Studies on production of Biosurfactant from isolated strain and its application for remediation of Xenobiotic

Thesis submitted for the degree of

Doctor of Philosophy (Science)

By

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DECLARATION

I hereby declare that the thesis entitled "Studies on production of Biosurfactant from isolated strain and its application for remediation of xenobiotic" submitted by me, for the award of the degree of *Doctor of Philosophy* to Jadavpur University is a record of bonafide work carried out by me under the supervision of Dr. Uma Ghosh, Professor, Department of Food Technology and Biochemical Engineering, Jadavpur University, Kolkata.

I further declare that the work reported in this thesis has not been submitted and will not be submitted, either in part or in full, for the award of any other degree or diploma in this institute or any other institute or university.

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Studies on production of Biosurfactant from isolated strain and its application for remediation of Xenobiotic

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ABSTRACT

PC1, the isolated strain later identified as *Bacillus oceanisediminis* H2 was tested for colony zonal diameter and maximal surface tension-lowering ability in NA plates with precoated anthracene. PC1 enters stationary phase after 28 hours of incubation, suggesting considerable biosurfactant production. The fermented medium had a lower maximum surface tension than the uninoculated medium at 5 days, pH 8, 37°C, and a 24hr old culture as a control. This shows the isolated strain can generate biosurfactants. The surface tension is 30.573 mN/m at 120 hours or 5 days. It also shows that the isolate strain produces biosurfactant effectively at both alkaline and acidic pH. Response surfaces show the degree of interaction between variables. A curved gradational drop in the surface tension of the fermented GlyMSM medium occurs as fermentation length and pH rise, with an optimum of 34.61 mN/m at 40°C and pH 9.0. The carbon and nitrogen sources, glycerol (29.957 mN/m) and yeast extract (30.23 mN/m) supplied the lowest surface tension for fermented MSM. The final fermentation conditions were 120 hours, 40°C, pH 9, and *Bacillus oceanisediminis* H2 growth for 24 hours. After four days of fermentation at 35°C and a pH of 8.99 utilising 4 g substrate and 3.99 ml solution, the largest reduction in surface tension was 36.428 mN/m. The FTIR and LC-MS studies revealed that the biosurfactant generated was lipopeptide-derived, namely surfactin (990 Da). The addition of Bacillus oceanisediminis H2 to tannery effluent wastewater decreased BOD and COD levels. Wastewater samples were obtained from the Calcutta Leather Complex, Bantala in Kolkata, India, and treated with a pure biosurfactant. Untreated samples' BOD and COD contents were reduced by 71.65% and 62.18 percent, respectively. Bacillus oceanisediminis H2 produces a biosurfactant.

Keywords: *Biosurfactant, Bacillus oceanisediminis H2, RSM, FTIR, LC-MS, Tannery wastewater.*

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Objectives

To investigate the use of biosurfactant as a powerful material in industrial waste bioremediation. Identification and isolation of a microbe that might be used to make a biosurfactant. Optimization of biosurfactant production environmental factors. Optimisation of biosurfactant production nutritional factors. Purification, characterization, and production of the biosurfactant. Use of the produced biosurfactant to remove xenobiotic substances as possible and remediate the industrial waste.

Introduction

According to the World Health Organization (WHO), bioremediation is an environmentally friendly and harmless process. As a cost-effective means to generate biosurfactants, microbial bioremediation techniques have recently attracted a lot of attention from researchers. Polycyclic aromatic hydrocarbons can be broken down with the help of these common chemicals (PAHs). Industrial effluents containing PAHs are a serious threat to the environment, contributing to human carcinogenicity, genotoxicity, and mutation (Bezza & Chirwa, 2016; Hu, Nakamura, Richardson, & Aitken, 2012).

Sources and occurrence of PAHs

PAHs are mostly produced by human activities such as biomass burning, incomplete fossil fuel combustion, oil spills, and other industrial practises. As a result, soil samples collected from coal storage areas, coke ovens and industrial gas plants have high levels of polycyclic aromatic hydrocarbons (PAHs) (Bezza & Chirwa, 2016; H. Li, Chen, & Jiang, 2014). Gasification and liquefaction procedures for the production of coke utilise petroleum and diesel, which may emit PAHs into the environment. There are a number of coking byproducts, such as creosote and coal tar, that include significant levels of PAHs (Fetter, Boving & Kreamer, 2017). (Khan, Zaidi, Wani, & Oves, 2009). As far as PAHs are concerned, tobacco smoke and food that has been charred are minor contributors. PAHs can also be produced by natural processes such as volcanic eruptions and forest fires (Blumer, 1976). They also have a geochemical basis since PAHs are generated during pyrolysis, which involves exposing deposits to high temperatures during sedimentation (Bamforth Selina M & Singleton Ian, 2005). Figure 1 displays a list of 30 potential PAH component structures (Richter, Grieco, & Howard, 1999).

Particulate matter (PAH) is the most significant source of pollution in the environment today. When contaminants are present, they can impede clean-up efforts, even though the polluted areas are small. 1 PAHs in soils can range from 1 to 300 grammes per kilogramme, depending on the source (Kanaly & Harayama, 2000). If you're looking for older coal gasification sites, the higher levels are listed there. Incomplete burning of materials such as coal and wood has resulted in quantities of PAHs in the

atmosphere ranging from 60 g/m3 to 3g/m3 (Freeman & Cattell, 1990; Ravindra, Sokhi, & Van Grieken, 2008; Yunker et al., 2002).

It remains to be determined whether or not PAHs identified in tannery industry wastes are responsible for the pollution. It's no secret that the world's tannery industry is one of the world's most important. Asia's meat-producing nations such as the United States, Australia, and Europe provide Korea, Japan, and Italy with the hides they desire. As opposed to North American countries, South American countries such as Argentina and Brazil process their own hides. Rio Grande do Sul produces more than half of all Brazilian leather (Basegio, Berutti, Bernardes, & Bergmann, 2002). A lot of research might be done in this area, and bioremediation can help restore the ecosystem's integrity by lowering human mortality.

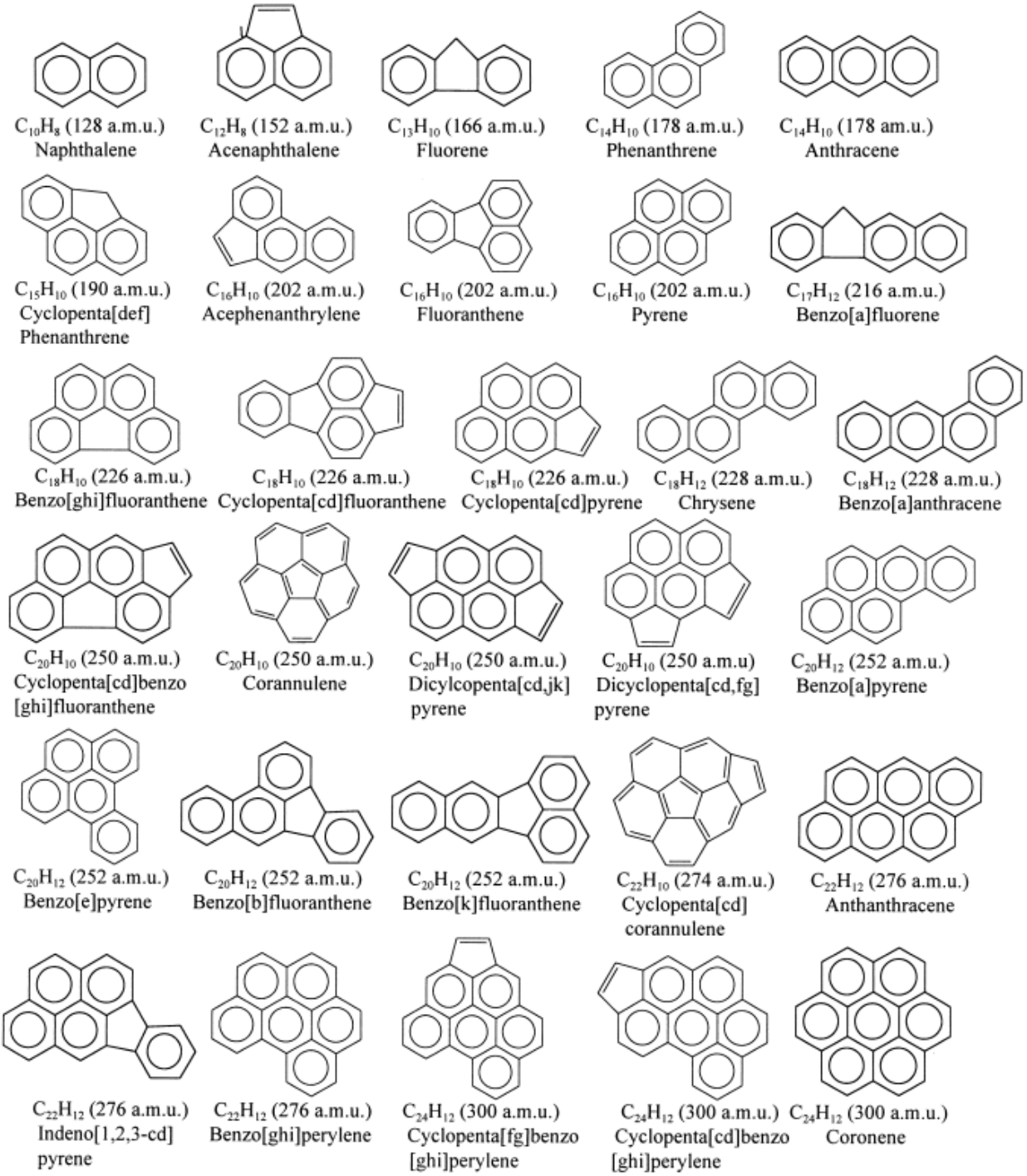


Fig 2: List of different members of the class of PAHs (Richter et al., 1999) .

PAHs persistence in the environment

The only approach to account for PAH emissions from tannery solidified wastes is to incinerate them, according to a research published by the European Commission in 2012. Adsorption of VOCs with Zeolite is also mentioned, as is catalytic oxidation of VOCs with platinum (Black et al., 2013). As these treatments are not cheap, tanning salons with deep pockets are the only ones who can afford to even contemplate them. But no mention of biological remediation has been made, which would not only cut the cost of abatement but also help remove these toxic, potentially cancer-causing compounds from the biospheres more efficiently.

Their persistence in the environment is influenced by their chemical structure, concentration and dispersion, and bioavailability. Longevity of PAHs is also affected by environmental parameters including soil type/structure/pH/temperature, oxygen, nutrient and water availability for the activity of the pollutant-degrading microbial population (Neff, 2002; Sutherland, Rafii, Khan, & Cerniglia, 1995; Van Hamme, Singh, & Ward, 2003).

Toxicity and hydrophobicity of the PAH molecule increase with increasing molecular weight

Another factor that affects biodegradability of PAH is the age of the contaminants in the soil/sediment matrix. As time went on, phenanthrene's mineralisation and biodegradability dropped drastically, according to study using it as a PAH model (Hatzinger & Alexander, 1995).

Additionally, contact with other contaminants, such as hydrocarbons and heavy metals, may influence the PAH residence duration in the environment. Biofilms are better at decomposing aliphatic hydrocarbons and BTEX compounds than PAHs because their structures are simpler. Anaerobicity arises as a result of the depletion of accessible oxygen in the environment. Anaerobic organic matter oxidation rates are up to an order of magnitude lower than aerobic rates in anaerobic environments (Bamforth Selina M & Singleton Ian, 2005; Eriksson, Sodersten, Yu, Dalhammar, & Mohn, 2003; Makkar & Rockne, 2003; Meckenstock, Safinowski, & Griebler, 2004; Song, Jing, Fleischmann, & Wilke, 2002). As a result of heavy metals in soil, anaerobic ecosystems are less able to digest contaminants.

However, according to the Center for Science and Environment's India Environment Portal, there are no such plans or initiatives for the remediation of these hazardous compounds ("India Environment Portal | News, reports, documents, blogs, data, and analysis on environment and development | India, South Asia," n/d). A unit to monitor pollution levels in various sectors, including tanneries, has been established by West Bengal State Pollution Control Board, but little progress in cleaning up PAHs from these enterprises has been made thus far. Because there is so little research done on cleaner remediation methods, it is a tremendously lucrative area of study.

PAH's corrosive power

For the most part, the chemical compounds that fall within this category in nature are either potential or probably human cancer-causing. Researchers in northern India have found that even rainfall contains enough PAHs to cause significant mortality in humans (Malik, Singh, & Singh, 2007). Researchers have established that PAHs can cause cancer, mutagenesis, and teratogenic consequences in mammals. Because benzo[a]pyrene is one of the most potently carcinogenic PAHs, the US Environmental Protection Agency has listed it as a priority contaminant (Renner, 1999). (Juhasz & Naidu, 2000).

Since PAHs are highly fat soluble, they are promptly taken up by the digestive system after consumption (Cerniglia, 1984). dermal absorption is an important route of PAH consumption, according to a study of 12 coke kiln workers (Brandt & Watson, 2003; VanRooij, Bodelier-Bade, & Jongeneelen, 1993). PAHs (especially pyrene) were absorbed by the skin at an estimated 75 percent of the total amount, demonstrating that the skin represents a substantial route of PAH exposure. Humans absorb PAHs quickly, which results in high levels of biomagnification in the food chain. A benzene ring within a molecule enhances the dangerous nature of PAHs (Cerniglia, 1992). Analyzing PAHs' LD_{50} values can help identify their relative toxicities (the lethal dose in 50 percent of cases). Per kilogram of the subject's weight, they are measured in milligrams (mg) of dangerous substance, which in 50% of cases will result in mortality. If the hazardous substance was administered orally or intraperitoneally to the test animal, as well as the species of the test animal (rat, mouse), this information must be included (Bamforth Selina M & Singleton Ian, 2005).

As with PAHs, they're suspected carcinogens, but they're not deemed genotoxic until they're transformed to reactive quinones by mammalian enzymes, at which point they become genotoxic. In order to do this, the cytochrome P450 monooxygenase enzyme must be activated. This enzyme catalysis the oxidation of the aromatic ring to generate epoxide and diol–epoxide reactive intermediates. Before interacting with or damaging DNA, these intermediates are believed to undergo at least four separate oxidation and/or hydrolysis steps. Cancer can be caused by DNA mutations caused by DNA adducts (Bispo, Jourdain, & Jauzein, 1999; Harvey, 1996).

Table 1: Inhalation unit risk and chronic oral reference dose data of some respective PAHs.

ANALYSIS	**No. of rings**	**Inhalation Unit Risk ($\mu g/m^3)^{-1}$)**	**Chronic Oral Reference Dose (mg/kg-day)**	**Bio-accumulative**
Anthracene	3		0.3	+
Benzo(j)fluoranthene	5	0.00011		+
Benzo[a]pyrene	5	0.0006	0.0003	+
Benzo[b]fluoranthene	5	0.00006		+
Benzo[g,h,i]perylene	6			+
Benzo[k]fluoranthene	5	0.000006		+
Benzofluorene, 2,3-	4			+
Chrysene	4	0.0000006		+
Fluoranthene	4		0.04	+
Naphthalene	2	0.000034	0.02	-
Phenanthrene	3			+
Pyrene	4		0.03	+

Most of the time, benzene ring count increases with increased toxicity, but be cautious to look at the route of exposure and other relevant factors before drawing conclusions. (data taken from the RAIS ("The Risk Assessment Information System," n.d.)).

Bioremediation as a process for PAH decontamination

When using biological products like bacteria, bioremediation is a biotechnological strategy that assists in the breakdown of organic pollutants (Johnsen, Wick, & Harms, 2005). There is a method that can be used to get rid of organic contaminants that are refractory or xenobiotic, or to degrade them. When these contaminants are metabolized into native microbes outside of the polluted area, they are found to obtain energy from the metabolization process. It's important to note that bioaugmentation and biostimulation of polluted environments are two bioremediation strategies that require different settings.

Nutritional biostimulation (nutrient addition) has been proposed as the most effective way to speed up the healing process at treatment locations that lack nutrition (de Souza Pohren, de Oliveira Leite, de Angelis, & Vargas, 2016; Kuppusamy, Thavamani, Megharaj, & Naidu, 2016; Mohan, Kisa, Ohkuma, Kanaly, & Shimizu, 2006). However, adding nutrients to coal-tar-contaminated soils did not result in significant improvements in coal-tar PAH biodegradation (Taylor & Jones, 2001). It was found that adding biodiesel (which is easily biodegradable and has less phytotoxicity) to inorganic nutrients resulted in higher breakdown of PAH components compared to inorganic nutrient-only samples after 55 days. It was shown that the biodiesel treatments boosted PAH biodegradability by increasing tar solubilization and dispersion.

For bioremediation when soil contains extremely low natural PAH degraders populations, the best choice is bioaugmentation (contaminant degraders produced in a laboratory) (Castiglione et al., 2016). There are creatures that are anaerobic as well as aerobic that are capable of destroying PAHs (Kuppusamy, Thavamani, Megharaj, Lee, & Naidu, 2016). New study has shown that microorganisms isolated from soil that's polluted with old oil are very efficient at breaking down PAHs in both the soil and slurry phases (45-56 percent) according to scientists (X. Li et al., 2008). This suggests that pretreatments (preferably chemical preoxidation) are necessary to improve HMW PAH bioavailability and removal efficacy in field soils. The scientists also discovered that 2 to 3 ring PAHs decomposed more quickly and extensively than

4 and more ring PAHs. To make better use of the limited enzymatic and mechanical properties, a consortium of microbes was found to have an enlarged surface area.

Bioremediation of PAH-contaminated soils, sediments, and water can be achieved through in situ treatment or ex-situ procedures such as bio-piling and composting. Although they are more expensive than in situ approaches, bioreactors can also be used to remediate waste. The physical and chemical remedies for polluted land include landfilling, burning, and soil washing; bioremediation must be equally effective and cost-effective. Factors influencing PAH bioremediation (see "Factors Influencing PAH Bioremediation") might affect the applicability of bioremediation. By studying these factors, bioremediation can be improved for better results. Since PAHs with more than four rings are difficult to remove and take a long time to degrade, bioremediation of PAH-contaminated soils is rarely used in commercial settings (Bio-Logic, personal communication). The most popular treatments are nutrient addition (see 'Nutrient availability') and regular turning of dirty soil to aerate the soil and improve airflow. PAH levels are typically reduced from 3000 mg/kg to 1000 mg/kg during bioremediation (Bamforth Selina M & Singleton Ian, 2005).

Problems in Bioremediation

Techniques such as bioremediation are incredibly valuable, yet they are often useless when employed in isolation. If you want to reduce the amount of PAHs in your soil, tillage is an option, but it is expensive and takes a long time (Kuppusamy et al., 2017). Fast-growing plants can absorb contaminants while simultaneously stimulating beneficial soil microbes in composting systems, which use fresh organic waste or sewage sludge to induce microbial breakdown (phytoremediation). Composting and phytoremediation are affected by the weather, which slows down in cold temperatures. But it comes at a higher cost and takes more energy to remove soil for treatment in a bioreactor. Tillage and phytoremediation strategies, along with PAH-degrading bacteria, can be combined to increase efficiency. As a result, bioremediation has a disadvantage when it comes to heavily polluted soils (>10 000 mg per kg). When soil is treated with biosurfactants (produced from bacteria and fungi), it may help the process

by increasing PAH bioavailability, but this would require work to develop cost-effective biosurfactants (Kuppusamy et al., 2017).

In comparison to typical chemical treatments, enzyme-mediated bioremediation offers a greener, more efficient method of cleanup. In the soil, PAHs are converted by microbial enzymes into less toxic chemicals. Enzymes with this property can work at low temperatures and across a wide pH range. While fungus extract laccase is of particular interest, it's currently prohibitively pricey (Kuppusamy et al., 2017; Kuppusamy, Thavamani, Megharaj, & Naidu, 2016).

Metabolism of PAHs

Bacterial and fungal PAH metabolism (ligninolytic and non-ligninolytic) in aerobic settings is mediated by at least three distinct processes (Fig. 2). An aromatic ring oxidation is followed by the systematic breakdown of the compound into PAHs and/or CO_2. The anaerobic metabolism of PAHs is thought to result in the hydrogenation of the aromatic ring. There are several areas in nature where PAH-degrading microorganisms can be found, such as soil (bacteria and non-ligninolytic fungi) and woody materials (ligninolytic fungi). PAH-degrading bacteria can be found in many soils and sediments that have been contaminated with PAHs. It was discovered that mangrove sediment in Hong Kong included bacteria that breakdown phenanthrene (Tam, Guo, Yau, & Wong, 2002). These isolates were able to break down phenanthrene in both pure and mixed cultures under a variety of salinities. In anaerobic environments, such as urban wastewater (Chang, Chang & Yuan, 2003) and marine sediments (John D. Coates, Woodward, Allen, Philp & Lovley, 1997), a wide variety of PAH-degrading bacteria can thrive. Fungi that are capable of degrading wood are rare in soils and are mainly found in woody products. If you enrich the soil with lignin-rich materials such as straw or wood chips, these fungi will grow faster. A thorough list of PAH-degrading microorganisms is provided by Mueller et al (Mueller, Cerniglia, & Pritchard, 1996).

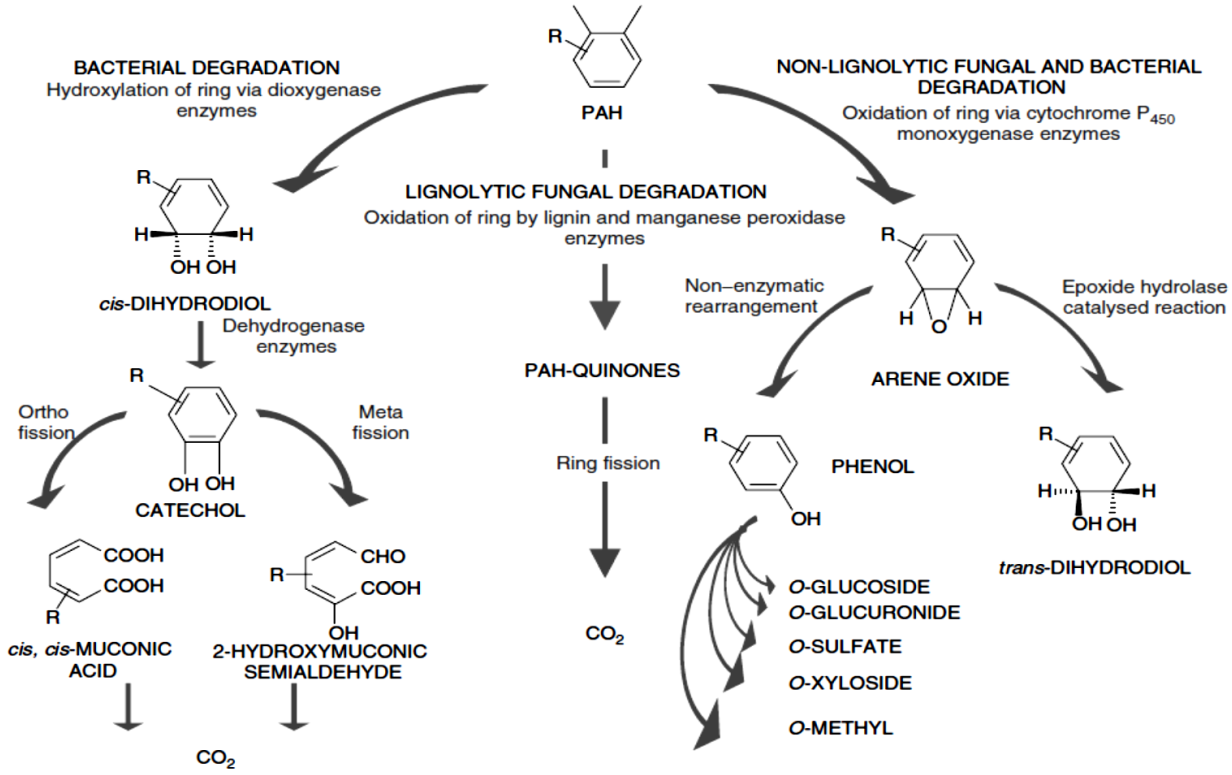


Fig 3: Fungi and bacteria can degrade PAHs via three primary routes. (Cerniglia, 1992).

There are several anaerobic environments where PAH pollution occurs, such as aquifers (Bakermans et al. 2002; Bewley et al. 2001; Meckenstock et al. 2000; Annweiler et al. 2000), and marine sediments (Bakermans et al. 2002). (J. D. Coates et al., 1998; John D. Coates et al., 1997; Sherafatmand & Ng, 2015; Singh, Kawamura, Yanase, & Barrie, 2017). Soils, sediments and groundwater that have been contaminated can develop into anaerobic zones (Lv, Lin, Su, & Zhang, 2016). Why? Because microbial infiltration caused by organic pollution causes aerobic respiration to reduce oxygen molecular concentrations. Anaerobic zones form when oxygen is not replenished at the same rate as it is being removed. Microorganisms can break down PAHs without molecular oxygen, although it has only just been discovered. When it comes to PAH bioremediation, previous research has mostly focused on the thermodynamically better aerobic bioremediation method that absorbs oxygen into the aromatic rings prior to dehydrogenation and subsequent PAH chain breakdown (see earlier for details of the mechanisms of aerobic degradation of PAHs). To oxidise these aromatic compounds in the absence of molecular oxygen, electron acceptors such as nitrate, ferrous iron, and sulphate are required; recent studies have found PAH degradation

occurs both in denitrifying (Rockne et al., 2000; Rockne & Strand, 1998) and sulfate-reducing (J. D. Coates et al., 1997; Meckenstock et al., 1998) conditions. Even though anaerobic naphthalene breakdown was recently discovered, the processes of anaerobic PAH degradation are still mostly hypothetical, according to current study (Meckenstock et al., 2000; Zhang et al., 2000), which is summarised in Fig 3. The aromatic ring is carboxylated to 2-naphthoic acid in the first step, which may activate

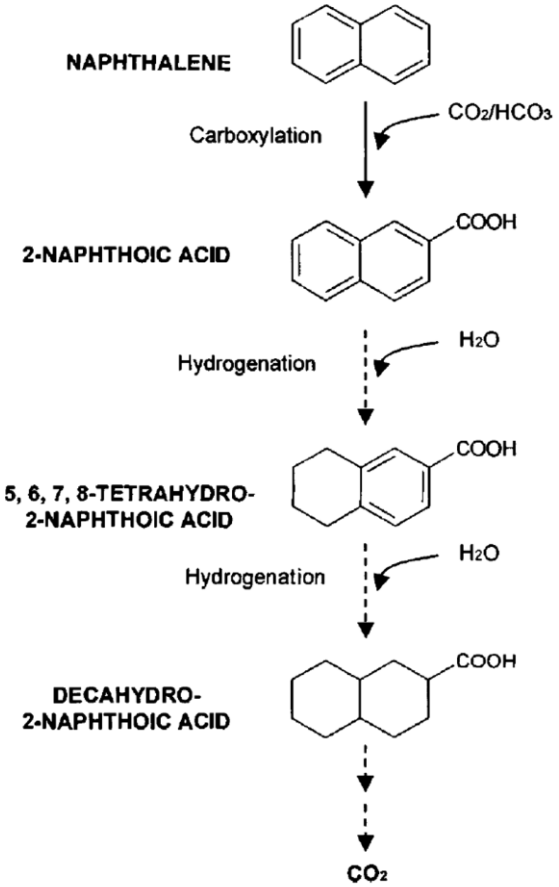


Fig 1: Simplified proposed pathway for the anaerobic metabolism of naphthalene under sulfate-reducing conditions (Meckenstock et al., 2000; Zhang et al., 2000).

the aromatic ring before hydrolysis. Through a sequence of hydrogenation processes, 2-naphthoic acid is reduced to decaclin-2-carboxylic acid, which is then transformed to decahydro-2-naphthoic acid. Other processes for anaerobic naphthalene degradation may exist, but they have yet to be discovered. The earliest stage of anaerobic naphthalene breakdown under sulfate-reducing conditions, for example, is thought to be a hydroxylation process that produces a naphthol intermediate (Bedessem, Swoboda-Colberg, & Colberg, 1997).

Emerging technologies

Recently, electrokinetic remediation, enzyme-mediated bioremediation, multi-process phytoremediation, and vermiremediation have been applied in the treatment of PAH-contaminated soils (Kuppusamy et al., 2017). Notably, vermiremediation (Ekperusi & Aigbodion, 2015) is a relatively new technique for cleaning up PAH-contaminated soils. Researchers have a number of options for combining novel remedial methods with existing physical, chemical, and/or biological treatment options in order to achieve maximum PAH removal efficacy. Recent improvements in bioremediation technologies are shown in Figure 4.

