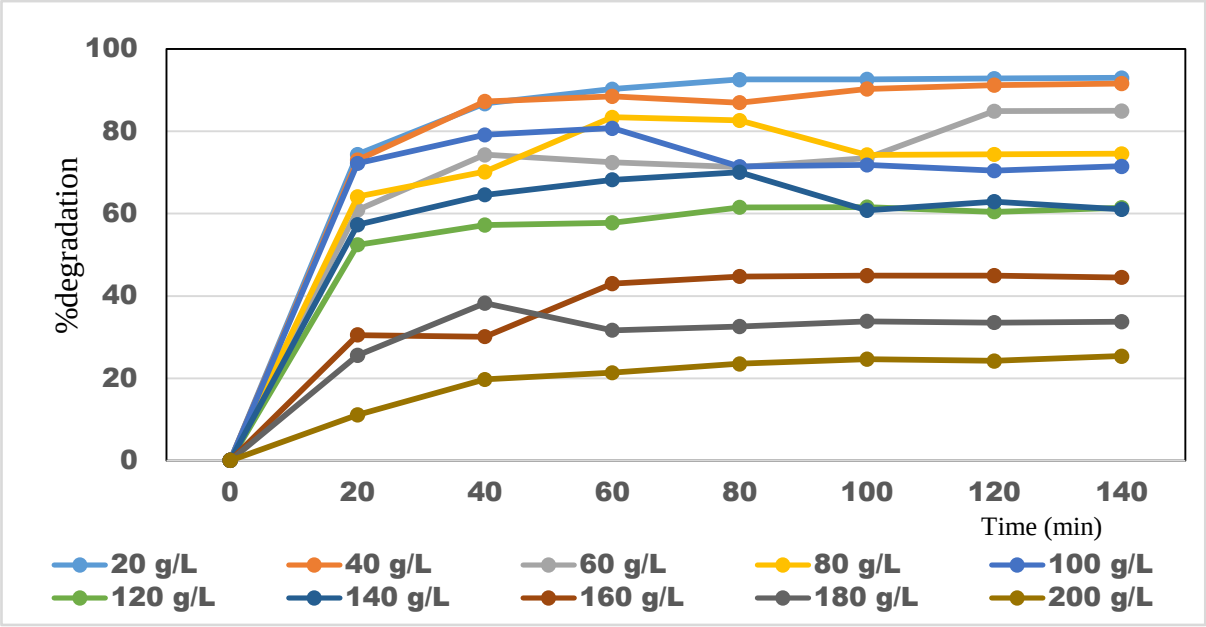

Fig. 28: Degradation study of IGD using PSTD 06 under varying salinity.

4.9. Study of the effect of heavy metals on IGD Degradation by PSTD 06:

4.9.1. Effect of Arsenic

Figure 29 represents the dye degradation patterns of IGD by PSTD 06 under arsenic stress at various concentrations. PSTD 06 seems to be tolerant to arsenic stress and hence showing over 90% degradation even at 5g/l As concentration. Minute decrement in the efficiency of IGD degradation was observed with the sharp increment of the arsenic concentration in the media.