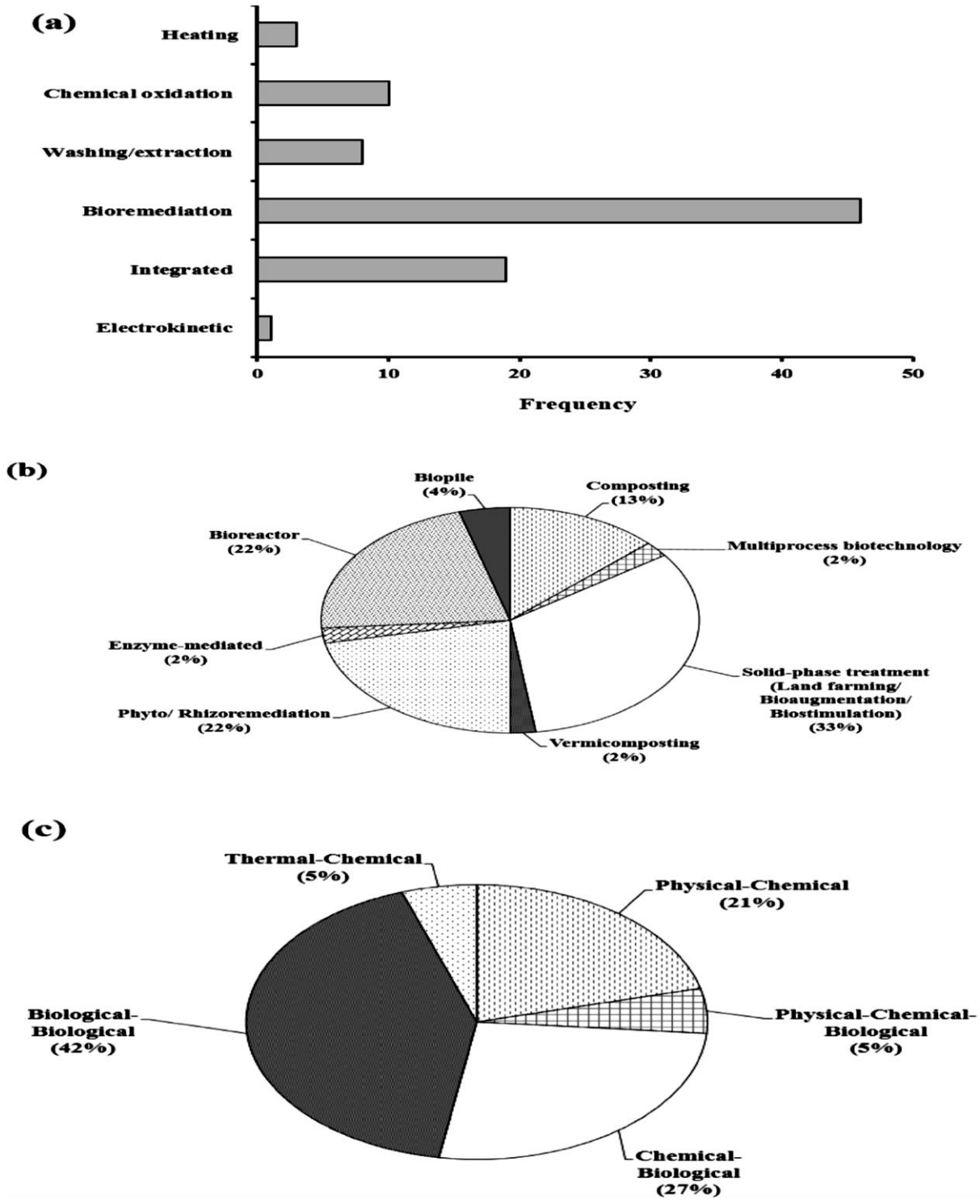


Fig 4: Current trend of application of technologies – (a) The usage of technology within the remediation of PAH-contaminated soils (b) occurred within a range of frequencies. (c) Technology integrated within that range of frequencies was also measured. (Kuppusamy et al., 2017).

Future aspects of Bioremediation techniques

Some believe that the future of biotechnology is in using new remedial treatments, such as nanoremediation, transgenic techniques, and photo-hetero microbial systems. A number of pollutants, both organic and inorganic, have been successfully removed utilising these newer techniques (Kuppusamy et al., 2017; Kuppusamy, Thavamani, Megharaj, Lee, et al., 2016; Kuppusamy, Thavamani, Megharaj, & Naidu, 2016). A further investigation may be necessary in order to build a quick and reliable low-cost PAH cleaning method.

Conclusion

This is due to the fact that PAHs have a long half-life and are poisonous, which has inspired a lot of laboratory-based study on the capacity of a variety of microorganisms (fungi and bacteria) to change these complicated aromatic chemicals. Biochemical pathways for aerobic PAH transformation have been identified, and microorganisms capable of reducing PAH concentrations are known to exist in a variety of environments. To combat PAH contamination in soils, researchers are exploring the possibility of using microorganisms. Recent study has shown that microorganism-based approaches can be utilized to do so. When PAH pollution is deep, bioremediation approaches such as land farming and biopiling are successful. In contrast, a recent field study indicated that increasing subsurface aeration can aid in bioremediation of polluted aquifers. PAH biodegradation under anaerobic conditions is also encouraging, enabling for further breakthroughs in in situ remediation of polluted sub surfaces. Our present understanding of PAH contamination makes bioremediation feasible. More research is needed to assess and exploit the potential for in situ microbial populations to metabolize PAHs (especially the larger molecular weight PAHs) at sites with less-than-ideal conditions, such as low pH and/or high temperatures, even though the inherent limitations of bioremediation are well understood. There are a number of commercially feasible options, including heating, extraction, oxidation, and bioremediation. All sites with PAH contamination are different, and the decision on how to continue must consider all relevant factors, such as the cleaning policy's restrictions, available financial resources and public approval. It is now vital to study the future options suggested in this analysis, as well as to gain a deeper understanding of how microbial communities collaborate, given the interest in green remediation and the preference for bioremediation. In contaminated areas, community fingerprinting and environmental genomics approaches can be used to study the structure and functioning of microbial communities on multiple geographical and temporal dimensions, as well as their responses to diverse stimuli (Megharaj, Ramakrishnan, Venkateswarlu, Sethunathan, & Naidu, 2011). Restoring PAH-contaminated soils to their native state is impractical, and not every site can be fully restored, even if background conditions are cleared. For longer-term PAH-

contaminated sites, this implies that a function-directed risk-based green remediation approach may be adequate. Another important area of study involves the development of effective strategies for cleaning up PAH-contaminated subterranean areas using anaerobic remediation.

References

Bakermans, C., Hohnstock-Ashe, A. M., Padmanabhan, S., Padmanabhan, P., & Madsen, E. L. (2002). Geochemical and physiological evidence for mixed aerobic and anaerobic field biodegradation of coal tar waste by subsurface microbial communities. *Microbial Ecology*, *44*(2), 107–117.

Bamforth Selina M, & Singleton Ian. (2005). Bioremediation of polycyclic aromatic hydrocarbons: current knowledge and future directions. *Journal of Chemical Technology & Biotechnology*, *80*(7), 723–736. https://doi.org/10.1002/jctb.1276

Basegio, T., Berutti, F., Bernardes, A., & Bergmann, C. P. (2002). Environmental and technical aspects of the utilisation of tannery sludge as a raw material for clay products. *Journal of the European Ceramic Society*, *22*(13), 2251–2259.

Bedessem, M. E., Swoboda-Colberg, N. G., & Colberg, P. J. (1997). Naphthalene mineralization coupled to sulfate reduction in aquifer-derived enrichments. *FEMS Microbiology Letters*, *152*(2), 213–218.

Bewley, R. J., & Webb, G. (2001). In situ bioremediation of groundwater contaminated with phenols, BTEX and PAHs using nitrate as electron acceptor. *Land Contamination & Reclamation*, *9*(4), 335–347.

Bezza, F. A., & Chirwa, E. M. N. (2016). Biosurfactant-enhanced bioremediation of aged polycyclic aromatic hydrocarbons (PAHs) in creosote contaminated soil. *Chemosphere*, *144*, 635–644. https://doi.org/10.1016/j.chemosphere.2015.08.027

Bispo, A., Jourdain, M. J., & Jauzein, M. (1999). Toxicity and genotoxicity of industrial soils polluted by polycyclic aromatic hydrocarbons (PAHs). *Organic Geochemistry*, *30*(8), 947–952.

Black, M., Canova, M., Rydin, S., Scalet, B. M., Roudier, S., & Sancho, L. D. (2013). Best available techniques (BAT) reference document for the tanning of hides and skins. *European Commission Database*, *46*.

Blumer, M. (1976). Polycyclic Aromatic Compounds in Nature. *Scientific American*, *234*(3), 34–45.

Brandt, H. C., & Watson, W. P. (2003). Monitoring human occupational and environmental exposures to polycyclic aromatic compounds. *Annals of Occupational Hygiene*, *47*(5), 349–378.

Castiglione, M. R., Giorgetti, L., Becarelli, S., Siracusa, G., Lorenzi, R., & Di Gregorio, S. (2016). Polycyclic aromatic hydrocarbon-contaminated soils: bioaugmentation of autochthonous bacteria and toxicological assessment of the bioremediation process by means of Vicia faba L. *Environmental Science and Pollution Research*, *23*(8), 7930–7941.

Cerniglia, C. E. (1984). Microbial metabolism of polycyclic aromatic hydrocarbons. In *Advances in applied microbiology* (Vol. 30, pp. 31–71). Elsevier.

Cerniglia, C. E. (1992). Biodegradation of polycyclic aromatic hydrocarbons. In *Microorganisms to combat pollution* (pp. 227–244). Springer.

Chang, B. V., Chang, S. W., & Yuan, S. Y. (2003). Anaerobic degradation of polycyclic aromatic hydrocarbons in sludge. *Advances in Environmental Research*, *7*(3), 623–628.

Coates, J. D., Woodward, J., & Allen, J. (1998). Anaerobic degradation of polycyclic aromatic hydrocarbons and alkanes in petroleum-contaminated marine harbor sediments. *Oceanographic Literature Review*, *2*(45), 375–376.

Coates, John D., Woodward, J., Allen, J., Philp, P., & Lovley, D. R. (1997). Anaerobic degradation of polycyclic aromatic hydrocarbons and alkanes in petroleum-contaminated marine harbor sediments. *Applied and Environmental Microbiology*, *63*(9), 3589–3593.

de Souza Pohren, R., de Oliveira Leite, D. A. N., de Angelis, D. de F., & Vargas, V. M. F. (2016). Performance of simulated bioremediation in real samples of soils contaminated with PAHs. *Water, Air, & Soil Pollution*, *227*(9), 330.

Ekperusi, O. A., & Aigbodion, I. F. (2015). Bioremediation of heavy metals and petroleum hydrocarbons in diesel contaminated soil with the earthworm: Eudrilus eugeniae. *SpringerPlus*, *4*(1), 540.

Eriksson, M., Sodersten, E., Yu, Z., Dalhammar, G., & Mohn, W. W. (2003). Degradation of polycyclic aromatic hydrocarbons at low temperature under aerobic and nitrate-reducing conditions in enrichment cultures from northern soils. *Applied and Environmental Microbiology*, *69*(1), 275–284.

Fetter, C. W., Boving, T., & Kreamer, D. (2017). *Contaminant Hydrogeology: Third Edition*. Waveland Press.

Freeman, D. J., & Cattell, F. C. (1990). Woodburning as a source of atmospheric polycyclic aromatic hydrocarbons. *Environmental Science & Technology*, *24*(10), 1581–1585.

Harvey, R. G. (1996). Mechanisms of carcinogenesis of polycyclic aromatic hydrocarbons. *Polycyclic Aromatic Compounds*, *9*(1–4), 1–23.

Hatzinger, P. B., & Alexander, M. (1995). Effect of aging of chemicals in soil on their biodegradability and extractability. *Environmental Science & Technology*, *29*(2), 537–545.

Hu, J., Nakamura, J., Richardson, S. D., & Aitken, M. D. (2012). Evaluating the Effects of Bioremediation on Genotoxicity of Polycyclic Aromatic Hydrocarbon-Contaminated Soil Using Genetically Engineered, Higher Eukaryotic Cell Lines. *Environmental Science & Technology*, *46*(8), 4607–4613. https://doi.org/10.1021/es300020e

India Environment Portal | News, reports, documents, blogs, data, analysis on environment & development | India, South Asia. (n.d.). Retrieved from http://www.indiaenvironmentportal.org.in/search/?q=tannery&reset=0

Johnsen, A. R., Wick, L. Y., & Harms, H. (2005). Principles of microbial PAH-degradation in soil. *Environmental Pollution*, *133*(1), 71–84.

Juhasz, A. L., & Naidu, R. (2000). Bioremediation of high molecular weight polycyclic aromatic hydrocarbons: a review of the microbial degradation of benzo [a] pyrene. *International Biodeterioration & Biodegradation*, *45*(1–2), 57–88.

Kanaly, R. A., & Harayama, S. (2000). Biodegradation of High-Molecular-Weight Polycyclic Aromatic Hydrocarbons by Bacteria. *Journal of Bacteriology*, *182*(8), 2059–2067. https://doi.org/10.1128/JB.182.8.2059-2067.2000

Khan, M. S., Zaidi, A., Wani, P. A., & Oves, M. (2009). Role of plant growth promoting rhizobacteria in the remediation of metal contaminated soils. *Environmental Chemistry Letters*, *7*(1), 1–19. https://doi.org/10.1007/s10311-008-0155-0

Kuppusamy, S., Thavamani, P., Megharaj, M., Lee, Y. B., & Naidu, R. (2016). Isolation and characterization of polycyclic aromatic hydrocarbons (PAHs) degrading, pH tolerant, N-fixing and P-solubilizing novel bacteria from manufactured gas plant (MGP) site soils. *Environmental Technology & Innovation*, *6*, 204–219. https://doi.org/10.1016/j.eti.2016.04.006

Kuppusamy, S., Thavamani, P., Megharaj, M., & Naidu, R. (2016). Bioaugmentation with novel microbial formula vs. natural attenuation of a long-term mixed contaminated soil—treatability studies in solid-and slurry-phase microcosms. *Water, Air, & Soil Pollution*, *227*(1), 25.

Kuppusamy, S., Thavamani, P., Venkateswarlu, K., Lee, Y. B., Naidu, R., & Megharaj, M. (2017). Remediation approaches for polycyclic aromatic hydrocarbons (PAHs) contaminated soils: Technological constraints, emerging trends and future directions. *Chemosphere*, *168*, 944–968.

Li, H., Chen, J., & Jiang, L. (2014). Elevated critical micelle concentration in soil–water system and its implication on PAH removal and surfactant selecting. *Environmental Earth Sciences*, *71*(9), 3991–3998. https://doi.org/10.1007/s12665-013-2783-3

Li, X., Bai, J., Li, Y., Li, X., Wang, Y., & Feng, X. (2008). Magnetic rotation imaging method to measure the geomagnetic field. *Progress in Natural Science*, *18*(1), 21–26. https://doi.org/10.1016/j.pnsc.2007.07.003

Lv, H., Lin, X., Su, X., & Zhang, Y. (2016). 14 C Isotopes and microbial community structures as evidence for biodegradation in a petroleum hydrocarbon-contaminated aquifer. *Environmental Earth Sciences*, *75*(2), 119.

Makkar, R. S., & Rockne, K. J. (2003). Comparison of synthetic surfactants and biosurfactants in enhancing biodegradation of polycyclic aromatic hydrocarbons. *Environmental Toxicology and Chemistry*, *22*(10), 2280–2292.

Malik, A., Singh, V. K., & Singh, K. P. (2007). Occurrence and distribution of persistent trace organics in rainwater in an urban region (India). *Bulletin of Environmental Contamination and Toxicology*, *79*(6), 639–645.

Meckenstock, R. U., Annweiler, E., Michaelis, W., Richnow, H. H., & Schink, B. (2000). Anaerobic naphthalene degradation by a sulfate-reducing enrichment culture. *Applied and Environmental Microbiology*, *66*(7), 2743–2747.

Meckenstock, R. U., Safinowski, M., & Griebler, C. (2004). Anaerobic degradation of polycyclic aromatic hydrocarbons. *FEMS Microbiology Ecology*, *49*(1), 27–36.

Megharaj, M., Ramakrishnan, B., Venkateswarlu, K., Sethunathan, N., & Naidu, R. (2011). Bioremediation approaches for organic pollutants: A critical perspective. *Environment International*, *37*(8), 1362–1375. https://doi.org/10.1016/j.envint.2011.06.003

Mohan, S. V., Kisa, T., Ohkuma, T., Kanaly, R. A., & Shimizu, Y. (2006). Bioremediation technologies for treatment of PAH-contaminated soil and strategies to enhance process efficiency. *Reviews in Environmental Science and Bio/Technology*, *5*(4), 347–374. https://doi.org/10.1007/s11157-006-0004-1

Mueller, J. G., Cerniglia, C. E., & Pritchard, P. H. (1996). Bioremediation of environments contaminated by polycyclic aromatic hydrocarbons. *Biotechnology Research Series*, *6*, 125–194.

Neff, J. M. (2002). *Bioaccumulation in marine organisms: effect of contaminants from oil well produced water*. Elsevier.

Ravindra, K., Sokhi, R., & Van Grieken, R. (2008). Atmospheric polycyclic aromatic hydrocarbons: source attribution, emission factors and regulation. *Atmospheric Environment*, *42*(13), 2895–2921.

Renner, R. (1999). EPA to strengthen persistent, bioaccumulative, and toxic pollutant controls—mercury first to be targeted. *Environmental Science & Technology*, *33*(3), 62A–62A.

Richter, H., Grieco, W. J., & Howard, J. B. (1999). Formation mechanism of polycyclic aromatic hydrocarbons and fullerenes in premixed benzene flames. *Combustion and Flame*, *119*(1), 1–22. https://doi.org/10.1016/S0010-2180(99)00032-2

Rockne, K. J., Chee-Sanford, J. C., Sanford, R. A., Hedlund, B. P., Staley, J. T., & Strand, S. E. (2000). Anaerobic naphthalene degradation by microbial pure cultures under nitrate-reducing conditions. *Applied and Environmental Microbiology*, *66*(4), 1595–1601.

Rockne, K. J., & Strand, S. E. (1998). Biodegradation of bicyclic and polycyclic aromatic hydrocarbons in anaerobic enrichments. *Environmental Science & Technology*, *32*(24), 3962–3967.

Sherafatmand, M., & Ng, H. Y. (2015). Using sediment microbial fuel cells (SMFCs) for bioremediation of polycyclic aromatic hydrocarbons (PAHs). *Bioresource Technology*, *195*, 122–130.

Singh, D. K., Kawamura, K., Yanase, A., & Barrie, L. A. (2017). Distributions of Polycyclic Aromatic Hydrocarbons, Aromatic Ketones, Carboxylic Acids, and Trace Metals in Arctic Aerosols: Long-Range Atmospheric Transport, Photochemical Degradation/Production at Polar Sunrise. *Environmental Science & Technology*, *51*(16), 8992–9004.

Song, Y. F., Jing, X., Fleischmann, S., & Wilke, B.-M. (2002). Comparative study of extraction methods for the determination of PAHs from contaminated soils and sediments. *Chemosphere*, *48*(9), 993–1001.

Sutherland, J. B., Rafii, F., Khan, A. A., & Cerniglia, C. E. (1995). Mechanisms of polycyclic aromatic hydrocarbon degradation. *Microbial Transformation and Degradation of Toxic Organic Chemicals*, 269–306.

Tam, N. F. Y., Guo, C. L., Yau, W. Y., & Wong, Y. S. (2002). Preliminary study on biodegradation of phenanthrene by bacteria isolated from mangrove sediments in Hong Kong. *Marine Pollution Bulletin*, *45*(1–12), 316–324.

Taylor, L. T., & Jones, D. M. (2001). Bioremediation of coal tar PAH in soils using biodiesel. *Chemosphere*, *44*(5), 1131–1136. https://doi.org/10.1016/S0045-6535(00)00344-1

The Risk Assessment Information System. (n.d.). Retrieved from https://rais.ornl.gov/

Van Hamme, J. D., Singh, A., & Ward, O. P. (2003). Recent advances in petroleum microbiology. *Microbiology and Molecular Biology Reviews*, *67*(4), 503–549.

VanRooij, J. G., Bodelier-Bade, M. M., & Jongeneelen, F. J. (1993). Estimation of individual dermal and respiratory uptake of polycyclic aromatic hydrocarbons in 12 coke oven workers. *Occupational and Environmental Medicine*, *50*(7), 623–632.

Yunker, M. B., Macdonald, R. W., Vingarzan, R., Mitchell, R. H., Goyette, D., & Sylvestre, S. (2002). PAHs in the Fraser River basin: a critical appraisal of PAH ratios as indicators of PAH source and composition. *Organic Geochemistry*, *33*(4), 489–515.

Zhang, X., Sullivan, E. R., & Young, L. Y. (2000). Evidence for aromatic ring reduction in the biodegradation pathwayof carboxylated naphthalene by a sulfate reducing consortium. *Biodegradation*, *11*(2–3), 117–124.

Chapter 1

Isolation, identification and characterization of the isolated strain.

1.1 Introduction

Polycyclic aromatic hydrocarbons (PAHs) are responsible for worldwide environmental pollution as a result of their excessive toxicity, mutability and carcinogenic results (Haritash & Kaushik, 2009). These are a category of risky natural compounds (VOCs) which for years had been deposited in the surroundings because of the reality that PAHs are insoluble or sparingly soluble in aqueous phases (Acree Jr & Abraham, 2002; Barro, Regueiro, Llompart, & Garcia-Jares, 2009). Microbial bioremediation of PAHs are the maximum convenient method, which need to be efficient, economical, smooth to fabricate and is exceptionally flexible relating to unfavorable levels of physicochemical treatment (Oller, Malato, & Sánchez-Pérez, 2011). Isolating microorganisms which is able to degrading PAHs is a primary step closer to information on the microbiology and outcome of PAHs withinside the surroundings, whilst enlisting the isolate is crucial to achieve a PAH degrading microbial community (B. Zhao, Wang, Mao, & Li, 2009). Due to their toxicity, the PAHs pose a excessive hazard to each the terrestrial and aquatic ecosystems. These group of compounds include a great range of individual compounds that now no longer only are poisonous and hydrophobic, however additionally get gathered withinside the organisms that metabolize them or receives mineralized in numerous forms which pose same hazard to the ecological balance (X. Zhang, Xu, Zhu, Lundaa, & Scherr, 2012). Decontamination of those compounds may be carried out through exclusive strategies extensively categorized as bioremediation (Z. Hua, Chen, Du, & Chen, 2004).

Microbial remediation has mentioned growing attention for some of the advantages over different technologies, inclusive of treatment by chemical methods, physical methods of separation, turning into solids and making it stable. These advantages encompass cost effectiveness, improved efficiency, and negligible counter results to the encircling environment. Bioremediation additionally has supplied an extensive application perspective in specific industrial aspects as said by Mukherjee et. Al. (Mukherjee & Bordoloi, 2011).

In bioremediation, the hydrophobicity of PAHs, hindering pollutant uptake via way of means of microorganisms, is certainly considered one among restrictive factors for the efficiency of the process. An approach that's evolved through which microorganisms tend to enhance the supply of PAHs is by generating and excreting biosurfactants that cause pseudosolubilization or entrapment the hydrocarbons (F. Hua & Wang, 2012; Rocha, Pedregosa, & Laborda, 2011).

Due to the low toxicity, biodegradability and, above all, the ability to reduce interfacial tension among oil and water process, the increasing microbial contaminant uptake and transformation, biosurfactants have received extensive interest in improving the effectiveness of bioremediation in recent years (X. Zhang & Xiang, 2010). *Bacillus, Pseudomonas, Acinetobacter, Achromobacter, Arthrobacter, Brevibacterium, Corynebacterium, Candida* and *Rhodotorula*, amongst others composed of surfactants (Rodrigues, Banat, Teixeira, & Oliveira, 2006). Although the five general groups of biosurfactants have been studied by Cameotra et al., 2010, it is assumed that glyco and phosphor lipids, polymeric and particulate surfactants occur (Cameotra, Makkar, Kaur, & Mehta, 2010). Glycolipids, as Das et al. reported in 2008, are some of the main bacterial biofactants. Biosurfactants are increasingly capable of emulsifying hydrophobic hydrocarbons. Superior water solubility and decreased surface tension are present in emulsified hydrocarbons. With the emulsification of oil materials from soil residue, the bioaccession and the next biological degradations of the hydrocarbons are increased (Cameotra et al., 2010; Das, Mukherjee, & Sen, 2008a). Therefore, it's far of super significance to device an isolation technique and the proper identification of native microorganisms from soil that degrades crude oil and has a potential for production of biosurfactants.

The objectives of this study is to isolate a biosurfactant generating bacterial strain from tannery processing industrial wastelands, identify them and employ a proper characterization for the isolate in phrases of its growth, biosurfactant manufacturing competencies and biochemical analyses.

1.2 Materials and Methods

1.2.1 Sample

Soil sample was collected from Tannery processing industrial wastelands, Kolkata, India. The pH of the sample was determined by standard method (Nakhleh & Krajcik, 1993).

1.2.2 Culture media

Nutrient Broth (NB) was made of yeast extract 2, extract of beef 1, peptone 5, NaCl 5, and further dissolved in distilled water Concentrations were measured in g/L. 20 g/L of agar was applied for the preparation of the plates or slants for nutrient agar (NA). (You et al., 2013)

The mineral salts media (MSM) is made up of 0.3% NH_4NO_3, 0.22% K_2HPO_4, 0.001% NaCl, 0.06% $MgSO_4$, 0.004% $CaCl_2$, 0.002% $FeSO_4$ and Trace elements, purified 0.5mL/L water, 0,32 g $ZnSO_4.7H_2O$ and 0,56g H_3BO_2, 0.3 g $CuSO_4.5\ H_2O$, 0,42g $CoCl_2.6\ H_2O$, 1.0g EDTA, 0.04g $NiCl_2.6\ H2O$ and 0,66 g KI in distilled water. Mineral salts media (MSM) 50mL MSM is combined with 2% (v/v) glycerol in output media and 0,2% (w/v) anthracene (AMSM) in select media as the special carbon source. MSM was loaded to 250mL shaking erlenmeyer flasks (Das et al., 2008). Once the sterilization process is complete, the flasks were sealed with anti-anthracene plastic film as by X. Zhang et al., 2012.

To bring the pH to 7.0, 1 N NaOH was used (Najafi et al., 2010) and sterilization was performed by autoclaving at 121°C for 15mins.

1.2.3 Isolation and screening

About 1g of soil sample was added to 10mL water which was serially diluted up to 10^{-12} concentration. This was then spread plated on NA aseptically with anthracene pre coated on the plates (Mujahid et al., 2015). The pre-coating was done by dissolving 0.5% anthracene in ice cold acetone and then evenly spreading the solution on NA plates until all acetone gets volatilized to leave behind whitish anthracene coat.

Prior to separate growth of each of the screened colonies the grown colonies in the anthracene precoated plates were looped out and incubated in separate flasks containing NB. All the isolates were incubated at 37°C in a rotary shaker at 180 rpm for NB incubations and without a shaker for plated NA incubates. Sub culturing was

performed, and after four such subcultures, it was spreaded on NA with anthracene precoat. The plates were visually seen for zonal clearance just about the colonies, pinpointing production of biosurfactant (Najafi et al., 2010). Colonies with large clearance zones were selected, and incubated in 20mL NB at 37°C in a rotary shaker at 180 rpm overnight. Total of three colonies were isolated and was named PC1, PC2 and PC3, and were maintained in NA slants at 4°C for further studies.

The 3 strains were incubated in a rotary shaker in AMSM at 37°C and 180rpm for 3 days for evaluation of biosurfactant producing ability, and PC1 was selected and screened out.

1.2.4 Biosurfactant production measurement

Culture from a NA slant in a test tube was utilized which was inoculated in 100mL GlyMSM in 500mL Erlenmeyer flask. The aliquot was centrifuged at 6000 rpm for a time period of 30mins which was done after 3 days of fermentation at a temperature of 37°C in a BOD incubator maintained at a steady rotation of 180 rpm. The ability of the strain for the production of biosurfactant calculated with the application of oil spreading procedure, drop collapse procedure and surface tension measurements which was in accordance with X. Zhang et al., 2012; Zhao et al., 2016 & P\laza et al., 2006.

1.2.5 Identification of the isolated strain

Gene sequencing was conducted by Xcelris Labs Limited, Ahmedabad, Gujarat, India under the technique of MID-S. It was determined to use the software BLAST (https://blast.ncbi.nlm.nih.gov/Blast.cgi), and EZbiocloud identifications (https://www.ezbiocloud.net/identify) to measure the phylogenetic neighbors. Neighbor joining (Yoon et al., 2017; You et al., 2013) tree-making algorithms with MEGA 7.0 have been developed for the phylogenetic trees (Kumar et al., 2016; Saitou & Nei, 1987; Tamura et al., 2004). The topologies of the phylogenetic trees were tested using the 1,000 replicated Bootstrap Resampling technique (Felsenstein, 1985).

1.2.6 Morphological, physiological and biochemical characteristics

Gram staining was done according to the method given by Gregersen, 1978. The isolate was observed under a light microscope (Olympus BH2) for morphological characteristics. Endospore staining (Harley, 2004), catalase test (J. Zhang et al., 2010),

oxidase test (Steel, 1961), coagulation assay (Yu et al., 2017), urease test (Vahabi et al., 2015) and nitrate reduction (Yang et al., 2015) analysis was done according to respective references.

1.2.7 Screening analysis for biosurfactant producers

Drop collapse test (Ahmad et al., 2016) and oil spreading test (Zhao et al., 2016) was performed with water as control and fermented cell free supernatant as test on a coconut oil greased glass slide. The drop (approx. 0.05mL) was observed and measured, it is considered that a displacement of ≥ 1 mm is considered positive for biosurfactant production.

1.2.8 Surface tension measurements

The culture broth surface tension of the supernatant was measured by a Traube's stalagmometer (Walter et al., 2010). The surface tension was calculated by counting the number of drops of the supernatant and comparing it to the number of drops of water. The following formula (Eqn 1.1) was applied for the calculation of relative surface tension to water (have a surface tension of 72 mN/m) (Cappello et al., 2016).

$$\sigma = \frac{\rho}{\rho_w} \cdot \frac{n_w}{n} \cdot \sigma_w \qquad \text{Eqn. 1.1}$$

Eqn 1.1: Stalagmometric method of surface tension measurement, where $\sigma \& \sigma_w$ = Surface tension of the supernatantand water respectively; $\rho \& \rho_w$ = density of the supernatant and water respectively; $n \& n_w$ = number of drops of the supernatant and water respectively.

1.3 Results and discussions

1.3.1 Isolation and screening of biosurfactant producing strain

Colonies with clear zones on anthracene coated on NA plates were observed (Fig 1.1). These 3 strains were named as PC1, PC2 and PC3, out of which PC1 showed colony diameter of 3.4cm which was the highest among the three and was thus selected.

Table 1.1: Colony diameter of the screened strains on anthracene coated NA plates.

Isolate	**Colony diameter (cm)**
PC1	3.4
PC2	1.2
PC3	0.9

Fig 1.1: Colonies of the screened strains on anthracene coated NA plates.

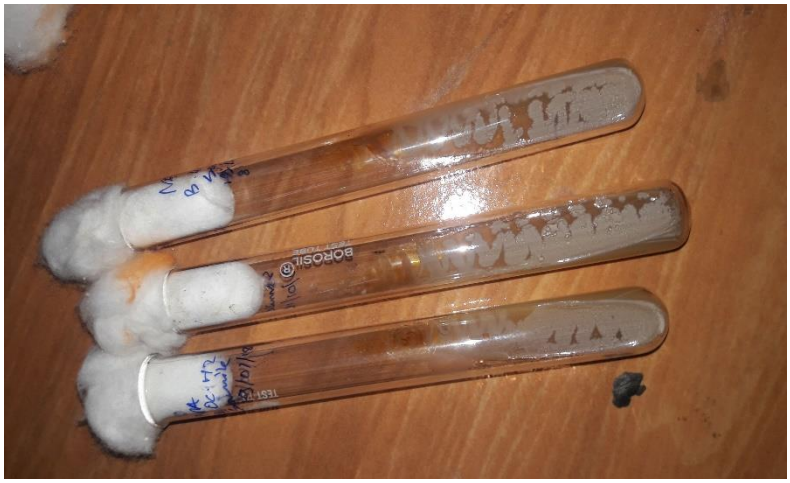


Fig 1.2: NA slants of PC 1, PC2, and PC3.

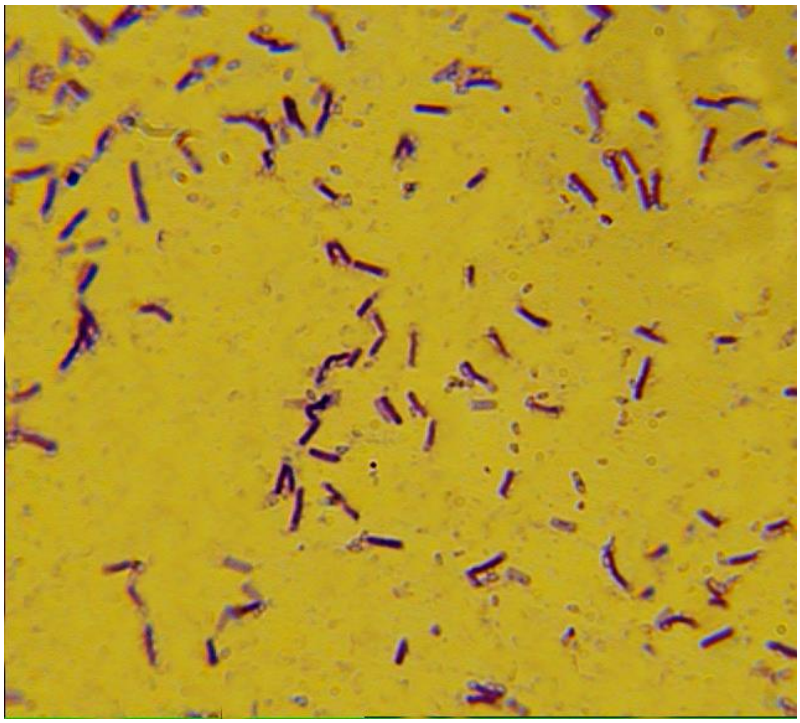


Fig 1.3: Microscopic image of PC1 after gram staining.

1.3.2 Surface Tension measurement

The control surface tension was measured to be 72 mN/m, for uninoculated GlyMSM it was found to be 68.24 mN/m, and for uninoculated AMSM it was 56.57 mN/m (Fig 1.4). The surface tension measurements are given in the Table 1.2 of the three isolated strains PC1, PC2 and PC3. PC1 was found to lower the surface tension of the GlyMSM and AMSM by almost 38%.

Table 1.2: Liquid media surface tension for isolated strains PC1, PC2 and PC3 in various conditions.

Isolates	Water (mN/m)	Uninoculated GlyMSM (mN/m)	Uninoculated AMSM (mN/m)	Inoculated GlyMSM (mN/m)	Inoculated AMSM (mN/m)
PC1	72 ± 3.6	68.24 ± 3.412	56.57 ± 2.8285	40.6 ± 2.03	33.8 ± 1.69
PC2	72 ± 3.6	68.24 ± 3.412	56.57 ± 2.8285	52.4 ± 2.62	41.8 ± 2.09
PC3	72 ± 3.6	68.24 ± 3.412	56.57 ± 2.8285	55.6 ± 2.78	49.4 ± 2.47

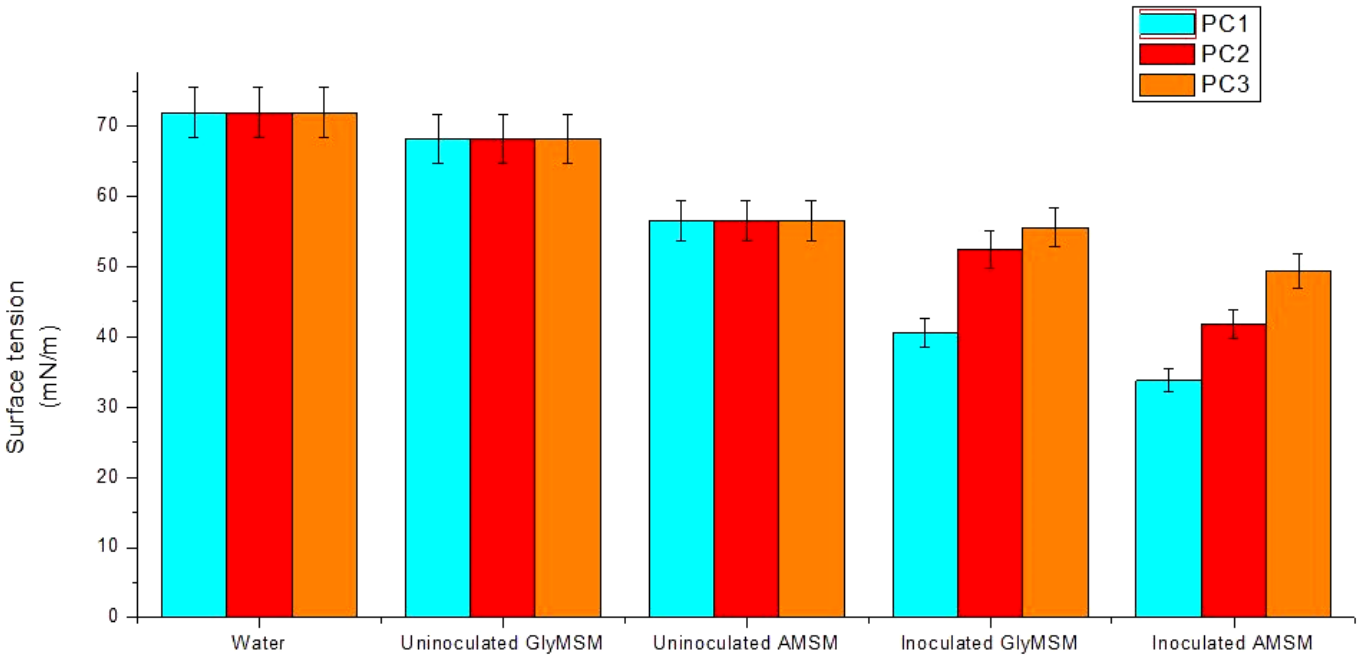


Fig 1.4.: Liquid media surface tension for isolated strains PC1, PC2 and PC3 in various conditions.

1.3.3 Biosurfactant production determination

1.3.3.1 Drop Collapse and Oil spreading method

PC1 was tested positive for drop collapse test as with about 50 µL of fermentation broth on a coconut oil greased glass slide, the drop was observed to collapse as it spread out more when compared with distilled water control (Fig 1.5).

The results show that with 50 µL of fermentation broth, PC1 strain could expel oil in notable amount (>4cm in diameter) and the average diameter was observed to be 4.1cm, significantly ($p < 0.05$) higher that the rest two isolates.

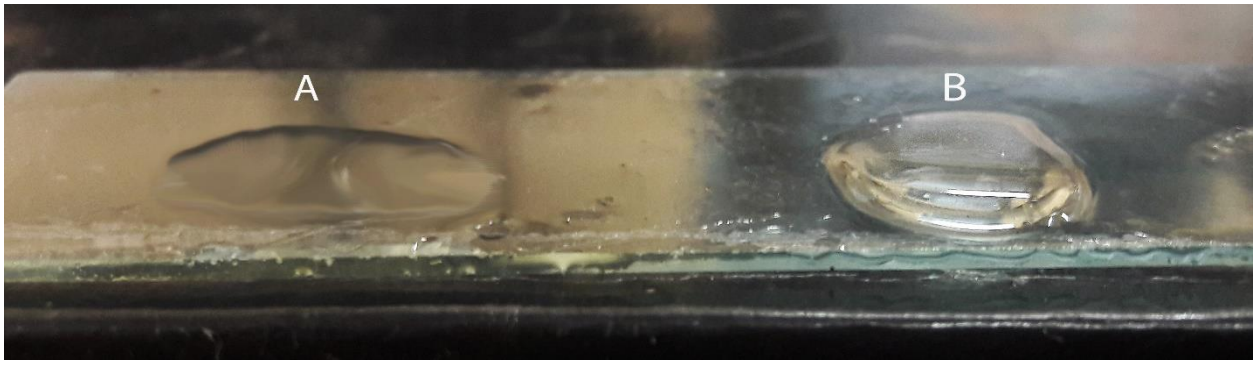


Fig 1.5.: Effect on fermented cell free broth supernatant drop on coconut oil greased glass slide after 1 minute; (A) Cell free supernatant drop, (B) Water, as control.

1.3.4 Biochemical characterization of isolated strain

Strain PC1 was observed to be gram positive. It was rod shaped, acapsulated, non-flagellate and motile (J. Zhang et al., 2010). These were observed under a light microscope (Olympus BH2). It was also seen to be endospore forming, catalase positive, oxidase positive, coagulase negative, urease negative and was a non-nitrate reducing strain (Table 1.3).

Table 1.3: Different biochemical characteristics shown by the isolate PC1.

Sl. No.	**Analytical tests**	**PC1**
1.	Gram staining	+
2.	Spore staining	+
3.	Motility assay	+
4.	Catalase assay	+
5.	Oxidase assay	+
6.	Coagulase	-
7.	Urease	-
8.	Nitrate reduction	-

Growth curve clearly showed that PC1 reached it maximal growth in about 28 hrs from the inoculation (Fig 1.6).

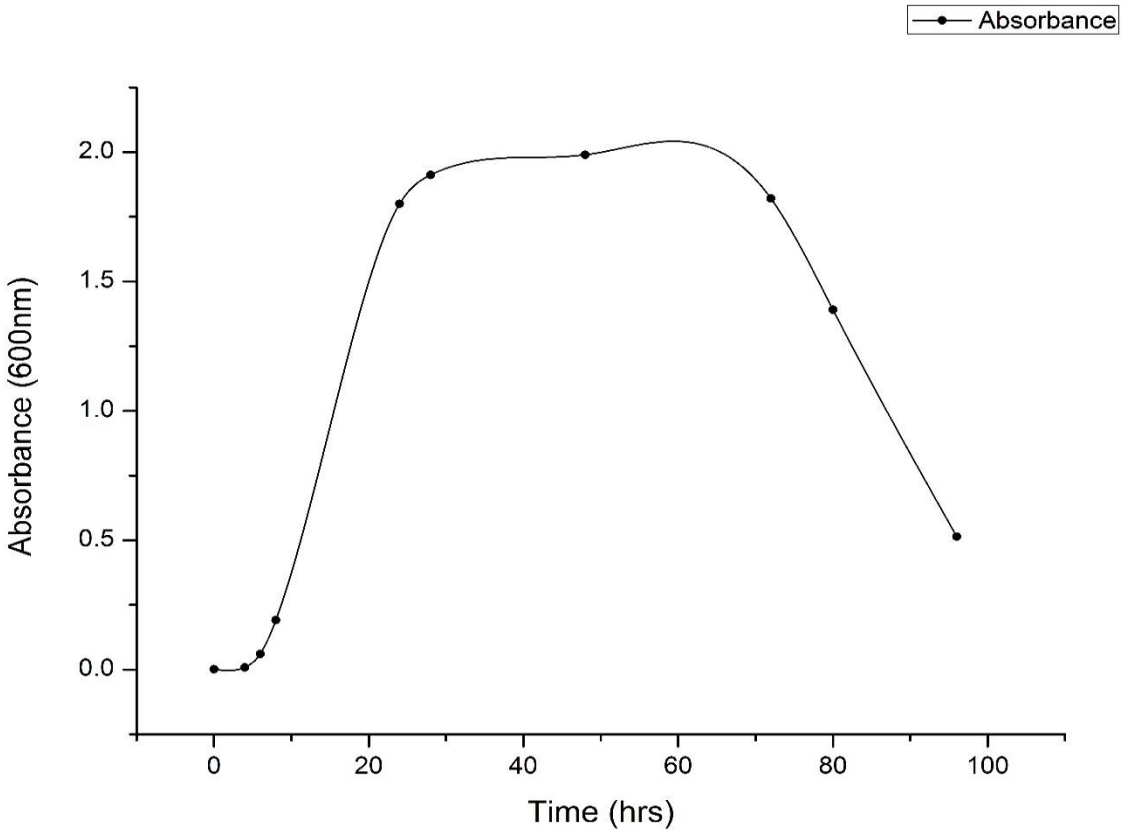


Fig 1.6.: Growth curve of isolated strain PC1.

BLAST analysis and EZ taxon analysis for phylogenetic analysis with PC1's close neighbors, identified it to be *Bacillus oceanisediminisH2* (Fig 1.7) which showed 97.91%

similarity with the query PC1 gene sequence. The bootstrap value was 1000 base repeats and 50 5 of the overlaps were eliminated for avoidance of repeated neighboring sequence results. Multiple sequence alignment (MSA) was performed with build in Clustal X in MEGA 7.0 (Kumar et al., 2016). A total number of 33 neighboring sequences were found through EZbiocloud's identify tools and then were selected for MSA with gave an evolutionary phylogenetic tree that related the query sequence.

The consensus 16S rRNA sequence of PC1 (obtained from Xcelris Labs Limited, Ahmedabad, Gujarat, India) is as follows:

5'-

ATGGCAAAGGTCTAGCGGACAGATGGGAGCTTGCTCCCTGAAGTCAGCGGCGGACGGGTG
AGTAACACGTGGGCAACCTGCCTGTAAGACTGGGATAATCCGGGAAACCGGGGCTAATAC
CGGATAATTCTTTCCCTCACATGAGGGAAAGCTGAAAGATGGTTTCGGCTATCACTTACAG
ATGGGCCCGCGGCGCATTAGCTAGTTGGTGAGGTAACGGCTCACCAAGGCAACGATGCGT
AGCCGACCTGAGAGGGTGATCGGCCACACTGGGACTGAGACACGGCCCAGACTCCTACGG
GAGGCAGCAGTAGGGAATCTTCCGCAATGGACGAAAGTCTGACGGAGCAACGCCGCGTGA
GTGATGAAGGTTTTCGGATCGTAAAACTCTGTTGTTAGGGAAGAACAAGTACCGGAGTAAC
TGCCGGTACCTTGACGGTACCTAACCAGAAAGCCACGGGTAACTACGTGCCAGCAGCCGC
GGTAATACGTAGGTGGCAAGCGTTGTCCGGAATTATTGGGCGTAAAGCGCGCGCAGGCGG
TTCCTTAAGTCTGATGTGAAAGCCCCCGGCTCAACCGGGGAGGGTCATTGGAAACTGGGGA
ACTTGAGTGCAGAAGAGAAGAGTGGAATTCCACGTGTAGCGGTGAAATGCGTAGAGATGT
GGAGGAACACCAGTGGTGAAGGCTGACTCTTTGGTCTGTAACTGACGCTGAGGCGCGAAA
GCGTGGGGAGCAAACAGGATTAGATACCCTGGTAGTCCACGCCGTAAACGATGAGTGCTA
AGTGTTAGAGGGTTTCCTCCCTTTTATTGCTGCAGCAAACGCATTAAAGCACTCCGACTGT
GGCAGTACGGACGCAAGGGTGAAAACTCAAAGGAATAGACGGGGGGCCCGCACAACGCGG
TGGAGCATGTGGTTTATTTCGAGCATCGCGAAGAACCTTTACCAGGTCGTTGACTTCTCTG
ACAACCCTAGAGATGGGTCGTTCCGCTATCCGGCGGACAGCATGACAGGTCGTTGCATGGT
TGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCTCAACGAGCGCAACCCTTGATC
TTAGTTGCCAGCATTCAGTTGGGCACTCTAAGGTGACTGCCGGTGACAAACCGGAGGAAG
GTGGGGATGACGTCAAATCATCATGCCCCTTATGACCTGGGCTACACACGTGCTACAATGG
ATGGTACAAAGGGCTGCGAGACCGCGAGGTTAAGCGAATCCCATAAACCATTCTCAGTTCG
GATTGCAGGCTGCAACTCGCCTGCATGAAGCCGGAATCGCTAGTAATCGCGGATCAGCATG
CCGCGGTGAATACGTTCCCGGGCCTTGTACACACCGCCCGTCACCCCACGAGAGTTTGTAA
CACCGGAAGTCGGTGGGGTAACCTTTTCTAGCCATTCCAAC - 3'

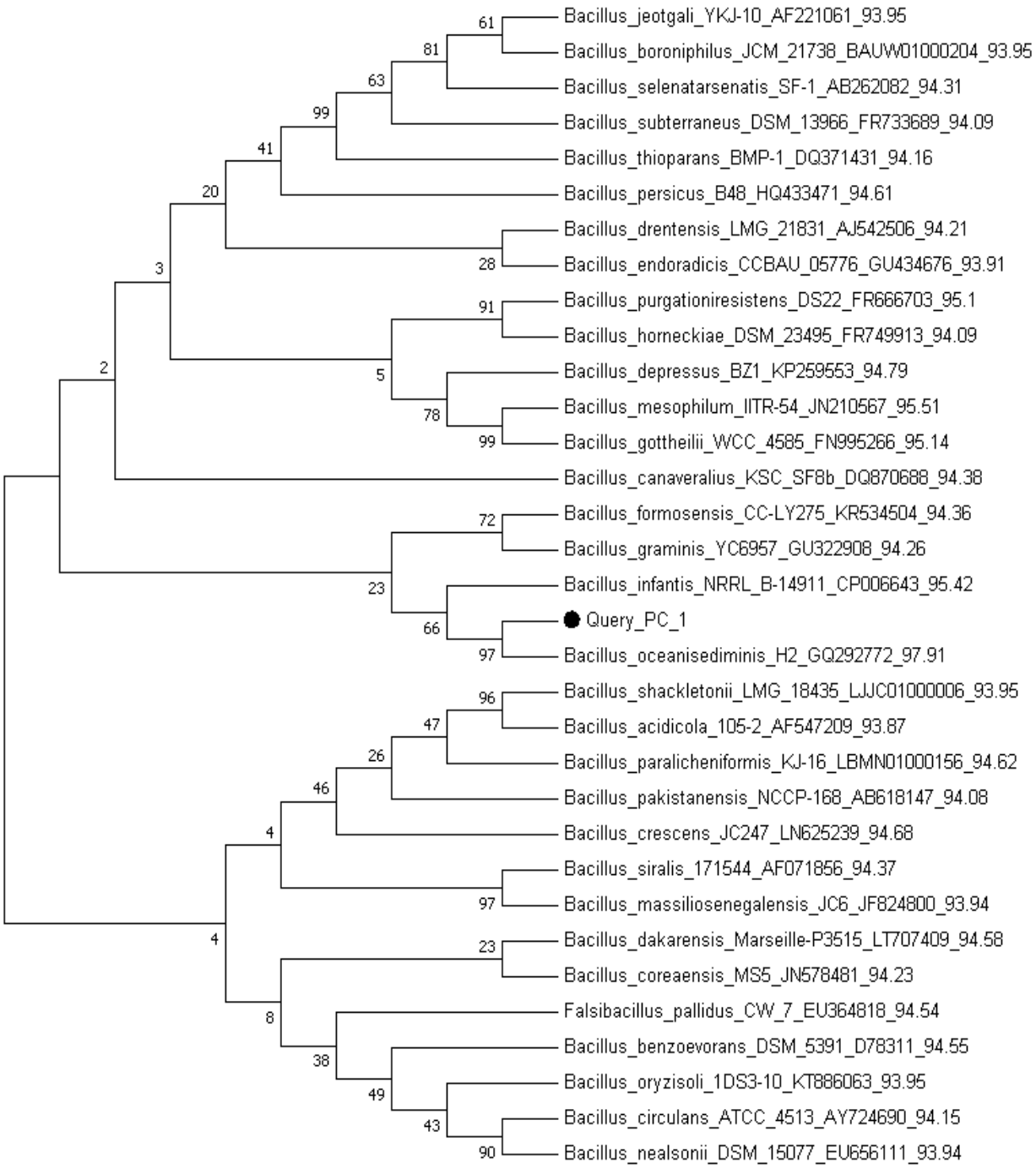


Fig 1.7. Evolutionary relationships of taxa based on 16S rRNA sequences of *Bacillus oceanisediminis H2*(PC1). The accession numbers and the similarity percentages are followed after the name of the taxa that are in close neighboring relationship with the query sequence.

The evolutionary history of the neighborhood was found using the Neighbor-joining process (Saitou & Nei, 1987). At one thousand replicates, as achieved by Felsenstein,

1985, is considered to be the complete genetic archive of PC1 (Felsenstein, 1985). Less than half of a bootstrap partitioning algorithm partitions the identical partition trees percentage of which cluster in the bootstrap (1000 replicates, accompanied by the associated taxa) (Felsenstein, 1985). The Maximum Probability method has been used to measure evolutionary distances (Tamura et al., 2004). The findings revealed that there were 33 nucleotide sequences in the study. Stored codon positions have been first, second, and last. Any gaps and and records lacking data have been omitted. There were an overall of 1125 positions in the last database. MEGA7.0 conducted evolutionary analysis (Kumar et al., 2016).

1.4 Conclusion

The isolated and screened strain PC1 was screened according to the colony's zonal diameter and maximum surface tension lowering ability when it was incubated in NA plates with precoated anthracene which was 3.4cm and 33.8 mN/m respectively. The selected strain PC1 was seen to be gram positive, endospore forming, motile, has catalase activity, oxidase activity but cannot coagulase, produce urease or reduce nitrate. The growth curve of PC1 shows that it reaches end of log phase in 28hrs and the stationary phase extends up to 72hrs from the time of incubation, suggesting extensive biosurfactant production.

1.5 References

Ahmad, Z., Arshad, M., Asghar, H. N., Sheikh, M. A., & Crowley, D. E. (2016). Isolation, screening and functional characterization of biosurfactant producing bacteria isolated from crude oil contaminated site. *International Journal of Agriculture and Biology*, *18*(3).

Cameotra, S. S., Makkar, R. S., Kaur, J., & Mehta, S. K. (2010). Synthesis of biosurfactants and their advantages to microorganisms and mankind. In *Biosurfactants* (pp. 261–280). Springer.

Cappello, S., Volta, A., Santisi, S., Morici, C., Mancini, G., Quatrini, P., Genovese, M., Yakimov, M. M., & Torregrossa, M. (2016). Oil-degrading bacteria from a membrane bioreactor (BF-MBR) system for treatment of saline oily waste: Isolation, identification and characterization of the biotechnological potential. *International Biodeterioration & Biodegradation*, *110*, 235–244.

Das, P., Mukherjee, S., & Sen, R. (2008). Improved bioavailability and biodegradation of a model polyaromatic hydrocarbon by a biosurfactant producing bacterium of marine origin. *Chemosphere*, *72*(9), 1229–1234.

Felsenstein, J. (1985). Confidence limits on phylogenies: An approach using the bootstrap. *Evolution*, *39*(4), 783–791.

Gregersen, T. (1978). Rapid method for distinction of Gram-negative from Gram-positive bacteria. *European Journal of Applied Microbiology and Biotechnology*, *5*(2), 123–127.

Harley, J. P. (2004). *Laboratory exercises in microbiology*. McGraw-Hill Science, Engineering & Mathematics.

Kumar, S., Stecher, G., & Tamura, K. (2016). MEGA7: Molecular evolutionary genetics analysis version 7.0 for bigger datasets. *Molecular Biology and Evolution*, *33*(7), 1870–1874.

Lee, Y.-J., Lee, S.-J., Jeong, H., Kim, H. J., Ryu, N., Kim, B.-C., Lee, H.-S., Lee, D.-W., & Lee, S. J. (2012). *Draft genome sequence of Bacillus oceanisediminis 2691*. Am Soc Microbiol.

Mujahid, T. Y., Wahab, A., Padhiar, S. H., Subhan, S. A., Baloch, M. N., & Pirzada, Z. A. (2015). Isolation and characterization of hydrocarbon degrading bacteria from petrol contaminated soil. *Journal of Basic and Applied Sciences*, *11*, 223–231.

Najafi, A. R., Rahimpour, M. R., Jahanmiri, A. H., Roostaazad, R., Arabian, D., & Ghobadi, Z. (2010). Enhancing biosurfactant production from an indigenous strain of Bacillus mycoides by

optimizing the growth conditions using a response surface methodology. *Chemical Engineering Journal*, *163*(3), 188–194.

Nakhleh, M. B., & Krajcik, J. S. (1993). A protocol analysis of the influence of technology on students' actions, verbal commentary, and thought processes during the performance of acid-base titrations. *Journal of Research in Science Teaching*, *30*(9), 1149–1168.

P\laza, G. A., Zjawiony, I., & Banat, I. M. (2006). Use of different methods for detection of thermophilic biosurfactant-producing bacteria from hydrocarbon-contaminated and bioremediated soils. *Journal of Petroleum Science and Engineering*, *50*(1), 71–77.