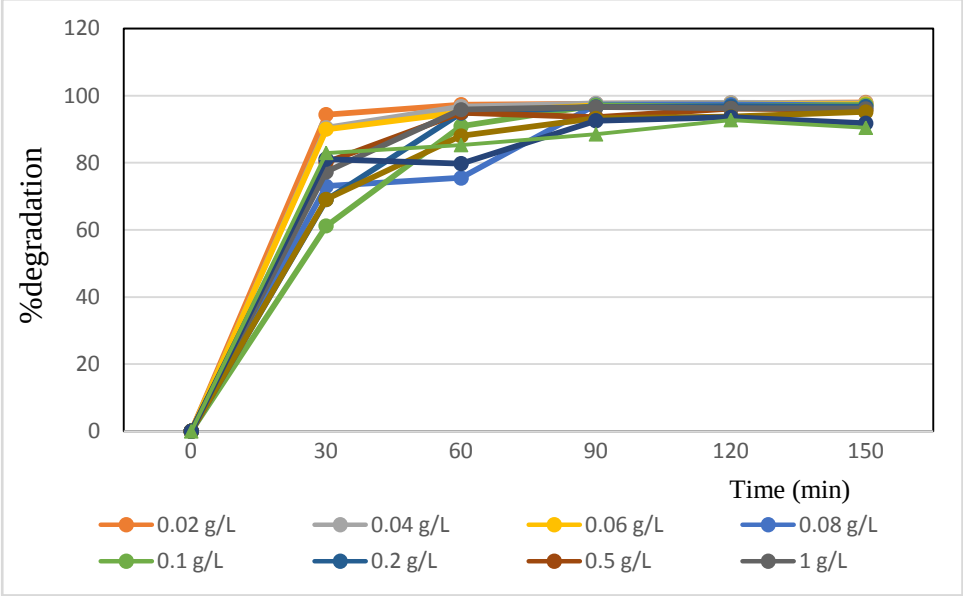

Fig. 29: IGD degradation pattern by PSTD 06 in presence of arsenic.

4.9.2. Effect of Lead

Higher stress of lead concentration seems to have significant inhibition on the degradation by PSTD 06. Less than 50% degradation of IGD was observed under lead stress of 0.6g/l and higher concentrations, whereas PSTD 06 showed above 90% degradation capability under lead stress of 0.4g/l and lower concentration.

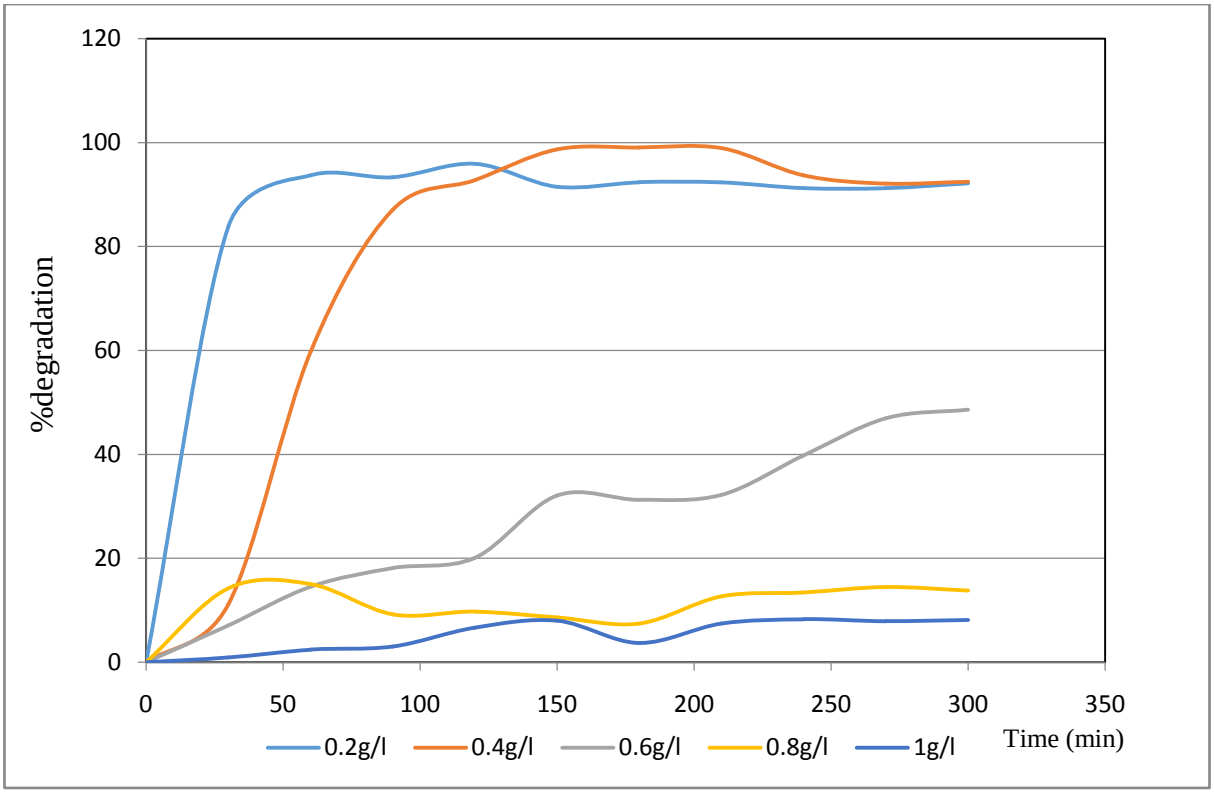

Fig. 30: IGD degradation pattern by PSTD 06 in presence of lead.

4.9.3. Effect of Mercury

Mercury stress of 0.25g/l and higher concentration shows less than 30% IGD degradation. 80-90% degradation was obtained for 0.125g/l and lower concentrations of mercury in growth media of PSTD 06.