Saitou, N., & Nei, M. (1987). The neighbor-joining method: A new method for reconstructing phylogenetic trees. *Molecular Biology and Evolution*, *4*(4), 406–425.

Steel, K. J. (1961). The oxidase reaction as a taxonomic tool. *Microbiology*, *25*(2), 297–306.

Tamura, K., Nei, M., & Kumar, S. (2004). Prospects for inferring very large phylogenies by using the neighbor-joining method. *Proceedings of the National Academy of Sciences*, *101*(30), 11030–11035.

Vahabi, A., Ramezanianpour, A. A., Sharafi, H., Zahiri, H. S., Vali, H., & Noghabi, K. A. (2015). Calcium carbonate precipitation by strain Bacillus licheniformis AK 01, newly isolated from loamy soil: A promising alternative for sealing cement-based materials. *Journal of Basic Microbiology*, *55*(1), 105–111.

Walter, V., Syldatk, C., & Hausmann, R. (2010). Screening concepts for the isolation of biosurfactant producing microorganisms. In *Biosurfactants* (pp. 1–13). Springer.

Yang, L., Quan, X., Xue, B., Goodwin, P. H., Lu, S., Wang, J., Du, W., & Wu, C. (2015). Isolation and identification of Bacillus subtilis strain YB-05 and its antifungal substances showing antagonism against Gaeumannomyces graminis var. Tritici. *Biological Control*, *85*, 52–58.

Yoon, S.-H., Ha, S.-M., Kwon, S., Lim, J., Kim, Y., Seo, H., & Chun, J. (2017). Introducing EzBioCloud: A taxonomically united database of 16S rRNA gene sequences and whole-genome assemblies. *International Journal of Systematic and Evolutionary Microbiology*, *67*(5), 1613.

You, Z.-Q., Li, J., Qin, S., Tian, X.-P., Wang, F.-Z., Zhang, S., & Li, W.-J. (2013). Bacillusabyssalis sp. Nov., isolated from a sediment of the South China Sea. *Antonie van Leeuwenhoek*, *103*(5), 963–969.

Yu, W., Kim, H. K., Rauch, S., Schneewind, O., & Missiakas, D. (2017). Pathogenic conversion of coagulase-negative staphylococci. *Microbes and Infection*, *19*(2), 101–109.

Zhang, J., Wang, J., Fang, C., Song, F., Xin, Y., Qu, L., & Ding, K. (2010). Bacillus oceanisediminis sp. Nov., isolated from marine sediment. *International Journal of Systematic and Evolutionary Microbiology*, *60*(12), 2924–2929.

Zhang, X., Xu, D., Zhu, C., Lundaa, T., & Scherr, K. E. (2012). Isolation and identification of biosurfactant producing and crude oil degrading Pseudomonas aeruginosa strains. *Chemical Engineering Journal*, *209*, 138–146.

Zhao, F., Liang, X., Ban, Y., Han, S., Zhang, J., Zhang, Y., & Ma, F. (2016). Comparison of methods to quantify rhamnolipid and optimization of oil spreading method. *Tenside Surfactants Detergents*, *53*(3), 243–248.

Chapter 2

Optimization of environmental parameters for biosurfactant production.

2.1. Introduction

Polycyclic aromatic hydrocarbons (PAH), because of their toxicity, mutability and cancer impacts, are world environmental pollutants (Haritash & Kaushik, 2009). These are classes of VOCs deposited in the environment for years due to the fact that PAHs are insoluble or sparingly soluble during aqueous phases (Barro et al., 2009; Nowicka et al., 2018). Microbial bioremediation of PAHs should be the most effective, cost-effective, and easy-to-produce method that refers to unfavorable physicochemical processes (Holkar et al., 2016). The isolation of bacteria that can degrade PAHs is a first step in understanding environmental microbiology and the fate of PAHs. Isolate listing is key to a microbial community degrading PAH (Tahseen, 2017). PAHs pose a high threat to land and sea environments as a result of their toxicity. These compounds contain a huge number of compounds, that are not only poisonous and hydrophobic, but also build up in or mineralized in different forms in the species, which are equivalent to the threat to ecological balance (Joy et al., 2017). Different methods widely classified as bioremediation may lead to the decontamination of these compounds (Kiran et al., 2016).

Microbial bioremediation has increasingly recognized the advantages of chemistry, physical separation, solidification and stabilization compared with other technologies. These advantages include cost saving, improved productivity and negligible environmental impacts. Bioremediation has also provided a broad approach to the application (Mukherjee & Bordoloi, 2011; Varjani & Upasani, 2017).

Bioremediation involves a restrictive factor in the effectiveness of the process by preventing PAHs from pollutant uptake by microorganisms. An approach to improving availability of PAHs produced by microorganisms is the production and disposals of biosurfactants that cause pseudosolubility or hydrocarbon entanglement (Hua & Wang, 2012; Rocha et al., 2011).

PAHs are strong environmental toxins composed of fused aromatic rings, or polycyclic aromatic hydrocarbons. PAHs are from raw, carbon tar and blacktop raw material (Ukiwe et al., 2013). PAHs listed in the contaminant applicant lists include the United States Environmental Protection Agency and the European Community (EC). The EPA has previously designated such toxins in water and sediment as priority contaminants (Hadibarata et al., 2009). PAHs are a significant source of toxins in humans and wildlife, some of them carcinogenic, mutagenic or teratogenic.

Benzene rings range from 2 and 7 in PAH-compounds. They lack mixtures of water affinity, with water solubility decreasing almost linearly with increasing molecular mass (Kuppusamy et al., 2017; Parrish et al., 2004). The number of rings in an atom influences PAHs' physical and molecular features. With their increased subatomic weight, chemical reactivity, water solubility and volatility of PAHs are all decreased. The dense blanket of pi-electrons surrounding the aromatic rings aids in high-resonance and resistance to molecular weight loss. It can be clarified by the poor solubility of water and fast soil regeneration of compounds (Kuppusamy et al., 2017; Parrish et al., 2004). The decay of PAH, its fate and surface transport are monitored by a number of physical and biological processes.

Microorganisms such as bacteria and algae (Hamamura et al., 2013) or fungi (Cerniglia & Sutherland, 2010) and algae are used as biological hydrocarbons (Chan et al., 2006; Ghosal et al., 2016). Fungi are viewed as a possible competitor for successful degradation of PAH. However, filamentous growth may evolve through the separation of extracellular hydrolyte enzymes on a wide range of substrates (Kuppusamy et al., 2017). Bioremediation includes inefficient degraders at a polluted site, either endogenous or microbial exogenous populations (Agrawal et al., 2019; Mishra et al., 2019).

The goal of this research will be the optimization of environmental parameters in the tannery processing industrial wastelands in Kolkata (India) for the derived biosurfactant from the isolated strain *Bacillus oceanisediminis* H2 found. The optimization procedure used here was one parameter variable that was optimized by the surface answering methodology (RSM).

2.2 Materials and Methods

2.2.1 Sample

Bacillus oceanisediminis H2 was collected and maintained as mentioned in chapter 1 (1.2.1)

2.2.2 Culture media

Nutrient Broth (NB) and Mineral salts media (MSM) was prepared as mentioned in chapter 1 (1.2.2).

MSM was supplemented with sole carbon source was 2% (v/v) glycerol (GlyMSM) in output media and 0.2% (w/v) anthracene (AMSM) in collection media (Das et al., 2008).

2.2.3 Surface tension measurements

The surface tension measurements were done as mentioned in chapter 1 (1.2.8)

2.2.4 Optimization of lowering of surface tension

The fermentation was done for optimization within a range of 24 – 168 hrs, at temperature range of 25 – 40 °C, with pH range of 5 – 9, and was done with bacterial culture whose age ranged from 8 – 56 hrs.

2.2.5 Optimisation of effect of surface tension by Response Surface Methodology (RSM)

These parameters, which may have an impact on surface tension, were investigated and systematically and optimized by central design (CCD). prior data about the process parameters and variable selections were chosen on an individual basis. Specific effects of time, temperature (°C), and pH on the fermentation on the production of biosurfactant were optimized to keep all other factors constant. Each variable was varied at 5 different levels, as shown in Table 2. This was done in two separate runs; in separate experiments, enzymatic activity was measured two times. The second order polynomial equation was used to determine surface tension, and the data were calculated using multiple regression (Banerjee & Ghosh, 2017). Later, an experiment was conducted using optimal values for the variables in response optimization to validate the expected value.

On the other hand, the surface tension measurement was found to be related to or dependent on the other variables. Data analysis based on the propertization for each degree of freedom was by polynomial least-squares was performed in order analysis of

variance for each degree of freedom (ANOVA). Using an empirical polynomial construction, a geometric mean, which was set at five levels, a central composite was expanded to second order was constructed. The quadratic model for optimum prediction was defined in accordance with Eq. 2.2 (Banerjee & Ghosh, 2017):

$$y = \beta_0 + \sum_{i=1}^{n} \beta\, x_i + \sum_{i=1}^{n} \beta_{ii}\, x_i^2 + \sum_{i=1}^{n-1} \sum_{i=1}^{n} \beta_{ij}\, x_i x_j \qquad \text{Eqn 2.2}$$

Eqn 2.2: Generalized quadratic polynomial equation in the multivariate form, where, *y* is the response variable, β is the regression coefficients of the model, and *x is* the coded levels of the independent variable.

The expanded response surface modeling uses the entire variable range to study response, locates the point where the response is optimized, and identifies the area where it is optimized. Information on how the various process factors work together to yield the best response can be obtained via a response surface plot.

Table 2.1: Experimental design for a three factor five level response surface analyses

Std	Run	Block	Time (Hours) :A	Temperature (°C) : B	pH : C
7	1	Block 1	24	40	9
10	2	Block 1	152.73	32.5	7
9	3	Block 1	-8.73	32.5	7
11	4	Block 1	72	19.88	7
5	5	Block 1	24	25	9
12	6	Block 1	72	45.11	7
6	7	Block 1	120	25	9
3	8	Block 1	24	40	5
17	9	Block 1	72	32.5	7
13	10	Block 1	72	32.5	3.64
16	11	Block 1	72	32.5	7
20	12	Block 1	72	32.5	7
15	13	Block 1	72	32.5	7
8	14	Block 1	120	40	9
19	15	Block 1	72	32.5	7
2	16	Block 1	120	25	5
14	17	Block 1	72	32.5	10.36
4	18	Block 1	120	40	5
18	19	Block 1	72	32.5	7
1	20	Block 1	24	25	5

2.2.6 Statistical analysis

The statistical importance of the second equation was tested by F-test, and the percentage of variance explained by the model was then indicated by R^2; that indicated by the F-value and multiple coefficient of determination had a function relationship of R^2. It focuses on complex models (i.e., multiple parameters) to search for factors that meet all the standards (in other words, maximizes models, because this paraphrase expresses multiple factors' effects on all responses and processes simultaneously). A 95% confidence limit was put on the experiment, and the result of that was that it was

found to be correct was found to be accurate. All the statistical analyses considering from the design of experiments to the optimization were performed using the statistical software 'Design Expert' (version 7.0.0; Stat-Ease, Inc. Minneapolis, MN USA). Everything was done using the Origin 9.0 software, and all of the statistical plots were completed.

2.3 Results and discussions

2.3.1 Optimization of surface tension lowering ability

The fermented media was seen to lower maximum surface tension when compared to its uninoculated counterpart at 5 days, pH 8, 37°C with 24hr old culture which was set to be the control. This shows that the isolated strain can be utilized for biosurfactant production as it significantly reduced the surface tension of the fermentable media.

The optimization was done by one variable at a time method which kept one parameter in the variable form and the other considered variables as constant. The effects of the parameters gave an optimum result for the time, temperature of the media, pH of the media and age of inoculum for the fermentation, which recorded the maximum lowering of surface tension of the fermented media.

In Fig 2.1 it can be observed that the surface tension measured over time has a minimum of 30.573 mN/m at 120 hrs or 5 days (Table 2.2). The surface tension starts to rise gradually up to 48hrs of fermentation suggesting no production of biosurfactants as the bacterial culture stays in the log phase or terminal log phase of its growth, which starts to decrease eventually till 120 hrs reaching a minimum surface tension record of 30.573 mN/m. Previous studies have shown to efficiently produce biosurfactant at 10 days' incubation time (Mouafi et al., 2016). This further ensures that the biosurfactant production occurs in the stationary phase of the bacterial growth.

Table 2.2: Effect of time of fermentation on the production of biosurfactant.

Time (hours)	**Surface Tension (mN/m)**
24	38.556 ± 1.9278
48	42.372 ±2.1186
72	36.297 ±1.81485
96	34.965 ±1.74825
120	30.537 ±1.52685
168	48.069 ±2.40345

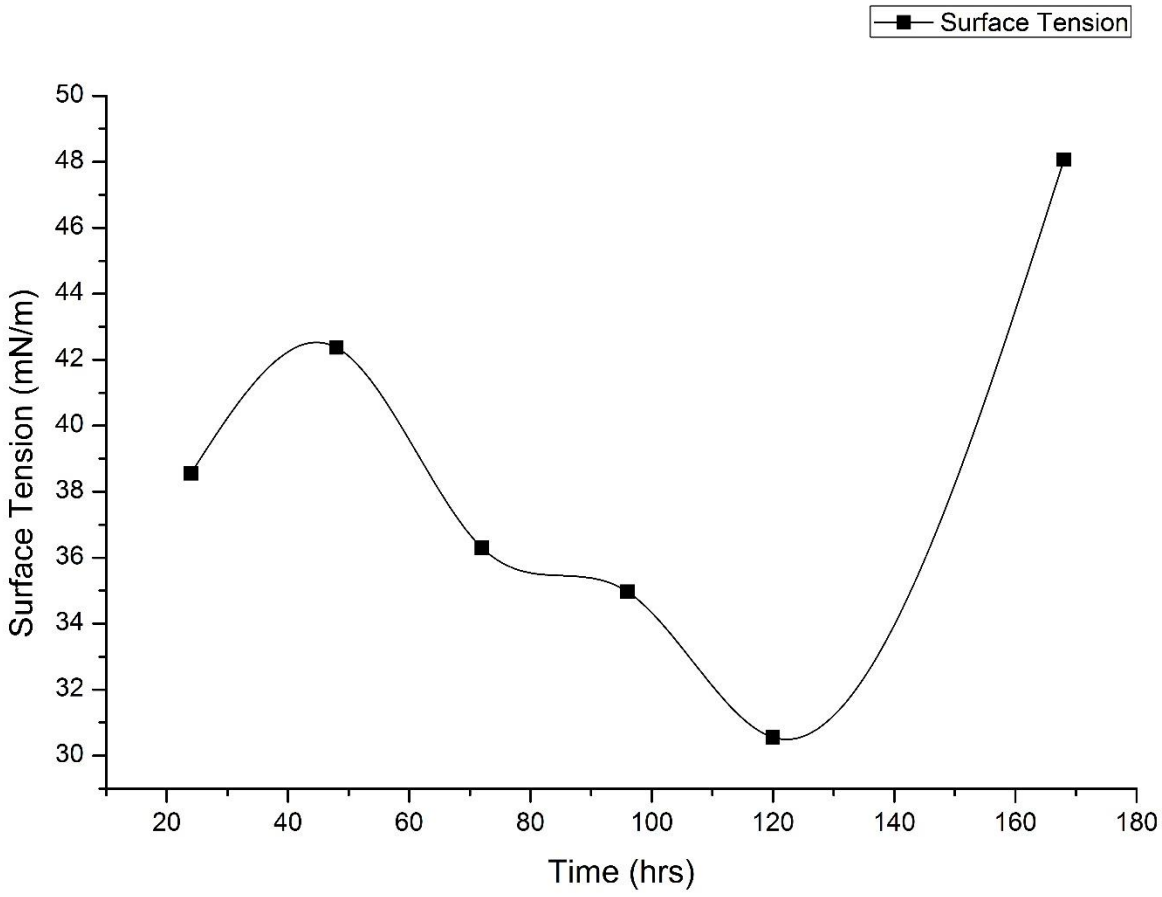


Fig 2.1. Effect of fermentation time on the production of biosurfactant.

In Fig 2.2 it can be observed that the surface tension measured at various temperatures has a minimum of 29.22 mN/m at 37°C (Table 2.3). The surface tension starts to decrease gradually with increase in the temperature of fermentation suggesting no production of biosurfactants as the bacterial culture grows not optimally at lower temperatures hence the increased surface tension of the fermented media. Previous reports have shown a maximum reduction in surface tension at 39.03°C (Najafi et al., 2010). This further ensures that the biosurfactant production occurs optimally at the optimal temperature for the bacterial growth.

Table 2.3: Effect of temperature of fermentation the production of biosurfactant.

Temperature (°C)	Surface tension (mN/m)
25	48.47 ± 2.4235
27	43.57 ± 2.1785
30	38.52 ± 1.926
35	37.21 ± 1.8605
37	29.22 ± 1.461
40	42.183 ± 2.10915

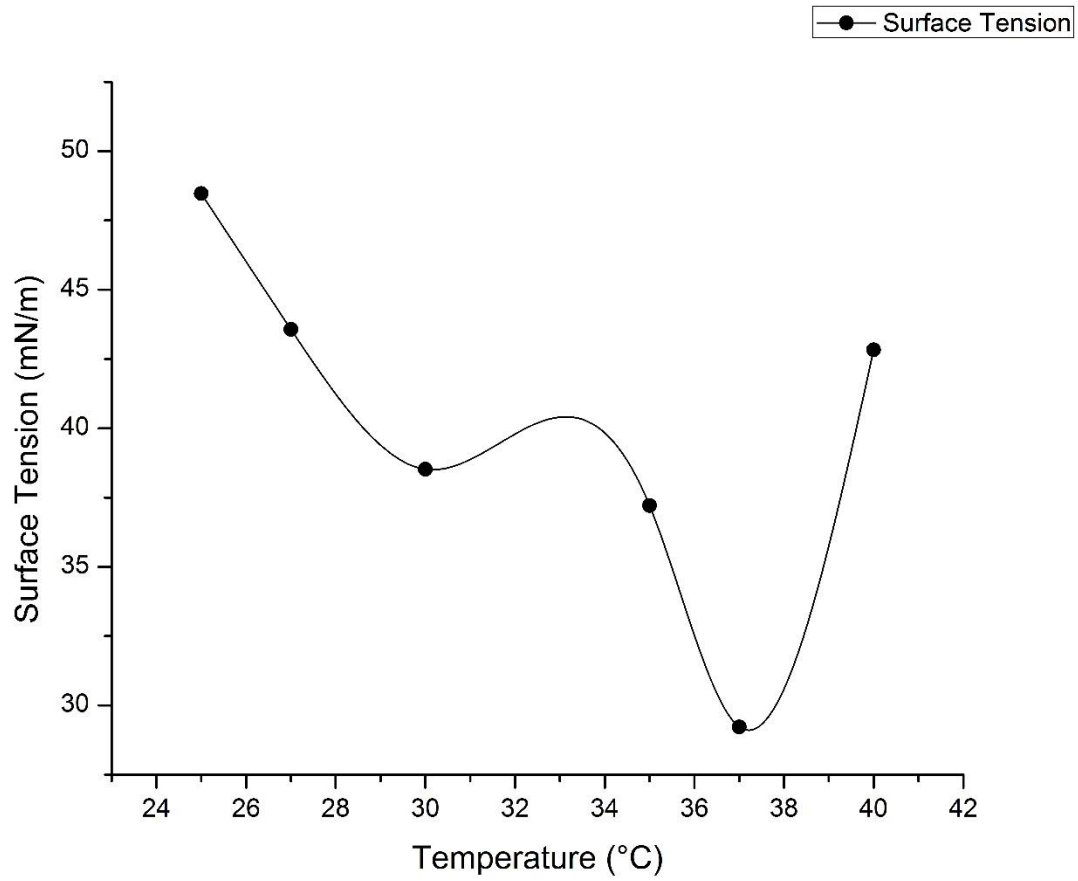


Fig 2.2. Effect of fermentation temperature on the production of biosurfactant.

In Fig 2.3 it can be observed that the surface tension measured at various pH of the fermentable media has a minimum of 30.51 mN/m at pH 8 (Table 2.4). Previous studies have shown to efficiently produce biosurfactant at pH 8 (Mouafi et al., 2016). The surface tension starts to decrease gradually with increase in pH of fermentable media

and gives close by results even at pH 6 (Table 2.4), suggesting production of biosurfactants is possible by the bacterial strain at both acidic and alkaline environments. It further suggests that the isolated strain not only produces biosurfactant optimally at alkaline pH but can also give a nearby efficient production at acidic pH.

Table 2.4: Effect of pH of fermentable media on the production of biosurfactant.

pH	**Surface tension (mN/m)**
5	48.177 ± 2.40885
6	37.44 ± 1.872
7	44.35 ± 2.2175
8	30.51 ± 1.5255
9	50.031 ± 2.50155

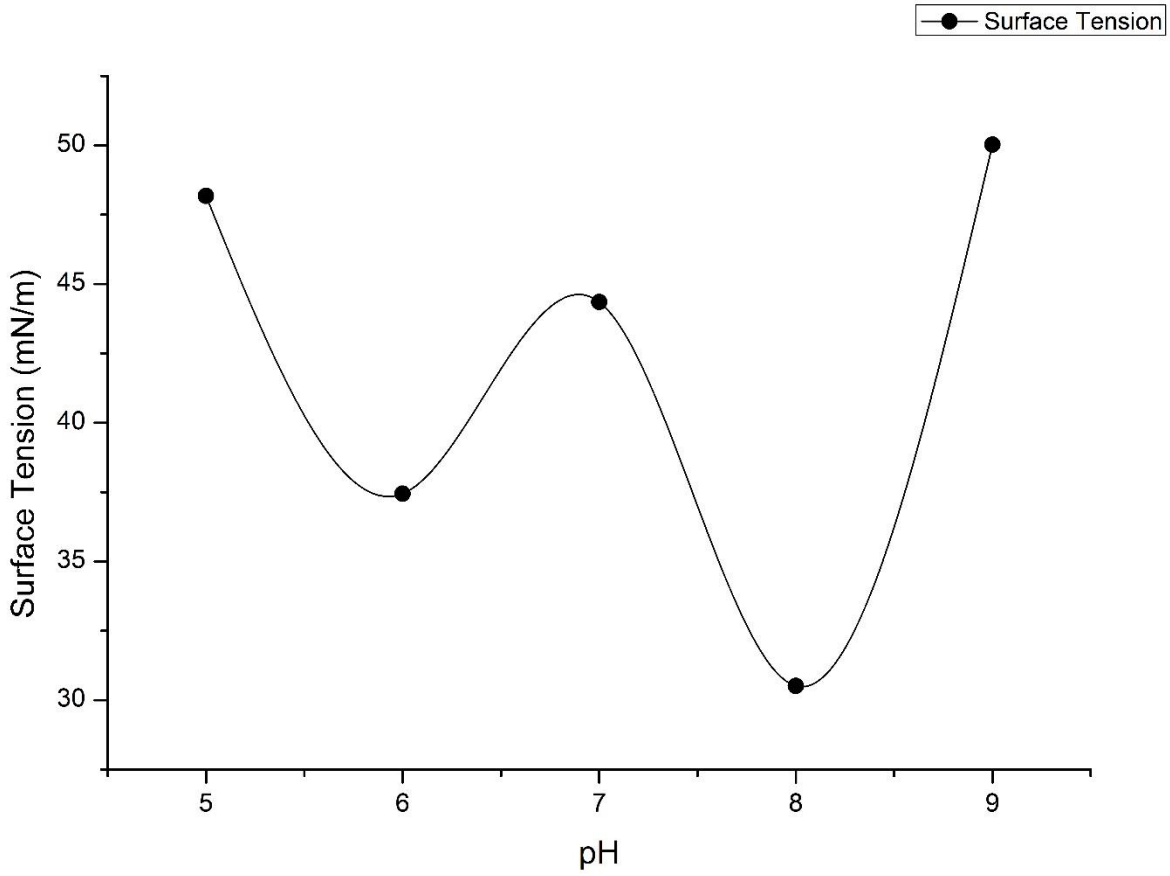


Fig 2.3. Effect of pH on the production of biosurfactant.

In Fig 2.4 it can be observed that the surface tension measured for the fermented media incubated with inoculum of the bacterial culture of different age has a minimum of 29.43 mN/m of 24 hours grown culture (Table 2.5). Previous studies have shown to efficiently produce biosurfactant for inoculum which was a 24hr grown culture (Ghribi et al., 2012). The surface tension starts to decrease gradually with a culture of age up to 24hours and then further increases. It further suggests that the isolated strain only produces biosurfactant optimally with 24 hours' culture.

Table 2.5: Effect of age of inoculum on the production of biosurfactant.

Age of inoculum (hours)	**Surface tension (mN/m)**
8	38.178 ± 1.9089
24	29.43 ± 1.4715
32	40.959 ± 2.04795
48	44.748 ± 2.2374
56	35.631 ± 1.78155

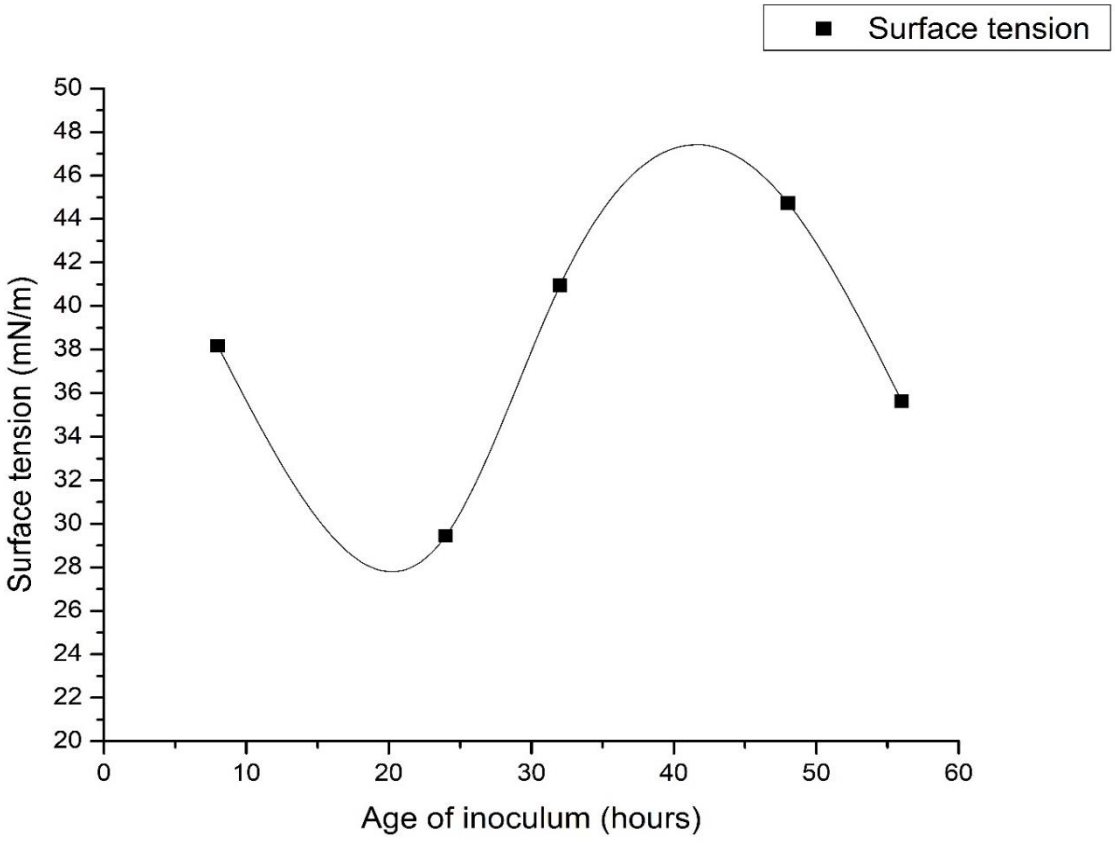


Fig 2.4: Effect of Age of inoculum on the production of biosurfactant.

2.3.2 Optimization of effect of surface tension by Response Surface Methodology (RSM)

The RSM central composite architecture investigated the optimum degree of the main factors and their impact on biosurfactant output. Table 2.3 shows experimental architecture and performance. The following second-order polynomial equation (Eqn. 2.3) was determined in order to describe surface tension calculation by applying multiple regression analyses on the test data:

$$Surface\ Tension = +46.65 - 4.07A + 0.89B - 3.69C + 1.91AB - 6.66AC - 2.89BC + 1.66A^2 - 2.14B^2 + 2.98C^2 \quad \text{Eqn 2.3}$$

Eqn 2.3: Multivariate polynomial equation obtained by multiple regression analysis for surface tension measurements. [A = Time; B = Temperature; C = pH.]

Where A, B, and C are the coded values for incubation time, incubation temperature, and substrate amount, respectively, and Y is the expected value of surface tension. The meaning of the fit of the second-order polynomial equation for the experimental results seen in Table 2.6 was tested using ANOVA. The model's F-value of 10.08 indicates that it is meaningful. The model terms are important if their P-values are less than 0.05. A, C, AC, BC, and B^2 are important model words in this situation. The model terms are not meaningful if the ANOVA parameters have $p > 0.10$. These non-significant model terms aid in the selection of terms that contribute the least statistically and practically to the design of experiments. The modified R^2 of 0.8113 was considered to be in fair alignment with the expected R^2 of 0.9007. This further confirms the theoretical model's suitability for the ongoing optimization project. The residuals were examined and no anomalies were found. As a result, the model can be inferred to be statistically sound. The response surfaces (Figs. 2.5, 2.6, and 2.7) show the type and degree of interaction between various variables.

Table 2.6: ANOVA for the response surface of a quadratic model*

Response	1	Surface Tension				
ANOVA for Response Surface Quadratic Model Analysis of variance table [Partial sum of squares - Type III]						
	Sum of		Mean	F	p-value	
Source	Squares	df	Square	Value	Prob > F	
Model	1125.59	9	125.07	10.08	0.0006	Significant
A-Time	226.17	1	226.17	18.22	0.0016	
B-Temperature	10.7	1	10.7	0.86	0.375	
C-pH	186.07	1	186.07	14.99	0.0031	
AB	29.11	1	29.11	2.35	0.1567	
AC	355.38	1	355.38	28.63	0.0003	
BC	67.05	1	67.05	5.4	0.0425	
A^2	39.6	1	39.6	3.19	0.1044	
B^2	66.19	1	66.19	5.33	0.0436	
C^2	127.82	1	127.82	10.3	0.0093	
Residual	124.13	10	12.41			Not significant
Lack of Fit	124.13	5	24.83			
Pure Error	0	5	0			
Cor Total	1249.72	19				

*Model p-value suggests that the experimental design is significant for the chosen design of experiment. The large F-values suggests that the experimental setups were quite significant. The multiple binary terms were significant which confirmed stable interaction between the process parameters. Lack of fit was shown to be non-significant which assured the model to be fit again.

Table 2.7: Experimental design and effect of various runs of experiment on the surface tension for a three factor five level response surface analyses

Std	Run	Block	Time (Hours) :A	Temperature (°C) : B	pH : C	Y : Surface Tension
7	1	Block 1	24	40	9	49.03
10	2	Block 1	152.73	32.5	7	49.77
9	3	Block 1	-8.73	32.5	7	58.33
11	4	Block 1	72	19.88	7	40.96
5	5	Block 1	24	25	9	59.93
12	6	Block 1	72	45.11	7	45.64
6	7	Block 1	120	25	9	30.14
3	8	Block 1	24	40	5	52.94
17	9	Block 1	72	32.5	7	46.49
13	10	Block 1	72	32.5	3.64	61.95
16	11	Block 1	72	32.5	7	46.49
20	12	Block 1	72	32.5	7	46.49
15	13	Block 1	72	32.5	7	46.49
8	14	Block 1	120	40	9	31.57
19	15	Block 1	72	32.5	7	46.49
2	16	Block 1	120	25	5	49.13
14	17	Block 1	72	32.5	10.36	53.62
4	18	Block 1	120	40	5	57.44
18	19	Block 1	72	32.5	7	46.49
1	20	Block 1	24	25	5	47.56

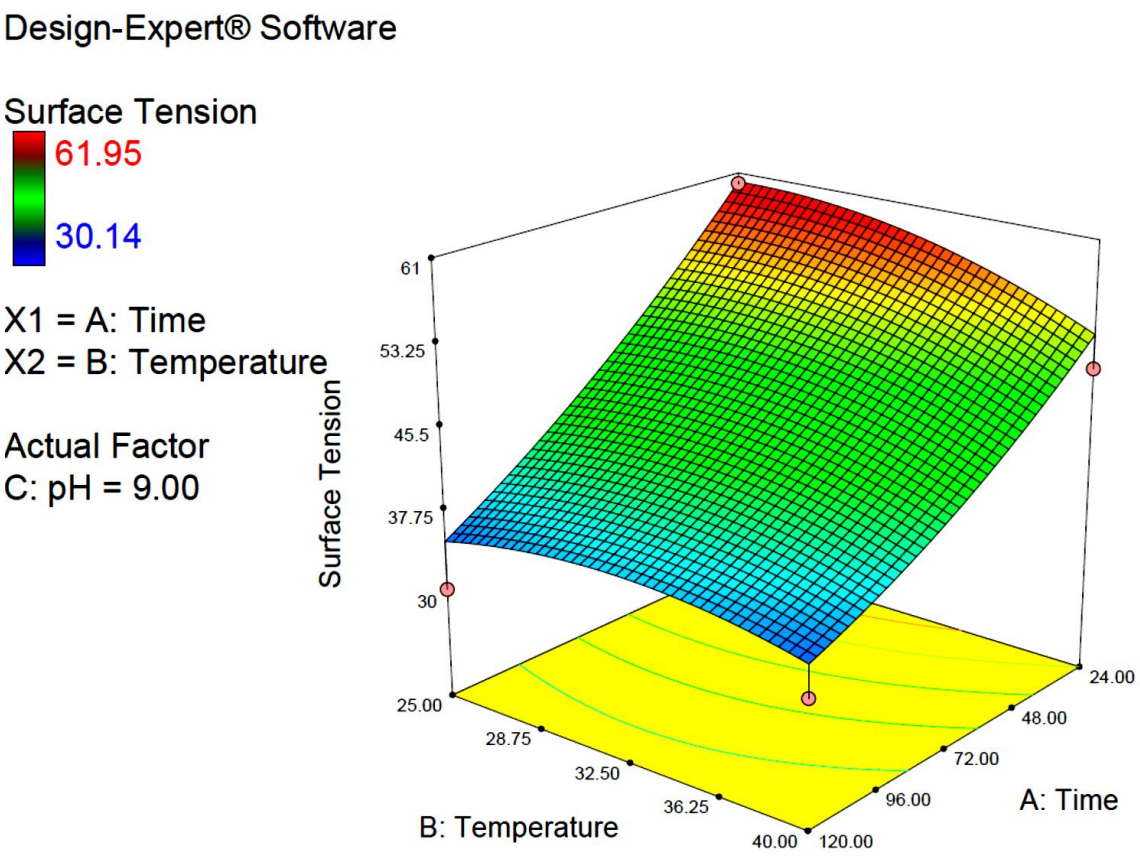


Fig 2.5: 3D response surface plot showing the effect of time and temperature on the production of biosurfactant.

In the Fig 2.5 the 3D surface shows a flat gradational decrease in the fermented GlyMSM media's surface tension with increase in time of fermentation and temperature of fermentation, which gave an optimum of 34.61 mN/m at 120 hours and 40°C respectively. The flat 3D surface suggests steady decrease in surface tension with increase in time of fermentation and temperature of fermentation, further suggesting no optimal results in the mid-range of the considered range of the two variables which were carried out at an optimum pH of 9.0.

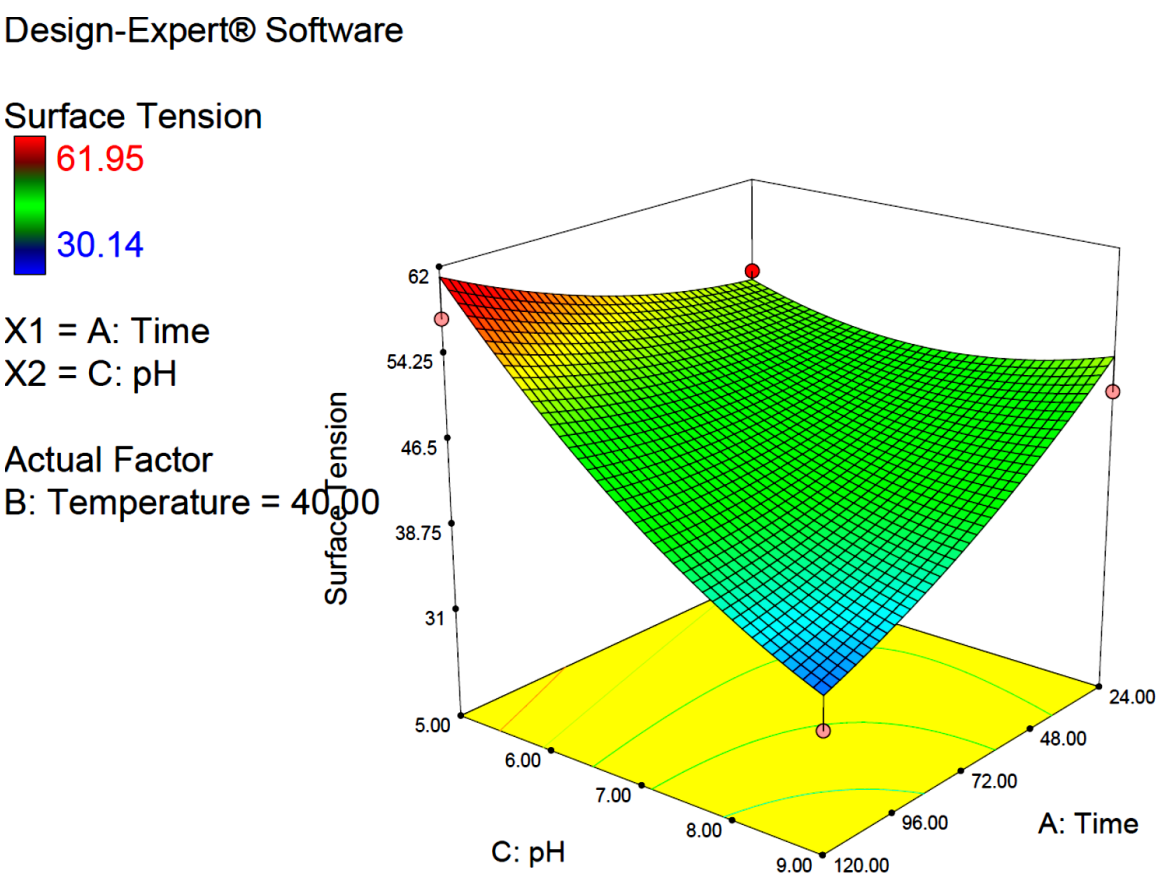


Fig 2.6: 3D response surface plot showing the effect of time and pH on the production of biosurfactant.

In the Fig 2.6 the 3D surface shows a curved gradational decrease in the fermented GlyMSM media's surface tension with increase in time of fermentation and pH of the fermentable media, which gave an optimum of 34.61 mN/m at 120 hours and pH 9.0 respectively. The curved downward 3D surface suggests steady decrease in surface tension with increase in time of fermentation and temperature of fermentation, further suggesting that the drop in surface tension is rapid as the fermentable media turns alkaline but no optimal results were recorded in the mid-range of the considered range of the two variables which were carried out at a temperature optimum of 40°C.

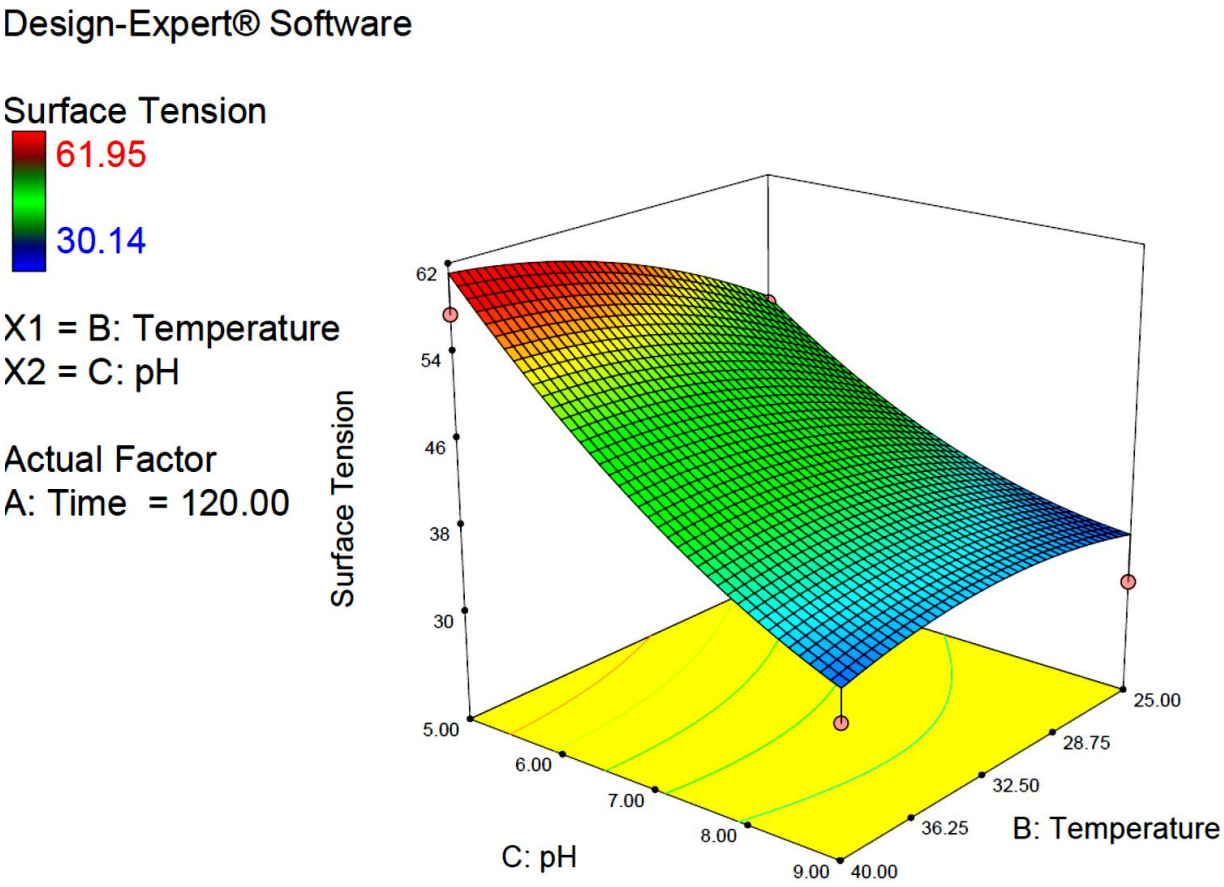


Fig 2.7: 3D response surface plot showing the effect of temperature and pH on the production of biosurfactant.

In the Fig 2.7 the 3D surface shows a curved gradational decrease in the fermented GlyMSM media's surface tension with increase in temperature and pH of the fermentable media, which gave an optimum of 34.61 mN/m at 40°C and pH 9.0 respectively. The curved upward 3D surface suggests steady decrease in surface tension with increase in temperature of fermentation and pH of the fermentable media but increase in the surface tension at around mid-ranges of the ranges chosen, further suggesting that the drop in surface tension is not so high at neutral pH and temperatures around 30°C. No optimal results were recorded in the mid-range of the considered range of the two variables which were carried out at an optimum time of fermentation of 120 hours.

2.3.3 Point prediction for validation of RSM

The quadratic model predicted that when the time, temperature, and pH were 120 hours, 40 degrees Celsius, and 9, respectively, the minimum lowering of surface tension would be 34.61 mN/m. The replication experiment was carried out in duplicate experiments to ensure that the expected findings were correct. The measured experimental titer of GA under optimal conditions was 30.102 mN/m, showing that the experimental and expected values of surface tension lowering were in good agreement. As a result, this finding supports the model's predictions and efficacy, showing that the optimized process parameters play a significant role in lowering surface tension in fermented media through the processing of a potent biosurfactant (Table 2.8). The results were in close communion with previous reports of optimum time at 240 hrs or 10 days (Mouafi et al., 2016), optimum temperature of 39.03°C (Najafi et al., 2010) and optimum pH at 8 (Mouafi et al., 2016).

Table 2.8: Point prediction for the validation of the RSM. (CI= Confidence Interval; SE= Standard Error; PI= Prediction interval) *

Factor	Name	Level	Low Level	High Level	Std. Dev.	Coding	
A	Time	119.9996229	24	120	0	Actual	
B	Temperature	39.99996611	25	40	0	Actual	
C	pH	8.999997718	5	9	0	Actual	
Response	Prediction	SE Mean	95% CI low	95% CI high	SE Pred	95% PI low	95% PI high
Surface Tension	34.61038991	2.883332975	28.18592	41.03486	4.552633	24.46649	44.75429

*The optimum level given by the statistical tool when the design of experiments was carried out between the low and high ranges. It points to the predicted lowest value of surface tension which lies in the range of 95% to 99% confidence limit. This also allows for validation studies.

2.4 Conclusion

The fermented media was seen to lower maximum surface tension when compared to its uninoculated counterpart at 5 days, pH 8, 37°C with 24hr old culture which was set to be the control. This shows that the isolated strain can be utilized for biosurfactant production. The surface tension measured over time is a minimum of 30.573 mN/m at 120 hrs or 5 days. This further ensures that the biosurfactant production occurs in the stationary phase of the bacterial growth. It further suggests that the isolate strain not only produces biosurfactant optimally at alkaline pH but can also give a nearby efficient production at acidic pH.

The response surfaces demonstrate the form and degree of interaction between various variables. With the time and temperature of fermentation, the flat 3D surface indicates a gradual decrease in surface tension. The 3D surface shows a curved gradational decrease in surface tension of the fermented GlyMSM media with an increase in time of fermentation and pH of the fermentable media, which gave an optimum of 34.61 mN/m at 40°C and pH 9.0. Although there is a clear downward curvature in the pH 3D surface tension with increasing temperature, there is a rise in surface tension at the mid-range. In the center of the two variables' considered set, no optimum results were found.

2.5 References

Agrawal, P. K., Shrivastava, R., & Verma, J. (2019). Bioremediation Approaches for Degradation and Detoxification of Polycyclic Aromatic Hydrocarbons. In R. N. Bharagava & P. Chowdhary (Eds.), *Emerging and Eco-Friendly Approaches for Waste Management* (pp. 99–119). Springer. https://doi.org/10.1007/978-981-10-8669-4_6

Banerjee, S., & Ghosh, U. (2017). Production and Characterization of Glucoamylase by Aspergillus niger. *Applied Food Biotechnology*, *4*(1), 19–26.

Barro, R., Regueiro, J., Llompart, M., & Garcia-Jares, C. (2009). Analysis of industrial contaminants in indoor air: Part 1. Volatile organic compounds, carbonyl compounds, polycyclic aromatic hydrocarbons and polychlorinated biphenyls. *Journal of Chromatography A*, *1216*(3), 540–566.

Cerniglia, C. E., & Sutherland, J. B. (2010). Degradation of Polycyclic Aromatic Hydrocarbons by Fungi. In K. N. Timmis (Ed.), *Handbook of Hydrocarbon and Lipid Microbiology* (pp. 2079–2110). Springer Berlin Heidelberg. https://doi.org/10.1007/978-3-540-77587-4_151

Chan, S. M. N., Luan, T., Wong, M. H., & Tam, N. F. Y. (2006). Removal and biodegradation of polycyclic aromatic hydrocarbons by Selenastrum capricornutum. *Environmental Toxicology and Chemistry*, *25*(7), 1772–1779. https://doi.org/10.1897/05-354R.1

Das, P., Mukherjee, S., & Sen, R. (2008). Improved bioavailability and biodegradation of a model polyaromatic hydrocarbon by a biosurfactant producing bacterium of marine origin. *Chemosphere*, *72*(9), 1229–1234.

Ghosal, D., Ghosh, S., Dutta, T. K., & Ahn, Y. (2016). Current State of Knowledge in Microbial Degradation of Polycyclic Aromatic Hydrocarbons (PAHs): A Review. *Frontiers in Microbiology*, *7*. https://doi.org/10.3389/fmicb.2016.01369

Ghribi, D., Abdelkefi-Mesrati, L., Mnif, I., Kammoun, R., Ayadi, I., Saadaoui, I., Maktouf, S., & Chaabouni-Ellouze, S. (2012). Investigation of Antimicrobial Activity and Statistical Optimization of *Bacillus subtilis* SPB1 Biosurfactant Production in

Solid-State Fermentation. *Journal of Biomedicine and Biotechnology*, *2012*, 1–12. https://doi.org/10.1155/2012/373682

Hadibarata, T., Tachibana, S., & Itoh, K. (2009). Biodegradation of chrysene, an aromatic hydrocarbon by Polyporus sp. S133 in liquid medium. *Journal of Hazardous Materials*, *164*(2), 911–917. https://doi.org/10.1016/j.jhazmat.2008.08.081

Hamamura, N., Ward, D. M., & Inskeep, W. P. (2013). Effects of petroleum mixture types on soil bacterial population dynamics associated with the biodegradation of hydrocarbons in soil environments. *FEMS Microbiology Ecology*, *85*(1), 168–178. https://doi.org/10.1111/1574-6941.12108

Haritash, A. K., & Kaushik, C. P. (2009). Biodegradation aspects of polycyclic aromatic hydrocarbons (PAHs): A review. *Journal of Hazardous Materials*, *169*(1–3), 1–15.

Holkar, C. R., Jadhav, A. J., Pinjari, D. V., Mahamuni, N. M., & Pandit, A. B. (2016). A critical review on textile wastewater treatments: Possible approaches. *Journal of Environmental Management*, *182*, 351–366.

Hua, F., & Wang, H. (2012). Uptake modes of octadecane by Pseudomonas sp. DG17 and synthesis of biosurfactant. *Journal of Applied Microbiology*, *112*(1), 25–37.

Joy, S., Rahman, P. K., & Sharma, S. (2017). Biosurfactant production and concomitant hydrocarbon degradation potentials of bacteria isolated from extreme and hydrocarbon contaminated environments. *Chemical Engineering Journal*, *317*, 232–241.

Kiran, G. S., Ninawe, A. S., Lipton, A. N., Pandian, V., & Selvin, J. (2016). Rhamnolipid biosurfactants: Evolutionary implications, applications and future prospects from untapped marine resource. *Critical Reviews in Biotechnology*, *36*(3), 399–415.

Kuppusamy, S., Thavamani, P., Venkateswarlu, K., Lee, Y. B., Naidu, R., & Megharaj, M. (2017). Remediation approaches for polycyclic aromatic hydrocarbons (PAHs) contaminated soils: Technological constraints, emerging trends and future directions. *Chemosphere*, *168*, 944–968. https://doi.org/10.1016/j.chemosphere.2016.10.115

Mishra, S., Chowdhary, P., & Bharagava, R. N. (2019). Conventional Methods for the Removal of Industrial Pollutants, Their Merits and Demerits. In R. N. Bharagava & P.

Chowdhary (Eds.), *Emerging and Eco-Friendly Approaches for Waste Management* (pp. 1–31). Springer. https://doi.org/10.1007/978-981-10-8669-4_1

Mouafi, F. E., Abo Elsoud, M. M., & Moharam, M. E. (2016). Optimization of biosurfactant production by Bacillus brevis using response surface methodology. *Biotechnology Reports*, *9*, 31–37. https://doi.org/10.1016/j.btre.2015.12.003

Mukherjee, A. K., & Bordoloi, N. K. (2011). Bioremediation and reclamation of soil contaminated with petroleum oil hydrocarbons by exogenously seeded bacterial consortium: A pilot-scale study. *Environmental Science and Pollution Research*, *18*(3), 471–478.

Najafi, A. R., Rahimpour, M. R., Jahanmiri, A. H., Roostaazad, R., Arabian, D., & Ghobadi, Z. (2010). Enhancing biosurfactant production from an indigenous strain of Bacillus mycoides by optimizing the growth conditions using a response surface methodology. *Chemical Engineering Journal*, *163*(3), 188–194.

Nowicka, E., Clarke, T. J., Sankar, M., Jenkins, R. L., Knight, D. W., Golunski, S., Hutchings, G. J., Willock, D. J., Francisco, M., & Taylor, S. H. (2018). Oxidation of Polynuclear Aromatic Hydrocarbons using Ruthenium-Ion-Catalyzed Oxidation: The Role of Aromatic Ring Number in Reaction Kinetics and Product Distribution. *Chemistry–A European Journal*, *24*(3), 655–662.

Parrish, Z. D., Banks, M. K., & Schwab, A. P. (2004). Effectiveness of Phytoremediation as a Secondary Treatment for Polycyclic Aromatic Hydrocarbons (PAHs) in Composted Soil. *International Journal of Phytoremediation*, *6*(2), 119–137. https://doi.org/10.1080/16226510490454803

Rocha, C. A., Pedregosa, A. M., & Laborda, F. (2011). Biosurfactant-mediated biodegradation of straight and methyl-branched alkanes by Pseudomonas aeruginosa ATCC 55925. *AMB Express*, *1*(1), 9.

Tahseen, R. (2017). *Effect of Biosurfactants on Remediation of Oil Contaminated Soil* [PhD Thesis]. Pakistan Institute of Engineering and Applied Sciences Nilore, Islamabad ….

Ukiwe, L., Egereonu, U., Njoku, P., Nwoko, C., & Allinor, J. (2013). Polycyclic Aromatic Hydrocarbons Degradation Techniques: A Review. *International Journal of Chemistry*, 5(4), p43. https://doi.org/10.5539/ijc.v5n4p43

Varjani, S. J., & Upasani, V. N. (2017). A new look on factors affecting microbial degradation of petroleum hydrocarbon pollutants. *International Biodeterioration & Biodegradation*, *120*, 71–83.

Chapter 3

Optimization of nutritional parameters for biosurfactant production.

3.1 Introduction

Amphiphilic compounds primarily derived by aerobic microorganisms such as bacteria, yeast and fungus are known as biosurfactants (De Almeida et al., 2016). It is extensively used in the food, pharmaceuticals, textiles, paper, and petroleum industries (Geys et al., 2014; Rebello et al., 2014) and household cleaning products, cosmetics, herbicides and pesticides. A number of Bacillus species develop lipopeptide biosurfactants. Surfactin, a lipopeptide produced by Bacillus subtilis strains, is one of the most significant biosurfactants (Gudiña et al., 2015).

Due to their many benefits, including the high compatibility with climate, biodegradability and ability to synthesize them from sustainable food supplies, biosurfactants have been extensively studied and applied (Geetha et al., 2018). They are also unique activities at high temperatures, pH and saltiness and can be summed up by sustainable supplies of food. They are also (De Almeida et al., 2016; Souza et al., 2018). Thanks to these advantages, biosurfactants have undergone multiple research and industrial applications (Kubicki et al., 2019).

The high cost of refining and purifying biosurfactants is usually not recommended (do Amaral Marques et al., 2019; Naughton et al., 2019; Singh et al., 2019). The biotechnological procedures underlying microbial surfactant production should concentrate on the addition of cheap substrate, such as agro-industry waste or by-products, to a crop broth to permit marketing (Devaraj et al., 2019; Naughton et al., 2019). Thus, biosurfactants made by strains of Bacillus were investigated using a variety

of substrates, such as molasses (Al-Bahry et al., 2013), cashew apple (de Oliveira et al., 2013), residual glycerol (Sousa et al., 2014), pineapple residues (Ehrhardt et al., 2015). While several forms of agro-industrial waste have been investigated for biosurfactant production as substrates, waste from the candy sector has not yet been analyzed.

The bulk of commercial candy waste is made up of sugars (glucose, sucrose and fructose), natural coloring, aromas and anti-weeding agents. As a result, waste must be disposed of correctly by primary and secondary treatment. A physical-chemical treatment in the static and settling tank sieve is used as the main treatment. Aerobic stabilizing lagoons, activated sludge reactors and settlers are all part of the biological secondary therapy. This is why these treatments are costly and time-consuming because of the huge investments in infrastructure. It is recommended to use this waste as a raw material in the processing of biosurfactants because it adds value to the waste while lowering the cost of production, because heat treatment is not necessary. Consequently, the use of hazardous waste bullets to treat biosurfactants is economically and environmentally attractive (Secato et al., 2017).

A typical technique is the Response Surface Methodology (RSM) for the development of modeling based on regression coefficients, using a variance analysis to assess its meaning. This statistical method, as can be seen, is largely used (Deepika et al., 2016; Khiari et al., 2018; Mouafi et al., 2016). RSM is a modelling based on the least quadrants, which takes advantage of the connection between experimental domain variables. In certain cases, therefore, no well-experimental results can be calculated accurately in sensitive models for variances in experimental errors.