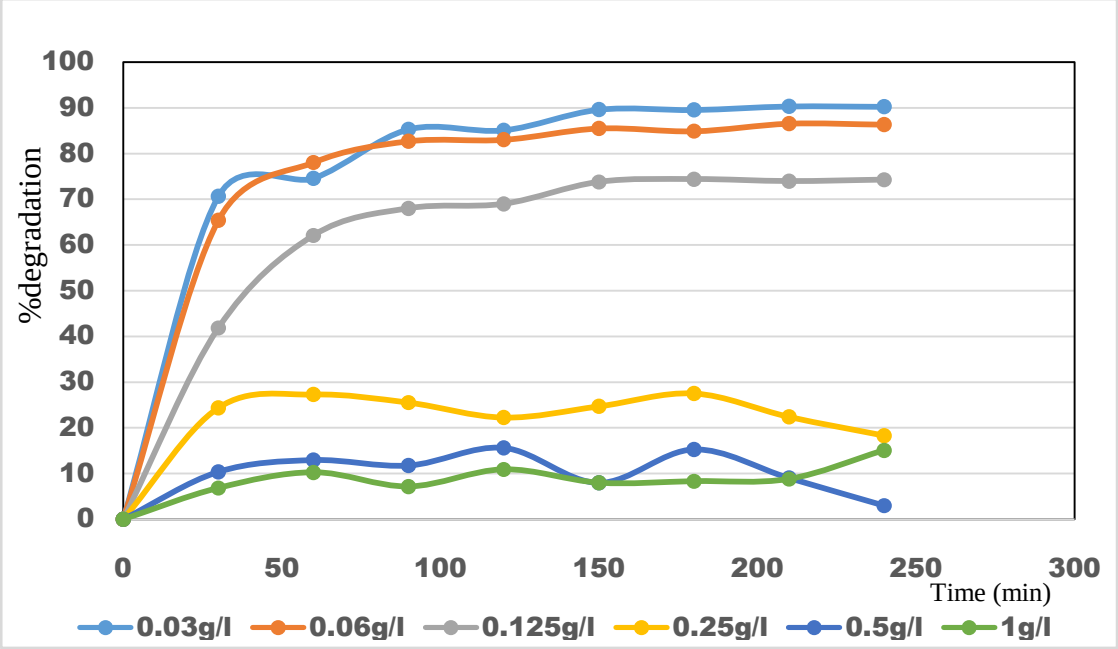

Fig. 31: IGD degradation pattern by PSTD 06 in presence of mercury.

4.9.4. Effect of Chromium

Chromium had prominent inhibition effect on the degradation activity of PSTD 06. The bacterium was able to degrade around 80% of IGD while tolerating stress of 0.04g/l and lower concentration of cadmium in media. Around 50% degradation was seen within the chromium stress range of 0.06-0.08g/l, and less than 50% degradation was there for 0.1g/l and higher concentration of chromium.