This study aims to establish how much surface pressure is reduced by *Bacillus oceanisediminis* H2 fermentation with different carbon and nitrogen sources as a result of the maximum biosurfactant synthesis. The relations between concentrations and the ability to reduce surface tensions were evaluated through experimental design techniques. The models were developed with one factor variable for optimizing and analyzing RSM optimum points to predict the surface tension of fermented media.

3.2 Materials and Methods

3.2.1 Sample

Bacillus oceanisediminis H2 was collected and maintained as mentioned in chapter 1 (1.2.1)

3.2.2 Culture media

Nutrient Broth (NB) and Mineral salts media (MSM) was prepared as mentioned in chapter 1 (1.2.2). Mineral salts media (MSM) was prepared and supplemented as mentioned in chapter 2 (2.2.2).

Potato peels (PP) were collected, cleaned, oven dried, and kept powdered, and the synthesis of biosurfactant was determined using Solid State Fermentation (SSF). M9 medium (Banerjee et al., 2019; K. Das & Mukherjee, 2007) (composition in g/L of the solution: Na_2HPO_4, 6.0; KH_2PO_4, 3.0; NH_4NO_3, 1.0; NaCl, 1.0; $CaCl_2$, 0.014; $MgSO_4.7H_2O$, 0.245) was added in different amounts depending on the ratio of substrate and M9 medium, and mixed thoroughly. The pH of the M9 medium was adjusted according to the experimental setup before sterilization. Under sterile circumstances, the flasks were cooled to room temperature before being infected with 5.0 ml of 24-hour growing bacterial suspension and incubated at 35°C for various incubation durations.

3.2.3 Surface tension measurements

The surface tension measurements were done as mentioned in chapter 1 (1.2.8).

3.2.4 Optimization of lowering of surface tension

The fermentation was done for one variable at a time optimization with each of the carbon sources and nitrogen sources as an additive in the MSM. The carbon sources used were Anthracene, Coconut oil, Glucose, Glycerol, Kerosene and Sucrose. The

nitrogen sources used were Ammonium Sulphate [$(NH_4)_2SO_4$], Yeast Extract, Peptone and also were used in combinations of Yeast Extract + Peptone.

Each of the carbon sources and the nitrogen sources were optimized initially for their optimal concentration at which the maximum lowering of surface tension occurred. Optimization was carried out within a range of 5 – 25 g/L for each of the additive sources.

3.2.5 Optimization of effect of surface tension by Response Surface Methodology (RSM)

Central composite architecture was used to assess and optimize the amounts of significant parameters as well as the interaction effects between different medium constituents that may affect the lowering of surface tension (CCD).

One element at a time, the method parameters and their ranges were chosen from previously collected data. The effects of Glycerol as a carbon source and Yeast Extract as a nitrogen source as an additive in MSM on fermentation that resulted in the development of biosurfactant that caused the surface tension to be reduced were optimized in this research. As seen in Table 3.1, each element in the design was investigated at five different levels ($-\alpha$, -1, 0, $+1$, $+\alpha$).

In 13 separate experimental runs, surface tension was calculated twice. A second order polynomial equation was used to analyze surface stress, and the data was fitted into the equation using a multiple regression method (Banerjee & Ghosh, 2017).

RSM was also done by the help of solid-state fermentation method where five independent factors were included (pH, temperature, incubation period, substrate amount, solution volume). The ranges of those factors were pH (5, 7, 9), temperature (35, 37, 39) °C, incubation periods (3, 4, 5) days, substrate amount (4, 5, 6) g (Asgher et al., 2020), solution volume (2, 3, 4) ml (Banerjee et al., 2019). The factors pH, temperature and incubation periods were previously optimized using one variable at a time (OVAT) method as mentioned in chapter 2 (2.2.4). The substrate amount and solution volume was done in accordance with (Asgher et al., 2020). Later, using the optimum values for variables determined by reaction optimization, an experiment was conducted to validate the expected value, and the optimum lowering of surface tension was verified.

Table 3.1: Experimental design for a two factor five level response surface analyses

Std	Run	Block	Factor 1 A: Glycerol g/L	Factor 2 B: Yeast Extract g/L
11	1	Block 1	15.00	15.00
2	2	Block 1	20.00	10.00
13	3	Block 1	15.00	15.00
1	4	Block 1	10.00	10.00
5	5	Block 1	7.93	15.00
4	6	Block 1	20.00	20.00
3	7	Block 1	10.00	20.00
8	8	Block 1	15.00	22.07
12	9	Block 1	15.00	15.00
6	10	Block 1	22.07	15.00
10	11	Block 1	15.00	15.00
7	12	Block 1	15.00	7.93
9	13	Block 1	15.00	15.00

The dependent variable or result was chosen as the surface tension measurement; y. Polynomial regression based on analysis of variance was used to match the experimental results empirically (ANOVA). A central composite configuration with five coded levels was used to match an analytical second-order polynomial model. As discussed in Chapter 2, the quadratic model was developed (2.2.5).

Response surface simulation aids in the investigation of the response over the whole space of variables and the identification of the area where it achieves its optimum value. A response surface plot will show the best combination of process variables that yields the best outcome.

3.2.6 Statistical analysis

The statistical significance of the second-order model equation was defined by F-test and R^2 was the multiple coefficient of determination to indicate the proportion of the variance that was described in the model obtained. The optimization method seeks to combine the factor levels that meet the specifications placed in each response and process variables simultaneously (i.e., optimization criteria). The response has been experimentally checked for the 95% to 99% conviction limits. The statistical program 'Design Expert' (version 7.0.0; Stat-Ease, Inc. Minneapolis, MN USA) used all of the mathematical analysis considering the design of tests for the optimization. In the program "Origin 9.0" all the statistical plots were made.

3.3 Results and Discussion

3.3.1 Optimization of surface tension lowering ability

The fermented media was seen to lower maximum surface tension when compared to its uninoculated counterpart at 5 days, pH 8, 37°C with 24hr old culture which was set to be the control. This shows that the isolated strain can be utilized for biosurfactant production as it significantly reduced the surface tension of the fermentable media.

The optimization was done by one variable at a time method which kept one parameter in the variable form and the other considered variables as constant. The effects of the parameters gave an optimum result for the different carbon sources *viz.* Anthracene, Coconut oil, Glucose, Glycerol, Kerosene and Sucrose and nitrogen sources *viz.* Ammonium Sulphate [$(NH_4)_2SO_4$], Yeast Extract, Peptone and also were used in combinations of Yeast Extract + Peptone as an additive for the fermentation media consisting MSM, which recorded the maximum lowering of surface tension of the fermented media.

Table 3.2: Effect of different carbon sources on the production of biosurfactant as per surface tension measurements.

Concentration (g/L)	**Surface tension (mN/m)**					
	Sucrose	**Kerosene**	**Glycerol**	**Glucose**	**Coconut oil**	**Anthracene**
5	50.211	50.27	42.47	47.47	45.245	42.56
10	47.631	45.63	34.29	37.569	37.569	41.56
15	43.183	42.23	29.65	35.139	33.53	39.67
20	44.197	43.54	31.24	38.64	33.91	40.12
25	46.36	44.5	32.04	42.56	39.6	40.98

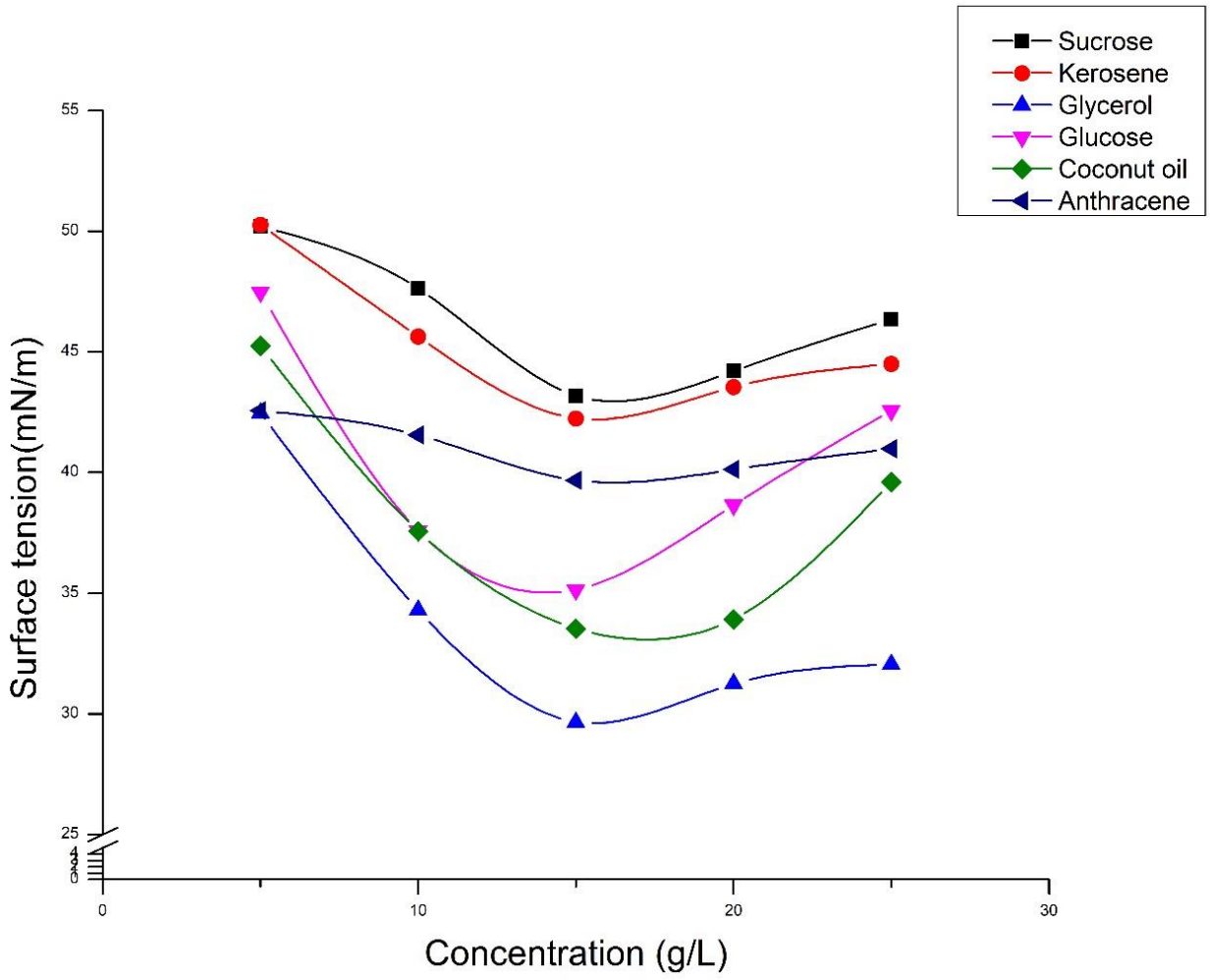


Fig 3.1: Effect of different concentrations of carbon sources on the production of biosurfactant.

In figure 3.1 different carbon sources' concentration (g/L) was optimized. It was supplemented with MSM in different concentrations for the fermentation with *Bacillus oceanisediminis* H2 with the previously optimized environmental factors *viz.* time, temperature, and pH were 120hrs, 40°C, and 9, respectively and with 24hours grown culture of *Bacillus oceanisediminis* H2. It can be clearly observed that the surface tension of the cell free supernatant of the fermented media was lowest at 43.183 mN/m, 42.23 mN/m, 29.65 mN/m, 35.139 mN/m, 33.53 mN/m, 39.67 mN/m for Sucrose, Kerosene, Glycerol, Glucose, Coconut oil, Anthracene respectively (Table 3.2) with 15 g/L of each of the carbon sources supplementation with the MSM. Thus, 15g/L was the optimum concentration of carbon sources at which the surface tension of the cell free

supernatant of the fermented media was lowest. This is in close communion with previous reports where 5% sucrose (Fadhile Almansoory et al., 2017), 1% kerosene (Faiq Ali et al., 2018), 2% glycerol in MSM (Cruz et al., 2018; Das et al., 2008), glucose in the range of 10-70 g/L (Heryani & Putra, 2017), 3% of coconut oil (George & Jayachandran, 2013), 2% of PAH (Deziel et al., 1996) has been used for biosurfactant production.

Table 3.3: Effect of different nitrogen sources on the production of biosurfactant as per surface tension measurements.

Concentration g/L	**Surface tension (mN/m)**				
	Yeast Extract	**Peptone**	**Ammonium Sulphate**	**Yeast Extract + Peptone (1:1 w/w)**	**Ammonium Sulphate + Yeast Extract (1:1 w/w)**
5	38.74	47.63	42.56	54.21	38.541
10	33.52	44.21	35.48	48.65	36.542
15	30.23	37.23	32.67	45.96	35.187
20	32.11	40.12	33.21	46.11	36.89
25	32.96	40.98	34.87	47.63	39.112

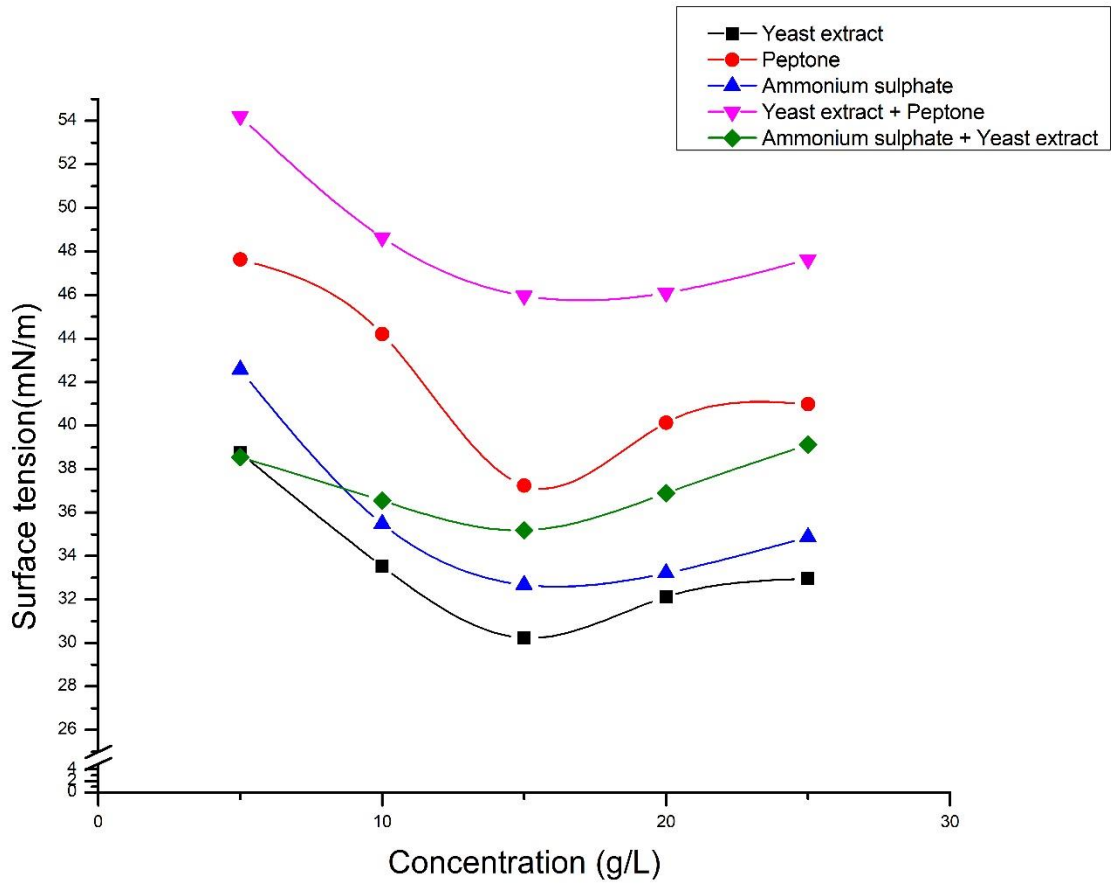


Fig 3.2: Effect of different concentrations of nitrogen sources on the production of biosurfactant.

In figure 3.2 different nitrogen sources' concentration in g/L was optimized. It was supplemented with MSM in different concentrations for the fermentation with *Bacillus oceanisediminis* H2 with the previously optimized environmental factors *viz.* time, temperature, and pH were 120hrs, 40°C, and 9, respectively and with 24hours grown culture of *Bacillus oceanisediminis* H2. It can be clearly observed that the surface tension of the cell free supernatant of the fermented media was lowest at 30.23 mN/m, 37.23 mN/m, 32.67 mN/m, 45.96 mN/m, 35.187 mN/m (Table 3.3) with 15 g/L of Yeast Extract, Peptone, Ammonium Sulphate [$(NH_4)_2SO_4$], Yeast Extract + Peptone, Ammonium Sulphate [$(NH_4)_2SO_4$] + Yeast Extract supplementation respectively with the MSM. Previous reports showed similar results of 7g/L yeast extract (Yaraguppi et al., 2020), 3% peptone (Sylvester et al., 2019), 2% (m/v) of Ammonium sulphate

[$(NH_4)_2SO_4$] (Jimoh & Lin, 2019) optimally used as a nitrogen source. Thus, 15g/L was the optimum concentration of nitrogen sources at which the surface tension of the cell free supernatant of the fermented media was lowest.

3.3.2 Optimization of effect of surface tension by Response Surface Methodology (RSM)

The optimal level of the key factors and the effect of their interactions on biosurfactant production were explored by the central composite design of RSM. Experimental design and results are shown in Table 3.4. By applying multiple regression analysis on the experimental data, the following second-order polynomial equation was established (Eq. 3.1) to explain the surface tension measurement:

$$\text{Surface Tension} = 29 \cdot 54 - 3 \cdot 46A^2 - 2 \cdot 64B^2 + 2 \cdot 53AB + 2 \cdot 86A^2 + 2.97B^2 \quad \text{(Eqn 3.1)}$$

Eq 3.1: Multivariate polynomial equation obtained by multiple regression analysis for surface tension measurements. [A = Glycerol; B = Yeast Extract]

Table 3.4: Experimental design for a two factor five level response surface analyses.

Std	Run	Block	**Factor 1** A: Glycerol g/L	**Factor 2** B: Yeast Extract g/L	**Response 1** Surface Tension mN/m
11	1	Block 1	15	15	29.54
2	2	Block 1	20	10	31.14
13	3	Block 1	15	15	29.54
1	4	Block 1	10	10	45.57
5	5	Block 1	7.93	15	37.89
4	6	Block 1	20	20	31.25
3	7	Block 1	10	20	35.56
8	8	Block 1	15	22.07	30.98
12	9	Block 1	15	15	29.54
6	10	Block 1	22.07	15	31.58
10	11	Block 1	15	15	29.54
7	12	Block 1	15	7.93	38.93
9	13	Block 1	15	15	29.54

Table 3.5 Experimental design for a five factor five level response surface analyses for SSF.

Std	Run	Factor 1 A: Time Days	Factor 2 B: Temperature deg C	Factor 3 C:pH	Factor 4 D: Substrate Amount g	Factor 5 E: Solution volume ml	Response 1 Surface Tension mn /m
18	1	5	35	5	4	4	45.96
36	2	4	41.75	7	5	3	43.75
1	3	3	35	5	4	2	48.71
47	4	4	37	7	5	3	41.81
14	5	5	35	9	6	2	41.64
41	6	4	37	7	5	0.62	46.44
17	7	3	35	5	4	4	44.93
38	8	4	37	11.75	5	3	48.70
34	9	6.37	37	7	5	3	51.72
43	10	4	37	7	5	3	41.81
16	11	5	39	9	6	2	43.43
12	12	5	39	5	6	2	40.74
40	13	4	37	7	7.37	3	35.07
25	14	3	35	5	6	4	43.01
39	15	4	37	7	2.62	3	41.08
50	16	4	37	7	5	3	41.81
28	17	5	39	5	6	4	35.72
49	18	4	37	7	5	3	41.81
32	19	5	39	9	6	4	45.34
11	20	3	39	5	6	2	41.05
27	21	3	39	5	6	4	43.17
13	22	3	35	9	6	2	45.67
33	23	1.62	37	7	5	3	51.73
21	24	3	35	9	4	4	36.66
5	25	3	35	9	4	2	37.50
22	26	5	35	9	4	4	37.12
9	27	3	35	5	6	2	46.51
35	28	4	32.24	7	5	3	40.26
30	29	5	35	9	6	4	38.94
44	30	4	37	7	5	3	41.81
4	31	5	39	5	4	2	51.56
23	32	3	39	9	4	4	48.91
37	33	4	37	2.24	5	3	53.96
48	34	4	37	7	5	3	41.81
6	35	5	35	9	4	2	42.11
31	36	3	39	9	6	4	47.37
42	37	4	37	7	5	5.37	42.47
10	38	5	35	5	6	2	48.06
8	39	5	39	9	4	2	44.88
20	40	5	39	5	4	4	47.26
19	41	3	39	5	4	4	46.08
29	42	3	35	9	6	4	46.11
7	43	3	39	9	4	2	42.13
3	44	3	39	5	4	2	45.24
26	45	5	35	5	6	4	42.41
46	46	4	37	7	5	3	41.81
45	47	4	37	7	5	3	41.81
15	48	3	39	9	6	2	42.31
24	49	5	39	9	4	4	48.51
2	50	5	35	5	4	2	53.89

ANOVA was carried out to verify the relevance of the experimental results as seen in Table 3.5 of the fit of the second order polynomial equation. The F-value model of 27.34 implies that the model is relevant. The chances of a "F-Value Model" of this size can only be 0.02% due to noise. "Prob > F" values of less than 0.0500 are important, indicating model words. In this case, the models A, B, AB, A^2, B^2 are meaningful. ANOVA parameters with p > 0.10 show that the terms of the model are not meaningful. These pointless models allow you to select terms that have a limited statistical and functional contribution to experimental design. The R^2 was found to be 0.9513, which corresponds to a rational 0.9165 Adj R^2 agreement (Table 3.5). This speaks more to the current optimization analysis that the experimental model is successful. In the analysis conducted on residuals, no anomalies were found. It can also be inferred that the model was sound statistically. The 3D response surface map (Fig. 3.3) and 2D response surface contour plot (Figure 3.4) show the type and degree of interaction through various variables.

Table 3.6: ANOVA for response surface quadratic model.

Source	Sum of Squares	df	Mean Square	F Value	p-value Prob > F	
Model	281.5	5	56.3	27.34	0.0002	significant
A-Glycerol	95.66	1	95.66	46.46	0.0002	
B-Yeast Extract	55.88	1	55.88	27.14	0.0012	
AB	25.6	1	25.6	12.44	0.0096	
A^2	56.75	1	56.75	27.56	0.0012	
B^2	61.21	1	61.21	29.73	0.001	
Residual	14.41	7	2.06			
Lack of Fit	14.41	3	4.8			not significant
Pure Error	0	4	0			
Cor Total	295.92	12				

Table 3.7: Results of ANOVA depicting model soundness. *

Std. Dev.	1.43	R-Squared	0.9513
Mean	33.12	Adj R-Squared	0.9165
C.V. %	4.33	Pred R-Squared	0.6536
PRESS	102.49	Adeq Precision	14.826

* "Pred R-Squared" of 0.6536 is not as similar as one would usually assume to the Adj R-Squared of 0.9165. This may mean a broad block effect or a future model or data problem. Model elimination, reaction transformation, outliers etc. are also matters to remember.

The signal-to-noise ratio is calculated by "Adeq Precision." It is ideal to have a ratio greater than 4. A suitable signal is shown by the resulting ratio of 14.826. This model can be used for concept space navigation.

Table 3.8: ANOVA for response from SSF on surface quadratic model.

Response 1			**Surface Tension**			
ANOVA for Response Surface Quadratic Model						
Analysis of variance table [Partial sum of squares - Type III]						
Source	Sum of Squares	DF	Mean Square	F Value	p-value Prob > F	
Model	905.1785	20	45.25892267	27.31801223	< 0.0001	Significant
A-Time	0.105986	1	0.105986021	0.063972522	0.8021	
B-Temperature	11.93556	1	11.93556386	7.204234222	0.0119	
C-pH	53.59553	1	53.59553422	32.34994058	< 0.0001	
D-Substrate Amount	45.25283	1	45.2528342	27.31433726	< 0.0001	
E-Solution Volume	17.27801	1	17.27800901	10.42890183	0.0031	
AB	0.000615	1	0.000615128	0.000371287	0.9848	
AC	4.193336	1	4.1933356	2.531072029	0.1225	
AD	50.15086	1	50.15086363	30.27075823	< 0.0001	
AE	32.34191	1	32.34190845	19.5213805	0.0001	
BC	111.7374	1	111.7374217	67.44403251	< 0.0001	
BD	52.34819	1	52.34819101	31.59705176	< 0.0001	
BE	49.88432	1	49.88431699	30.10987229	< 0.0001	
CD	97.77508	1	97.7750792	59.01644697	< 0.0001	
CE	41.61799	1	41.6179857	25.12036467	< 0.0001	
DE	0.326816	1	0.326815913	0.19726411	0.6602	
A^2	149.9733	1	149.9732764	90.52296336	< 0.0001	
B^2	0.321542	1	0.321542075	0.194080854	0.6628	
C^2	137.495	1	137.4950386	82.99117441	< 0.0001	
D^2	33.03732	1	33.03731653	19.94112462	<0.0001	
E^2	7.069559	1	7.069558572	4.267142834	0.0479	
Residual	48.04554	29	1.656742895			
Lack of Fit	48.04554	22	2.183888362			not significant
Pure Error	0	7	0			
Cor Total	953.224	49				

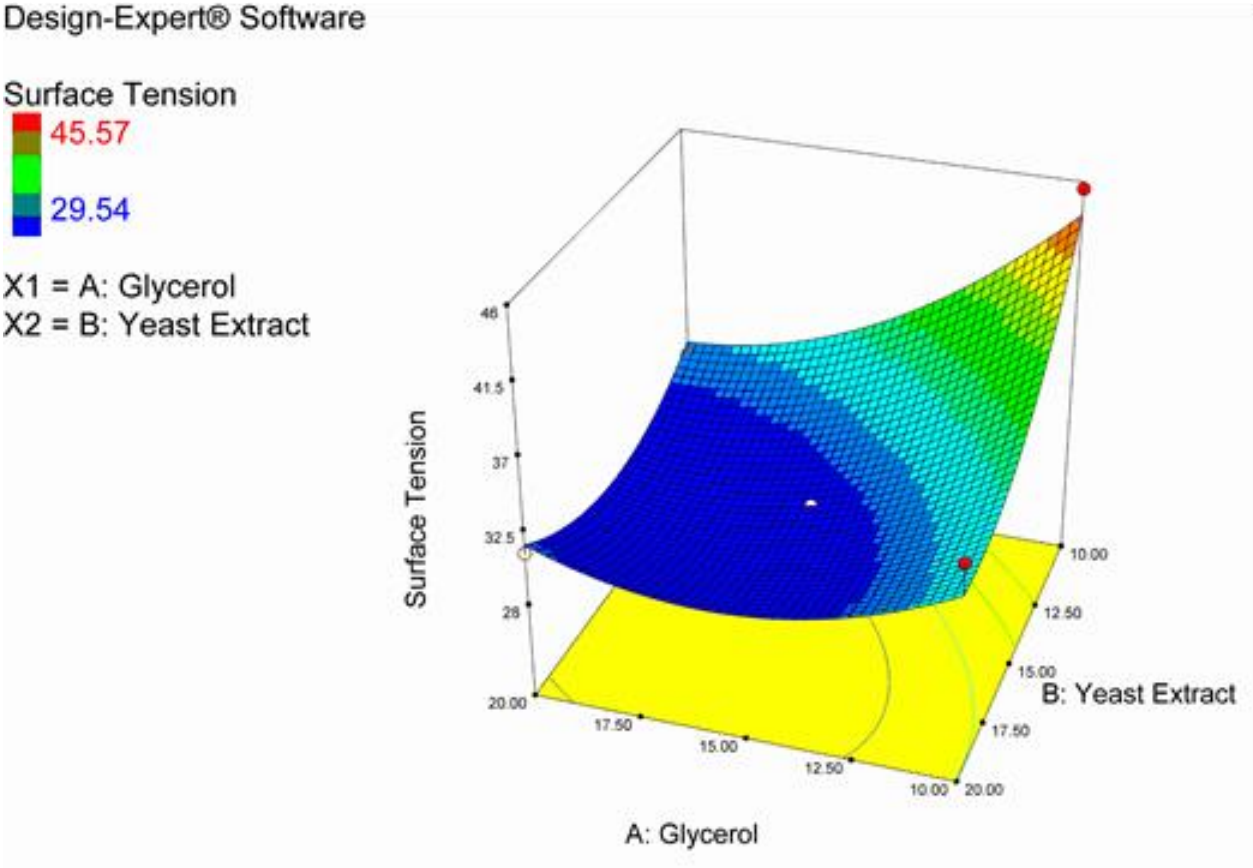


Fig 3.3: 3D response surface plot showing the effect of Glycerol and Yeast extract on surface tension of the fermented MSM media.

In Fig 3.3 the 3D response surface plot shows a minimizing type plot suggesting that the surface tension (response) lowering is in effect. The predicted surface tension was suggested to be 28.6488 mN/m which is in close approximation with the experimental run via one variable at a time optimization. The difference in this optimization study was its ability to optimize both the carbon and nitrogen source simultaneously suggesting even a more efficient lowering ability of surface tension when both the carbon and nitrogen sources are supplemented in the MSM for the fermentation to carry on. The plot also suggests that 15g/L for both glycerol (carbon source) and yeast extract (nitrogen source) was optimum giving a surface tension of 28.6488 mN/m.

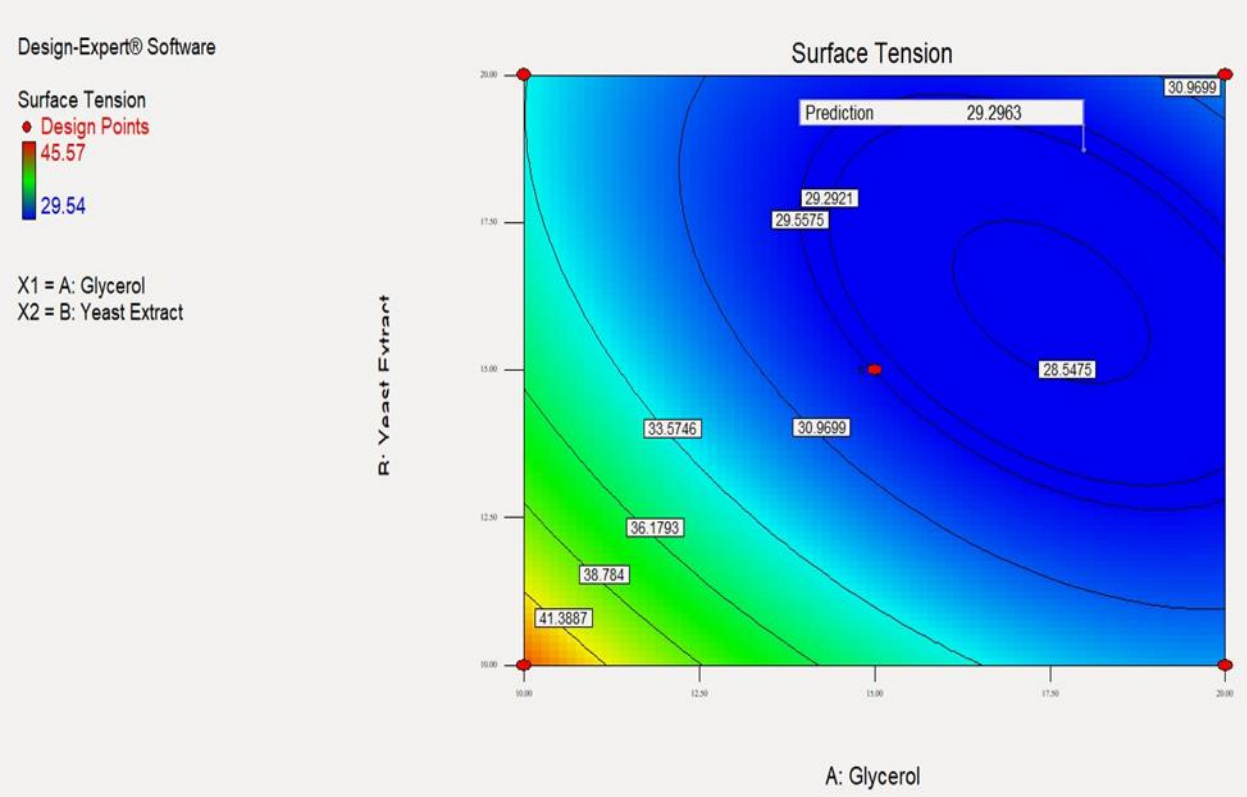


Fig 3.4: Contour response surface plot showing the effect of Glycerol and Yeast extract on surface tension of the fermented MSM media.

In Fig 3.4 the contour response surface plot shows a minimizing type plot suggesting that the surface tension (response) lowering is in effect. The predicted surface tension was suggested to be 28.6488 mN/m which is in close approximation with the experimental run via one variable at a time optimization. The difference in this optimization study was its ability to optimize both the carbon and nitrogen source simultaneously suggesting even a more efficient lowering ability of surface tension when both the carbon and nitrogen sources are supplemented in the MSM for the fermentation to carry on. The plot also suggests that 15g/L for both glycerol (carbon source) and yeast extract (nitrogen source) was optimum giving a surface tension of 28.6488 mN/m. The contour lines and the color of the contour suggests that the whole experimental study has a minimizing effect and as the plot becomes intense blue from red, it further suggests that the surface tension (response) is optimal in the lower measurements which is true for the optimum concentration of carbon and nitrogen sources at 15g/L for both. The predicted value for the optimum surface tension

according to the plot was 29.2963 mN/m which was in good agreement with the point prediction given according to the statistical software (Stat-Ease Design Expert 7.0.0).

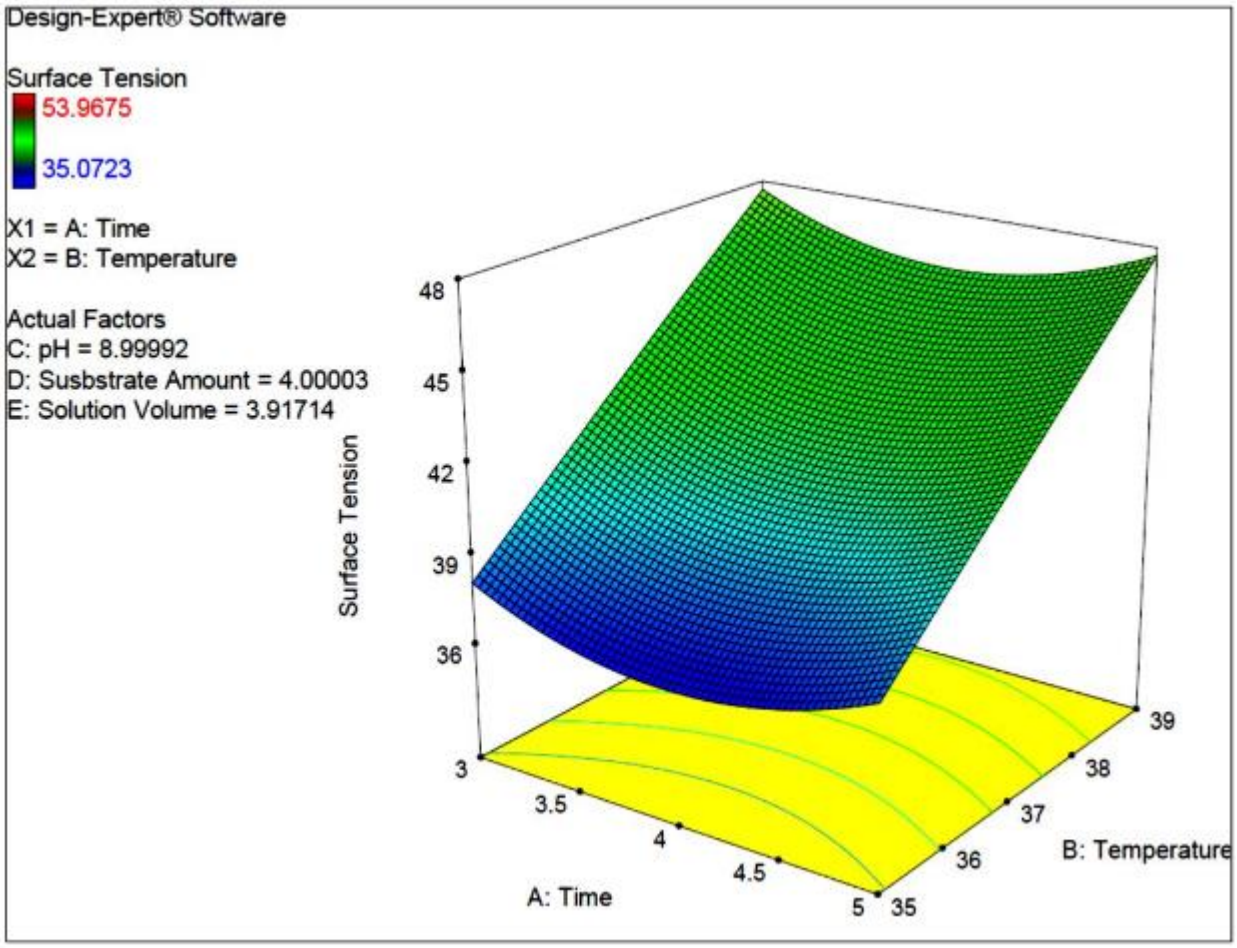


Fig 3.5: The effect of time and temperature on the surface tension of the SSF represented by a response surface plot.

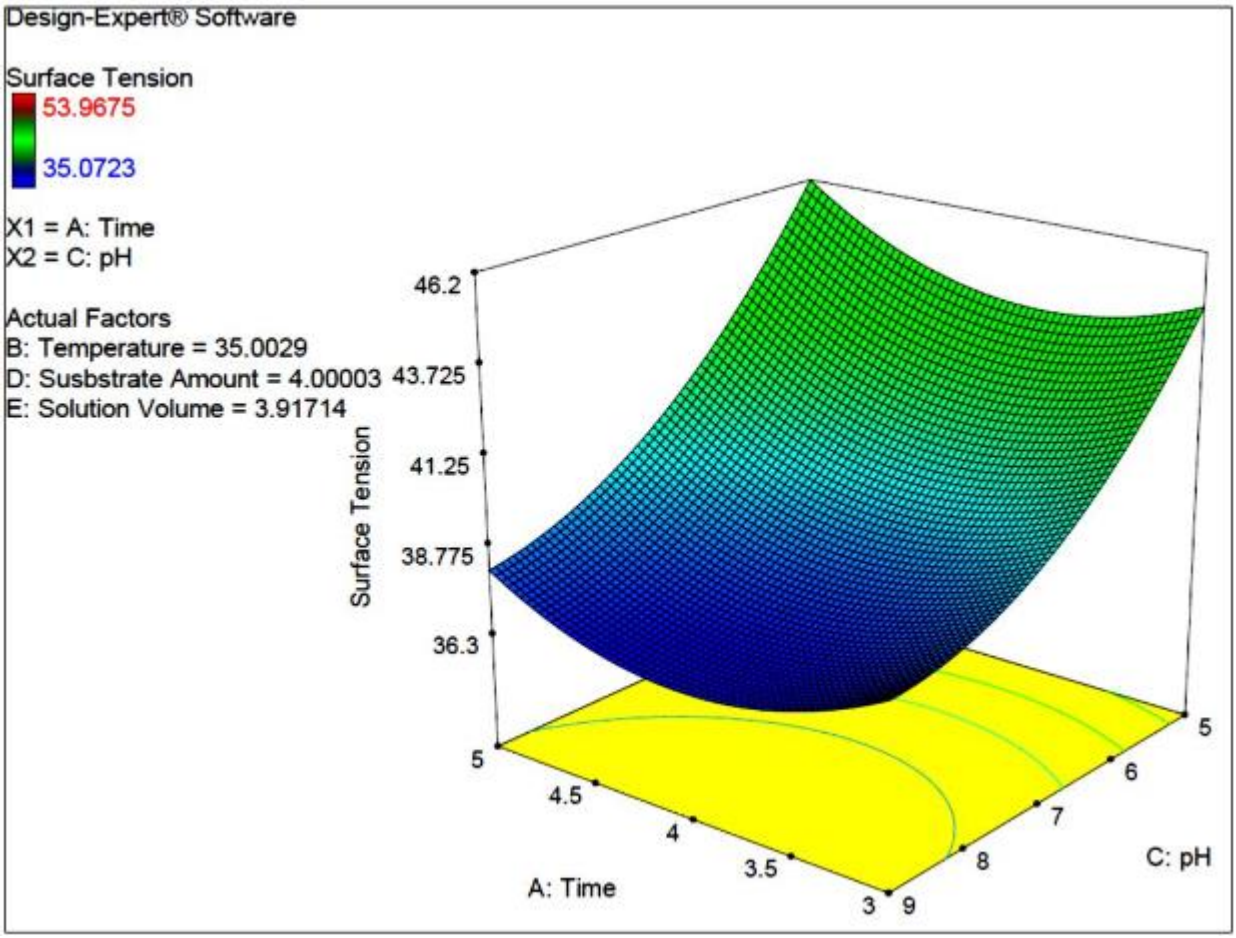


Fig 3.6: The effect of time and pH on the surface tension of the SSF represented by a response surface plot.

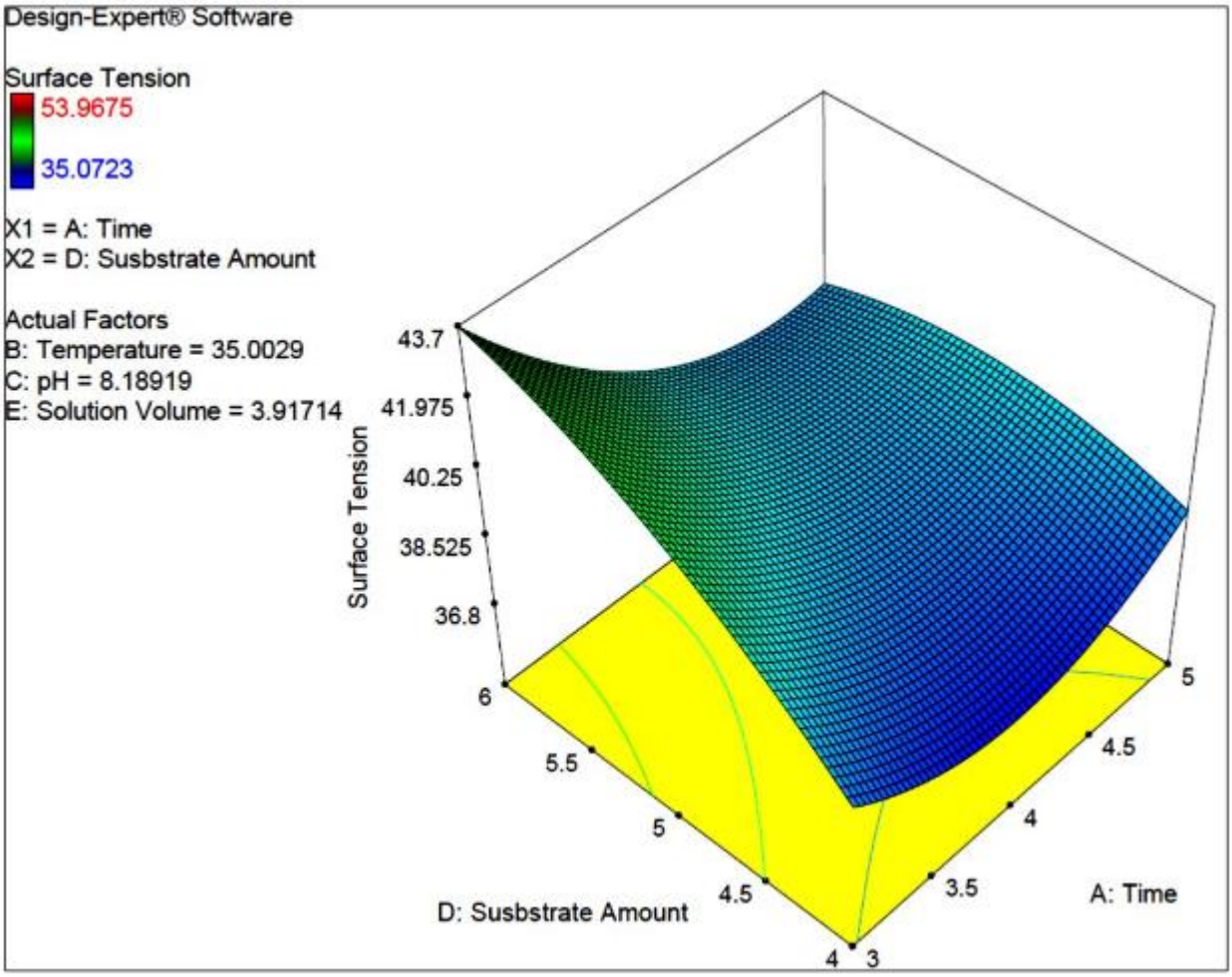


Fig 3.7: The effect of Substrate amount and Time on the surface tension of the SSF represented by a response surface plot.

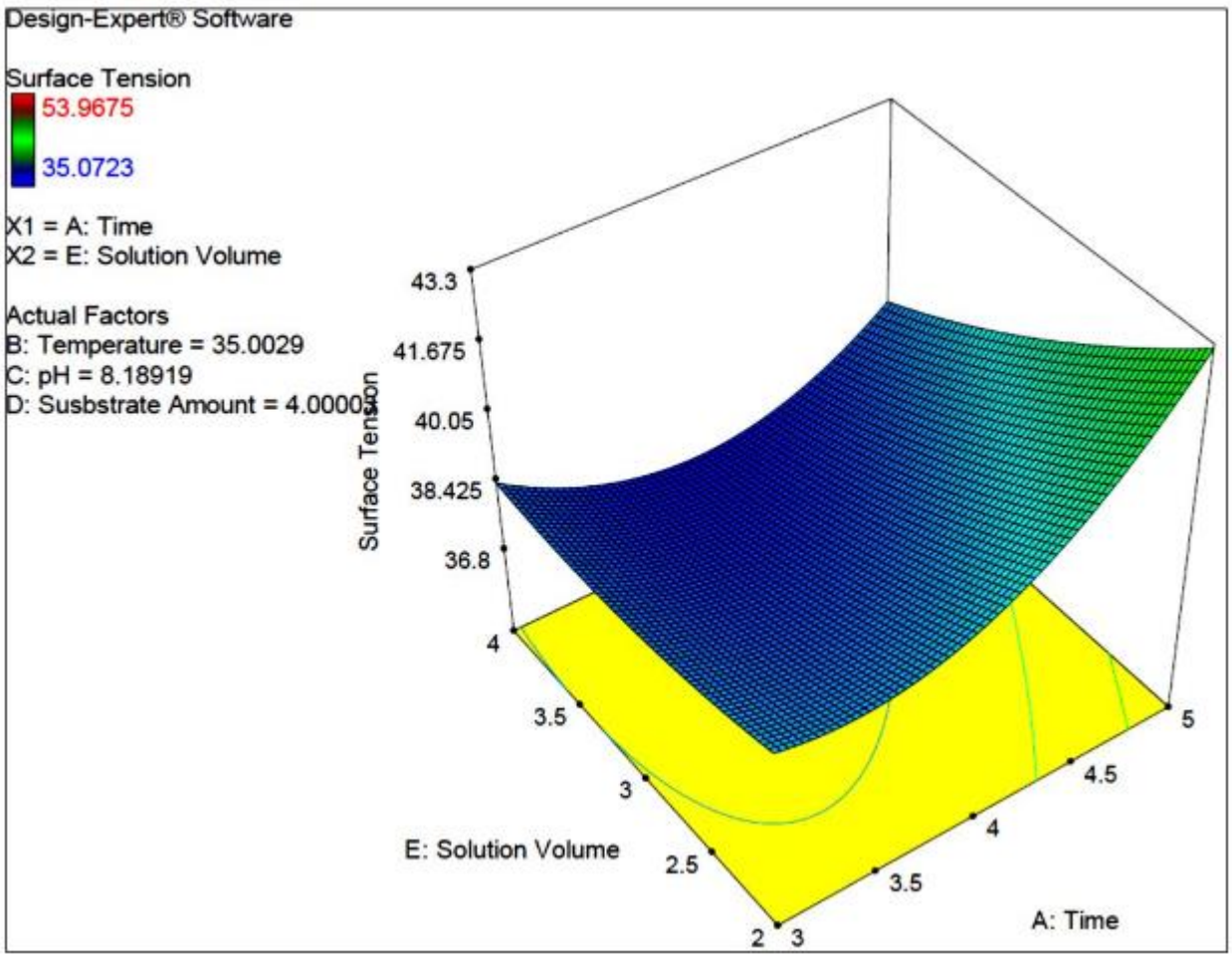


Fig 3.8: The effect of Solution volume and Time on the surface tension of the SSF represented by a response surface plot.

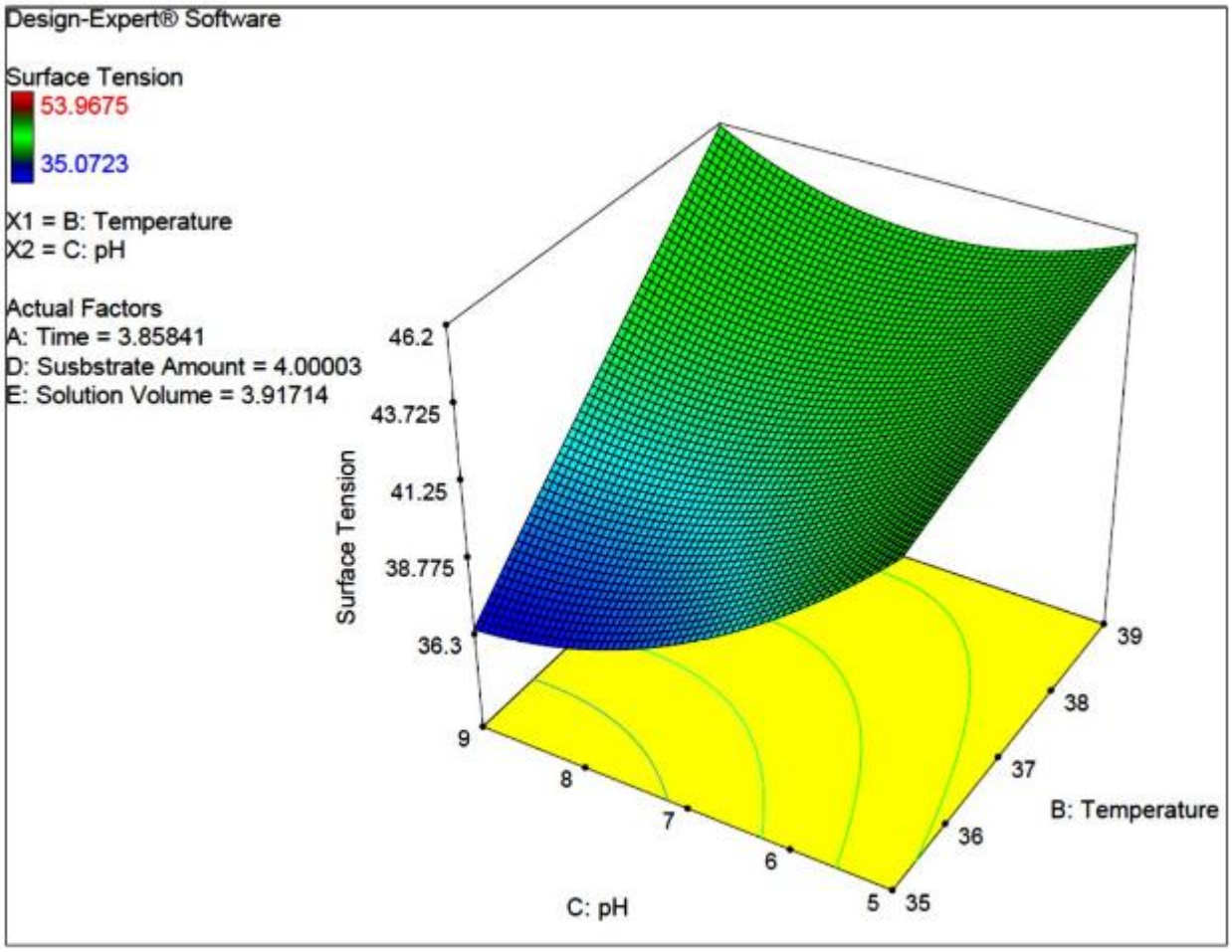


Fig 3.9: The effect of pH and Temperature on the surface tension of the SSF represented by a response surface plot.

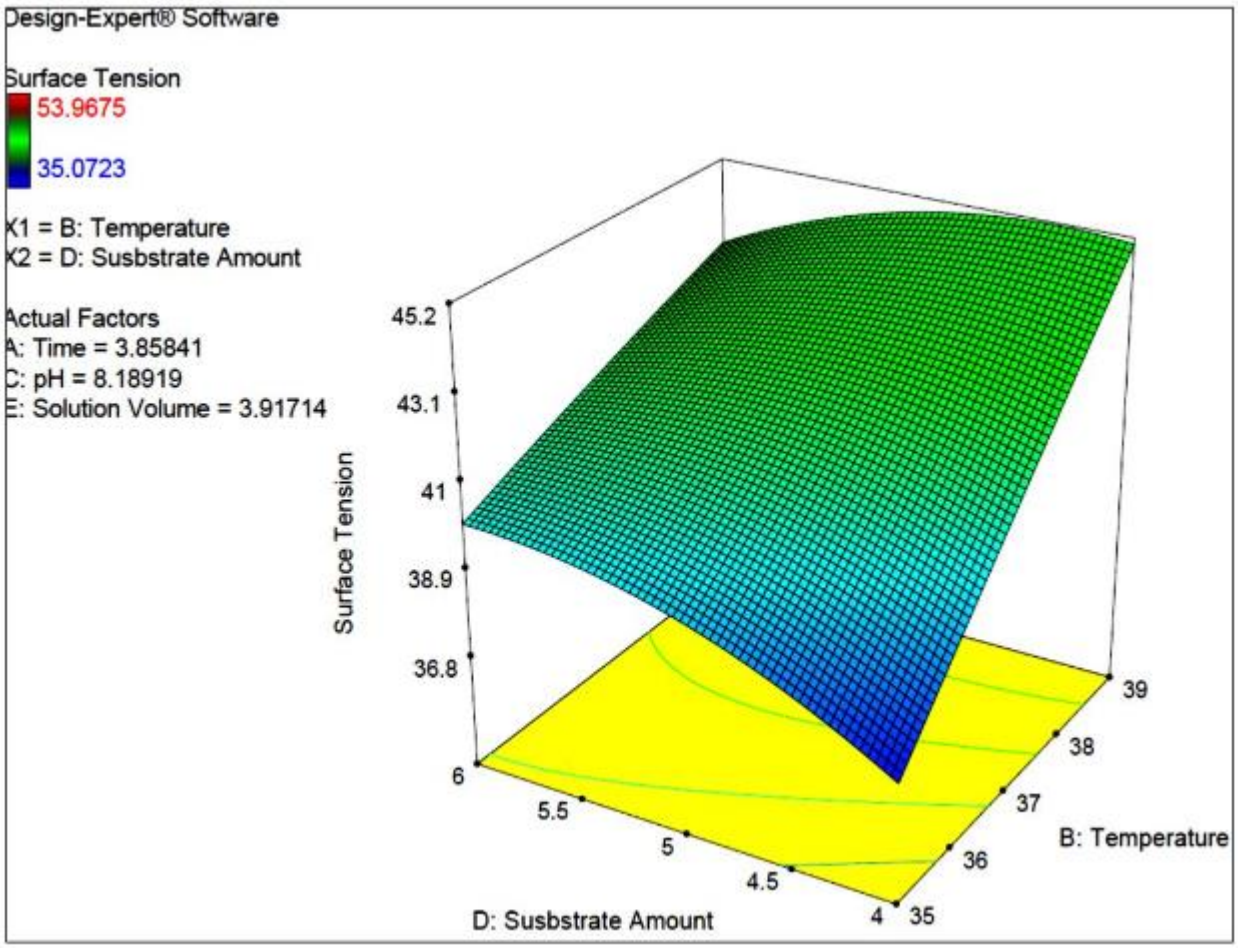


Fig 3.10: The effect of Substrate amount and Temperature on the surface tension of the SSF represented by a response surface plot.