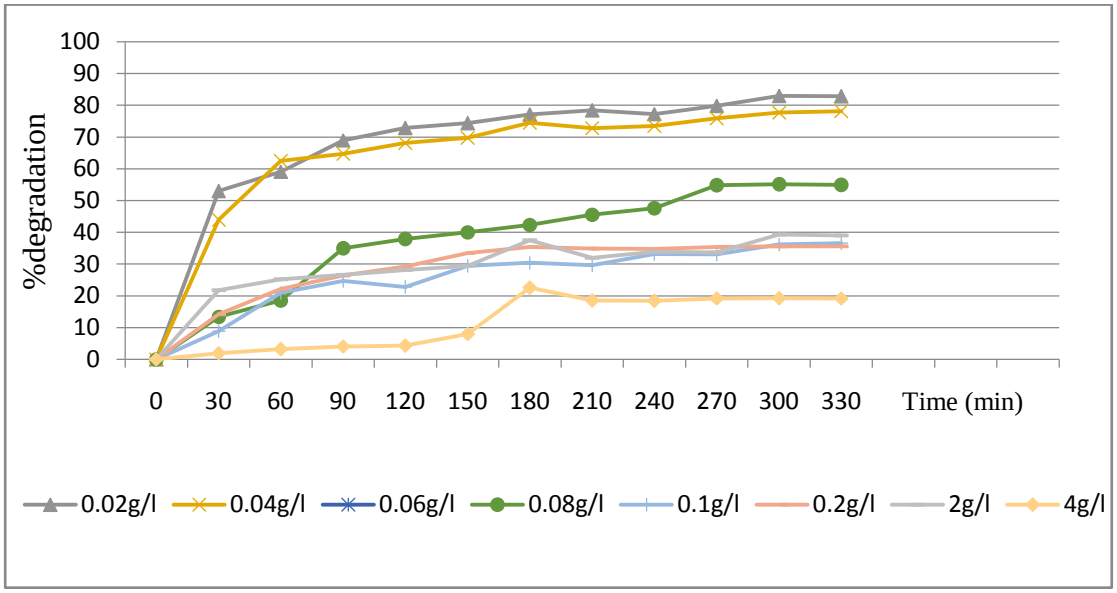

Fig. 32: IGD degradation pattern by PSTD 06 in presence of chromium.

4.9.5. Effect of Cadmium

Above 50% IGD degradation was achieved by PSTD 06 under cadmium stress within 0.025-0.5g/l cadmium stress, with around 90% and 80% degradation at 0.05-0.025g/l and 0.1-0.075g/l cadmium stress in the media. At higher concentration of cadmium, 1g/l the degradation percentage got significantly reduced almost to 30%.

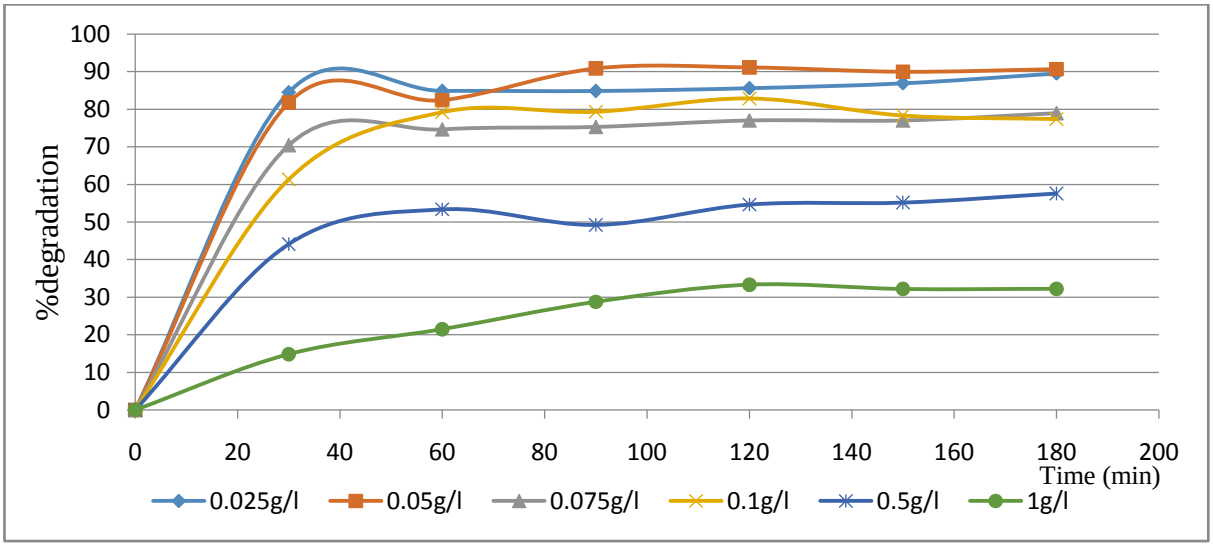

Fig. 33: IGD degradation pattern by PSTD 06 in presence of cadmium.

4.10. IGD Degradation study by PVA-Alginate beads of PSTD 06:

Figure 34 shows the result of 20hrs degradation study of IGD by PVA-Alginate beads of PSTD 06. The19 hr study showed that degradation rate got increased with the increased amount of beads used. Maximum amount of beads used for the study, equivalent to 18% (v/v) inoculums dose after 19 hr study showed above 90% degradation of IGD.

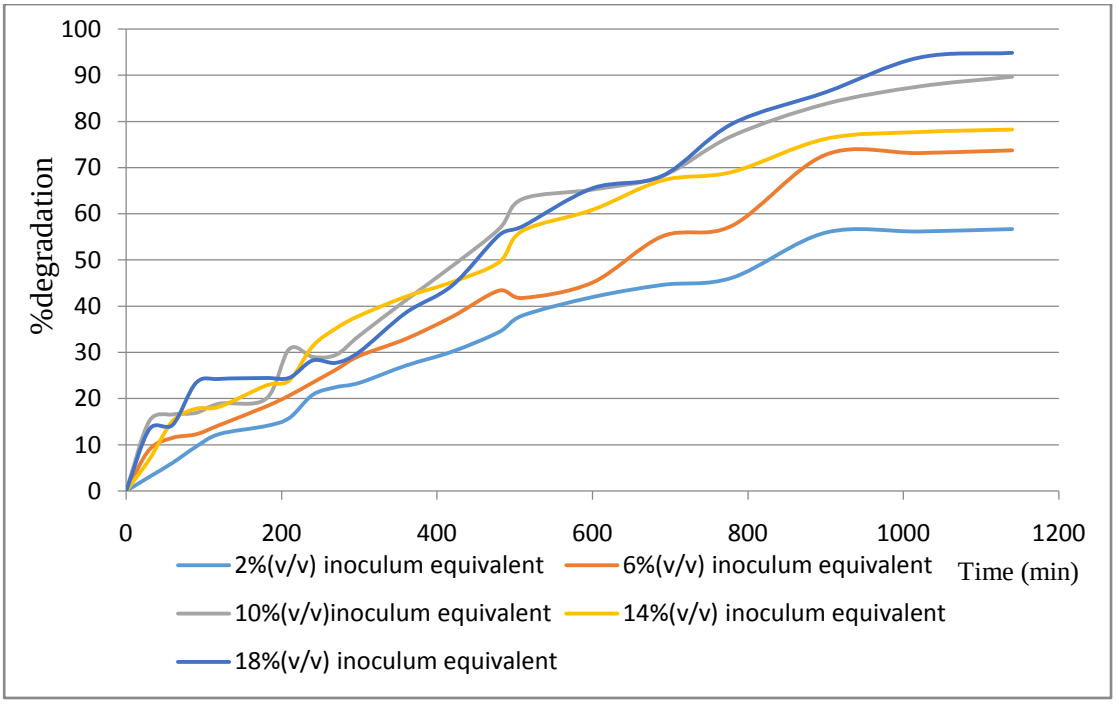

Fig. 34: 19 hrs IGD degradation study by PVA-Alginate beads of PSTD 06.

CHAPTER 5:
CONCLUSION AND FUTURE SCOPE

5.1. Summary of the work

5.2. Future directions

5.1. Summary of the work:

Dye degradation capability of the isolated strain of noble bacteria (PSTD 06) was explored after checking its biochemical and morphological characterization. The industrial green dye (IGD) showing the best and fastest decolorization was selected for the study. By analyzing the obtained end product (DP) with the help of UV-vis spectroscopy, TLC and FTIR, it was confirmed that the decolorization was due to bacterial degradation. Then toxicity studies confirmed that DP wasn't much toxic and hence can be released to the environment as the treated product of IGD. By studying the dye degradation under varying condition, it was observed that above 95% degradation was obtained when 10% (v/v) inoculum dose was applied at initial dye concentration of 20-60mg/l, incubation temperature was 40°C initial media pH within 4.5-10.5. Dextrose and ribose were found to be the best carbon sources, calcium nitrate and sodium azide were the best inorganic nitrogen sources and trypton was found to be the best organic nitrogen source for the dye degradation by PSTD 06. Study by varying salinity, showed that PSTD 06 was able to degrade above 90% at 40g/l and lower concentrations of salt. By studying the effects of heavy metal stress, it was observed that over 85% degradation of the dye was achieved by PSTD 06 while tolerating stress of 5g/l and lower concentration of As, 0.4g/l and lower concentration of Pb, 0.06g/l and lower concentrations of Hg, 0.02g/l and lower concentrations of Cr and 0.05g/l and lower concentrations of Cd. Ultimately PVA-alginate beads of PSTD 06 were formed to study IGD degradation for 19hrs to achieve over 95% degradation with beads equivalent to 18%(v/v) inoculum dose.

PSTD 06 was found to be degrading IGD efficiently at conditions prevailing in industrial effluents, so the bacterial strain can be used at commercial level for degradation of IGD.

5.2. Future directions:

1. Optimization of the degradation capability of beads, by varying the PVA alginate and bacterial culture ratios.
2. Other immobilization techniques can be utilized to study for any further improvement.
3. Impressive result was seen regarding the degradation of industrial orange dye, which can also be explored for future work.
4. Consortium can be made with other dye degrading bacteria, such that more than one dye can be degraded by the same consortium.

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