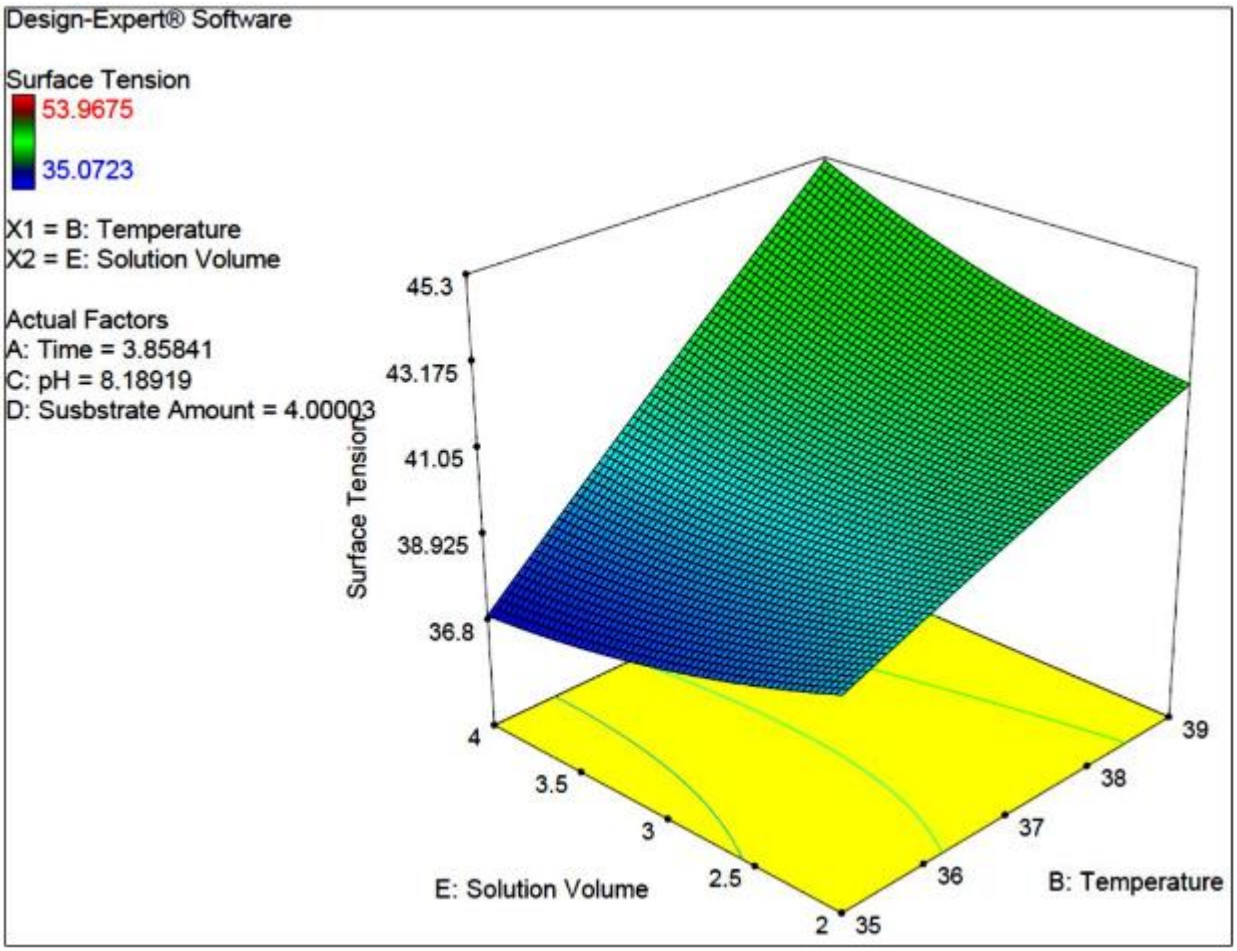


Fig 3.11: The effect of Solution volume and Temperature on the surface tension of the SSF represented by a response surface plot.

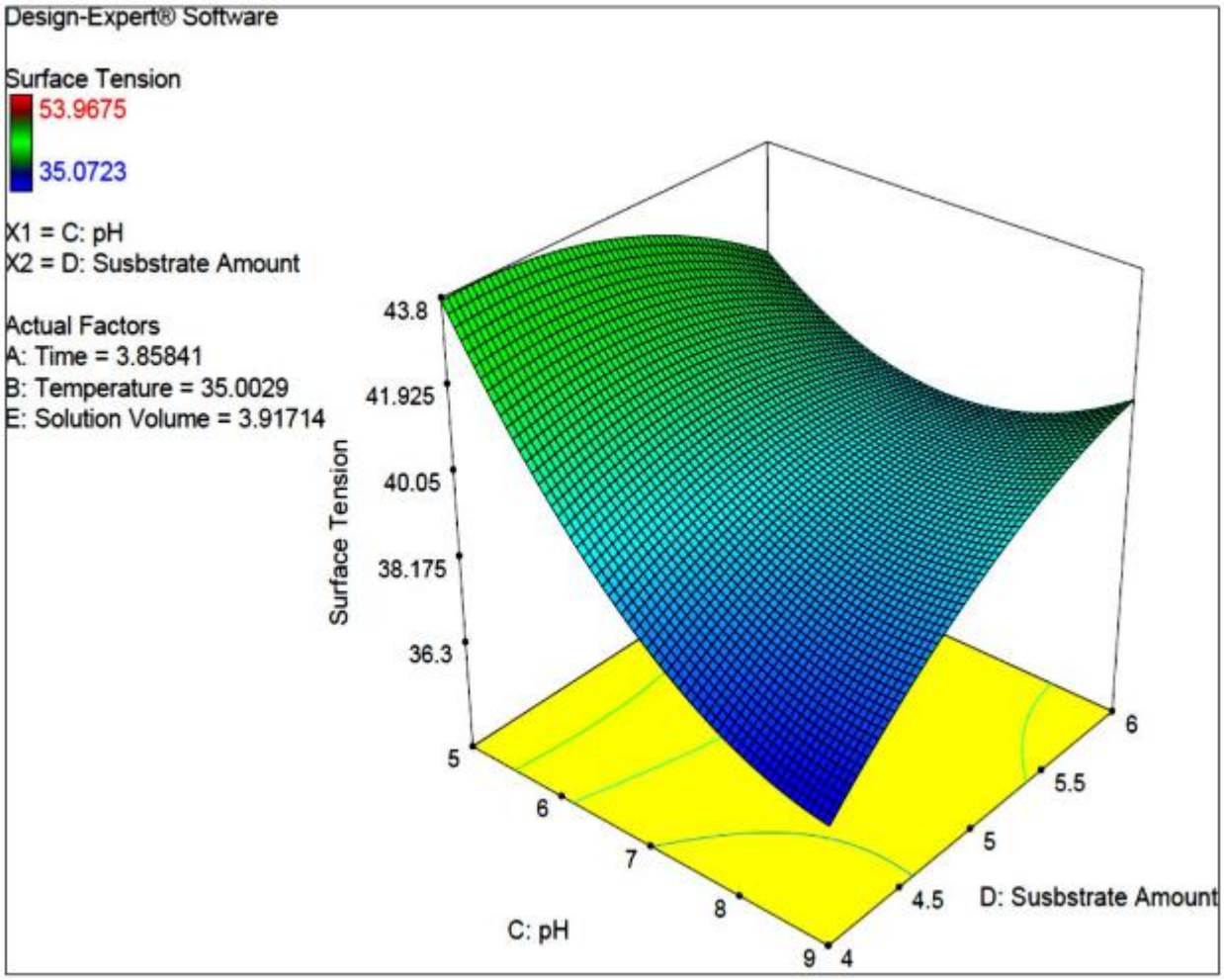


Fig 3.12: The effect of pH and Substrate amount on the surface tension of the SSF represented by a response surface plot.

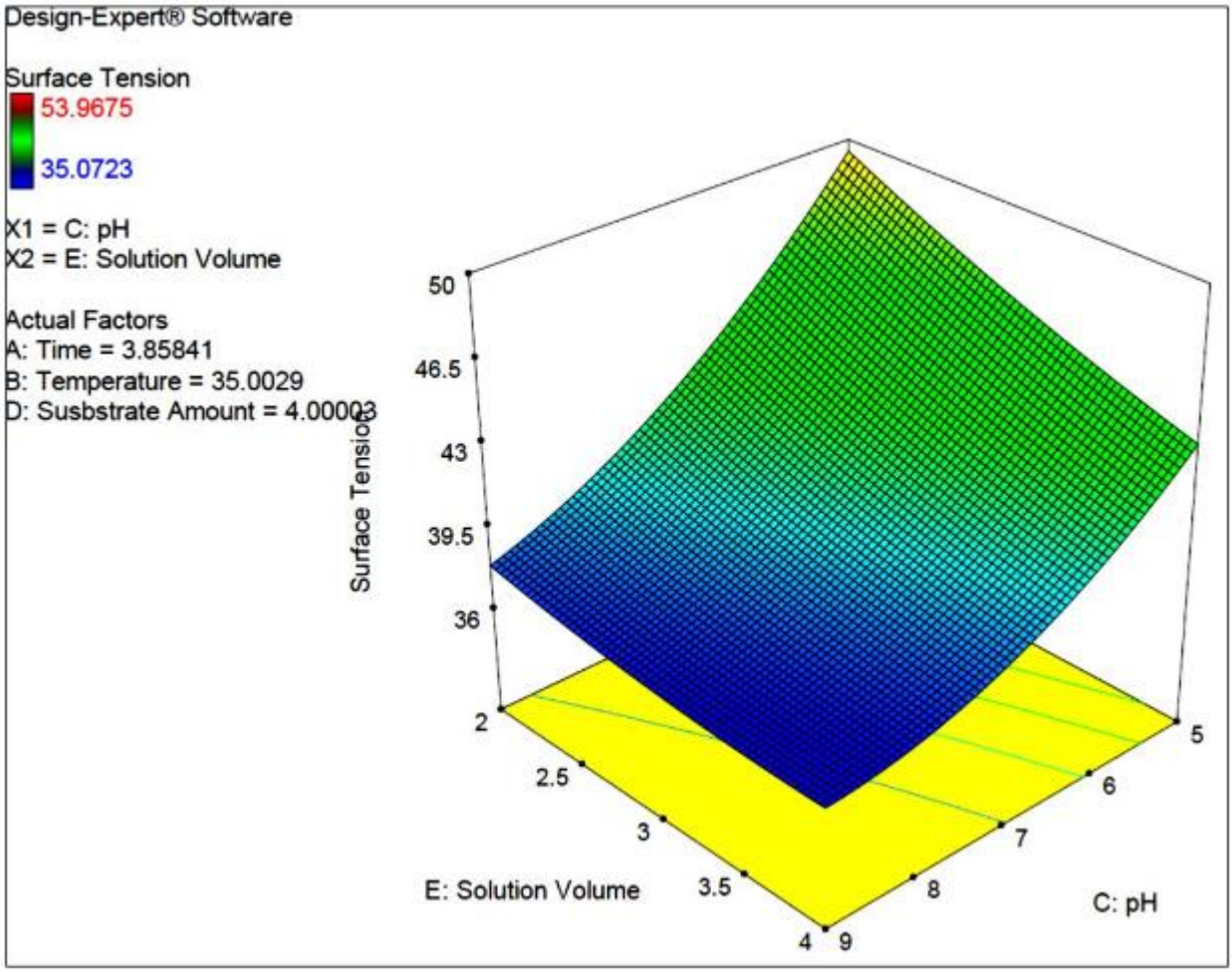


Fig 3.13: The effect of Solution volume and pH on the surface tension of the SSF represented by a response surface plot.

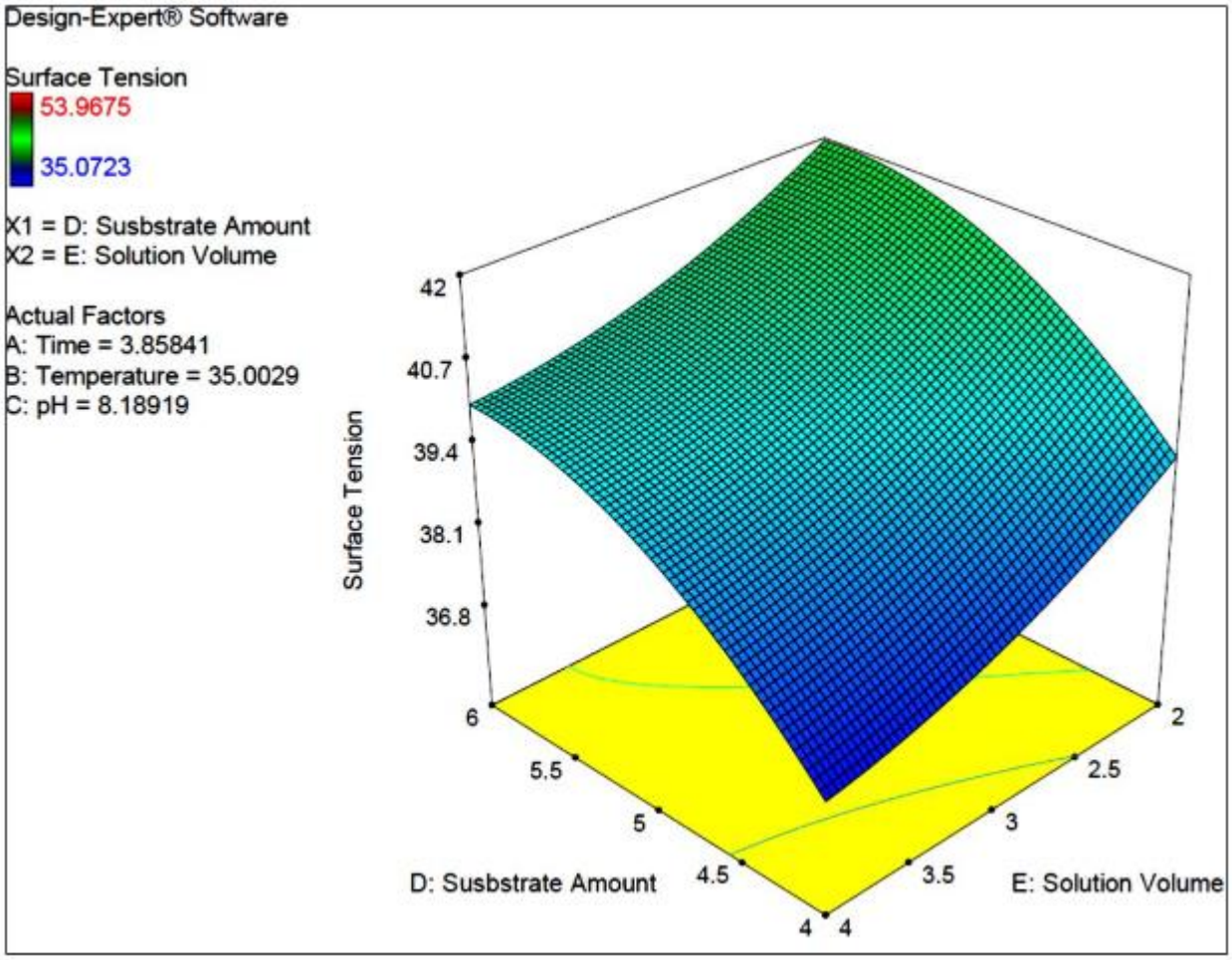


Fig 3.14: The effect of Solution volume and Substrate amount on the surface tension of the SSF represented by a response surface plot.

Optimisation of pH, temperature, incubation time, substrate amount, and solution volume using the quadratic model led to a surface tension value of 36.428 mN /m at the lowest pH and temperature settings in SSF using potato peels as substrate. (Fig 3.5 - 3.14).

3.3.3 Point prediction for validation of RSM

The quadratic model predicted that, because Glycerol and Yeast extracts were used as an MSM additive for fermentation with optimized environmental factors as found in the previous chapter (120 hours, 40°C and 9 hours, and 24 hours g), the minimum surface tension should be 28.6488 mN/m. The results are as follows: The validation experiment was conducted in duplicate experiments to evaluate the expected findings.

The measured test surface voltage calculation under optimized conditions was found to be 30.012 mN/m, meaning that the experimental and expected surface tension values were in good harmony. This finding also confirms the expected values and the feasibility of the model. The optimized method parameters substantially lead to lower surface tension by the creation of a powerful biosurfactant in fermented media (Table 3.9).

The quadratic model indicated that when the pH (5, 7, 9), temperature (35, 37, 39) ℃, incubation durations (3, 4, 5) days, substrate quantity (4, 5, 6) g, and solution volume (2, 3, 4) mL were all optimised, the lowest value of surface tension would be 36.428 mN /m (Table 3.10). A validation experiment was carried out in duplicate to confirm the expected findings. The measured experimental titer of surface tension at the optimum condition was 37.241 mN/m, indicating that experimental and predicted values of surface tension are in accord. As a result, the anticipated values and the model's efficacy were confirmed, suggesting that the optimized medium favored surface tension generation via solid state fermentation.

Table 3.9: Point prediction for the validation of the RSM. (CI= Confidence Interval; SE= Standard Error; PI= Prediction interval) *

Factor	**Name**	**Level**	**Low Level**	**High Level**	**Std. Dev.**	**Coding**	
A	Glycerol	18.47	10.00	20.00	0.000	Actual	
B	Yeast Extract	14.45	10.00	20.00	0.000	Actual	
Response	**Prediction**	**SE Mean**	**95% CI low**	**95% CI high**	**SE Pred**	**95% PI low**	**95% PI high**
Surface Tension	28.6488	0.64	27.14	30.16	1.57	24.94	32.36

Table 3.10: Point prediction for the validation of the RSM for SSF. (CI= Confidence Interval; SE= Standard Error; PI= Prediction interval) *

Factor	Name	Level	Low Level	High Level	Std. Dev.	Coding	
A	Time	4.00	3	5	0	Actual	
B	Temperature	35	35	39	0	Actual	
C	pH	8.99	5	9	0	Actual	
D	Substrate Amount	4	4	6	0	Actual	
E	Solution Volume	3.99	2	4	0	Actual	
Response	Prediction	SE Mean	95% CI low	95% CI high	SE Pred	95% PI low	95% PI high
Surface Tension	36.428	0.718	34.95	37.89	1.47	33.41	39.44

*The optimum level given by the statistical tool when the design of experiments was carried out between the low and high ranges. It points to the predicted lowest value of surface tension which lies in the range of 95% to 99% confidence limit. This also allows for validation studies.

3.3.4 Statistical analysis

All the optimization study by response surface methodology was done with the help of a statistical software Stat-Ease Design Expert 7.0.0 (Minneapolis, USA). The graphical statistics was done in Origin 9 (OriginLab Corporation, Northampton, USA).

3.4 Conclusion

In the present analysis, a single variable used to optimize carbon sources and nitrogen sources in conjunction with RSM for optimization of all sources was used and was in near accordance with the RSM optimization data. RSM has been demonstrated as an important instrument to optimize the calculation of surface pressure of the fermented biosurfactant media. Glycerol (29.957 mN/m) and Yeast extract (30.23 mN/m), respectively, supplemented the carbon source and nitrogen source with an MSM which measured the lowest surface tension. The final parameters of the fermentation process were used by environmentally optimized variables such as 120 hours, 40°C, pH 9, and with *Bacillus oceanisediminis* H2 growing for 24 hours. The optimization study's expected value was 29.2963 mN/m and was further confirmed at 28.6844mN/m. The single variable was in direct contact with the optimization strategy and RSM optimization. me. Potato peel, an agricultural residue was utilized as the solid-state fermentation (SSF) media, which served as both carbon and nitrogen sources. The maximum drop in surface tension was recorded to be 36.428 mN/m, for the fermentation time being 4 days, temperature being 35°C, pH 8.99, with 4 g substrate and 3.99 ml solution.
This experiment thus provided the basis for further research of large-scale fermentation for the development of biosurfactants that reduced surface tension using this isolated strain, *Bacillus oceanisediminis* H2.

3.5 References

Al-Bahry, S. N., Al-Wahaibi, Y. M., Elshafie, A. E., Al-Bemani, A. S., Joshi, S. J., Al-Makhmari, H. S., & Al-Sulaimani, H. S. (2013). Biosurfactant production by Bacillus subtilis B20 using date molasses and its possible application in enhanced oil recovery. *International Biodeterioration & Biodegradation*, *81*, 141–146.

Asgher, M., Arshad, S., Qamar, S. A., & Khalid, N. (2020). Improved biosurfactant production from Aspergillus niger through chemical mutagenesis: Characterization and RSM optimization. *SN Applied Sciences*, *2*(5), 966. https://doi.org/10.1007/s42452-020-2783-3

Banerjee, S., Basak, S., & Ghosh, U. (2019). Optimization of bio-surfactant production by solid state fermentation using response surface methodology. *Research & Reviews: Journal of Food Science and Technology*, *8*(2), 21–32.

Banerjee, S., & Ghosh, U. (2017). Production and Characterization of Glucoamylase by Aspergillus niger. *Applied Food Biotechnology*, *4*(1), 19–26.

Cruz, J. M., Hughes, C., Quilty, B., Montagnolli, R. N., & Bidoia, E. D. (2018). Agricultural Feedstock Supplemented with Manganese for Biosurfactant Production by Bacillus subtilis. *Waste and Biomass Valorization*, *9*(4), 613–618. https://doi.org/10.1007/s12649-017-0019-6

Das, P., Mukherjee, S., & Sen, R. (2008). Improved bioavailability and biodegradation of a model polyaromatic hydrocarbon by a biosurfactant producing bacterium of marine origin. *Chemosphere*, *72*(9), 1229–1234.

De Almeida, D. G., Soares Da Silva, R. de C. F., Luna, J. M., Rufino, R. D., Santos, V. A., Banat, I. M., & Sarubbo, L. A. (2016). Biosurfactants: Promising molecules for petroleum biotechnology advances. *Frontiers in Microbiology*, *7*, 1718.

de Oliveira, D. W. F., França, Í. W. L., Félix, A. K. N., Martins, J. J. L., Giro, M. E. A., Melo, V. M. M., & Gonçalves, L. R. B. (2013). Kinetic study of biosurfactant production

by Bacillus subtilis LAMI005 grown in clarified cashew apple juice. *Colloids and Surfaces B: Biointerfaces*, *101*, 34–43.

Deepika, K. V., Kalam, S., Sridhar, P. R., Podile, A. R., & Bramhachari, P. V. (2016). Optimization of rhamnolipid biosurfactant production by mangrove sediment bacterium Pseudomonas aeruginosa KVD-HR42 using response surface methodology. *Biocatalysis and Agricultural Biotechnology*, *5*, 38–47.

Devaraj, S., Sabapathy, P. C., Nehru, L., & Preethi, K. (2019). Bioprocess optimization and production of biosurfactant from an unexplored substrate: Parthenium hysterophorus. *Biodegradation*, 1–10.

Deziel, E., Paquette, G., Villemur, R., Lepine, F., & Bisaillon, J. (1996). Biosurfactant production by a soil pseudomonas strain growing on polycyclic aromatic hydrocarbons. *Applied and Environmental Microbiology*, *62*(6), 1908.

do Amaral Marques, N. S. A., e Silva, T. A. de L., da Silva Andrade, R. F., Júnior, J. F. B., Okada, K., & Takaki, G. M. C. (2019). Lipopeptide biosurfactant produced by Mucor Circinelloides UCP/WFCC 0001 applied in the removal of crude oil and engine oil from soil. *Acta Scientiarum. Technology*, *41*, e38986.

Ehrhardt, D., Secato, J., & Tambourgi, E. B. (2015). Biosurfactant production by bacillus subtilis using the residue from processing of pineapple, enriched with glycerol, as substrate. *Chemical Engineering Transactions*, *43*, 277–282.

Fadhile Almansoory, A., Abu Hasan, H., Idris, M., Sheikh Abdullah, S. R., Anuar, N., & Musa Tibin, E. M. (2017). Biosurfactant production by the hydrocarbon-degrading bacteria (HDB) Serratia marcescens: Optimization using central composite design (CCD). *Journal of Industrial and Engineering Chemistry*, *47*, 272–280. https://doi.org/10.1016/j.jiec.2016.11.043

Faiq Ali, M., J. M-Ridha, M., & Hussein Taly, A. (2018). Optimization Kerosene Bio-degradation by a Local Soil Bacterium Isolate Klebsiella pneumoniae Sp. Pneumonia. *Journal of Pure and Applied Microbiology*, *12*(4), 2049–2057. https://doi.org/10.22207/JPAM.12.4.41

Geetha, S. J., Banat, I. M., & Joshi, S. J. (2018). Biosurfactants: Production and potential applications in microbial enhanced oil recovery (MEOR). *Biocatalysis and Agricultural Biotechnology*, *14*, 23–32.

George, S., & Jayachandran, K. (2013). Production and characterization of rhamnolipid biosurfactant from waste frying coconut oil using a novel *Pseudomonas aeruginosa* D. *Journal of Applied Microbiology*, *114*(2), 373–383. https://doi.org/10.1111/jam.12069

Geys, R., Soetaert, W., & Van Bogaert, I. (2014). Biotechnological opportunities in biosurfactant production. *Current Opinion in Biotechnology*, *30*, 66–72.

Gudiña, E. J., Fernandes, E. C., Rodrigues, A. I., Teixeira, J. A., & Rodrigues, L. R. (2015). Biosurfactant production by Bacillus subtilis using corn steep liquor as culture medium. *Frontiers in Microbiology*, *6*, 59.

Heryani, H., & Putra, M. D. (2017). Kinetic study and modeling of biosurfactant production using Bacillus sp. *Electronic Journal of Biotechnology*, *27*, 49–54. https://doi.org/10.1016/j.ejbt.2017.03.005

Jimoh, A. A., & Lin, J. (2019). Enhancement of Paenibacillus sp. D9 Lipopeptide Biosurfactant Production Through the Optimization of Medium Composition and Its Application for Biodegradation of Hydrophobic Pollutants. *Applied Biochemistry and Biotechnology*, *187*(3), 724–743. https://doi.org/10.1007/s12010-018-2847-7

Khiari, K., Tarabet, L., Awad, S., Loubar, K., Mahmoud, R., & Tazerout, M. (2018). Optimization of Pistacia lentiscus Oil Transesterification Process Using Central Composite Design. *Waste and Biomass Valorization*, 1–7.

Kubicki, S., Bollinger, A., Katzke, N., Jaeger, K.-E., Loeschcke, A., & Thies, S. (2019). Marine Biosurfactants: Biosynthesis, Structural Diversity and Biotechnological Applications. *Marine Drugs*, *17*(7), 408.

Mouafi, F. E., Elsoud, M. M. A., & Moharam, M. E. (2016). Optimization of biosurfactant production by Bacillus brevis using response surface methodology. *Biotechnology Reports*, *9*, 31–37.

Naughton, P. J., Marchant, R., Naughton, V., & Banat, I. M. (2019). Microbial biosurfactants: Current trends and applications in agricultural and biomedical industries. *Journal of Applied Microbiology*, *127*(1), 12–28.

Rebello, S., Asok, A. K., Mundayoor, S., & Jisha, M. S. (2014). Surfactants: Toxicity, remediation and green surfactants. *Environmental Chemistry Letters*, *12*(2), 275–287.

Secato, J. F. F., dos Santos, B. F., Ponezi, A. N., & Tambourgi, E. B. (2017). Optimization Techniques and Development of Neural Models Applied in Biosurfactant Production by <i>Bacillus subtilis</i> Using Alternative Substrates. *Advances in Bioscience and Biotechnology*, *08*(10), 343–360. https://doi.org/10.4236/abb.2017.810025

Singh, P., Patil, Y., & Rale, V. (2019). Biosurfactant production: Emerging trends and promising strategies. *Journal of Applied Microbiology*, *126*(1), 2–13.

Sousa, M., Dantas, I. T., Feitosa, F. X., Alencar, A. E. V., Soares, S. A., Melo, V. M. M., Gonçalves, L. R. B., & Sant'ana, H. B. (2014). Performance of a biosurfactant produced by Bacillus subtilis LAMI005 on the formation of oil/biosurfactant/water emulsion: Study of the phase behaviour of emulsified systems. *Brazilian Journal of Chemical Engineering*, *31*(3), 613–623.

Souza, K. S. T., Gudiña, E. J., Schwan, R. F., Rodrigues, L. R., Dias, D. R., & Teixeira, J. A. (2018). Improvement of biosurfactant production by Wickerhamomyces anomalus CCMA 0358 and its potential application in bioremediation. *Journal of Hazardous Materials*, *346*, 152–158.

Sylvester, O., Onyekonwu, M., & Okpokwasili, G. (2019, August 5). *Isolation and Screening of Hydrocarbon Utilizing Bacteria for Biosurfactant Production: Application for Enhanced Oil Recovery*. SPE Nigeria Annual International Conference and Exhibition. https://doi.org/10.2118/198784-MS

Yaraguppi, D. A., Bagewadi, Z. K., Muddapur, U. M., & Mulla, S. I. (2020). Response surface methodology-based optimization of biosurfactant production from isolated Bacillus aryabhattai strain ZDY2. *Journal of Petroleum Exploration and Production Technology*, *10*(6), 2483–2498. https://doi.org/10.1007/s13202-020-00866-9

Chapter 4

Production, Purification and Characterization of the produced biosurfactant.

4.1 Introduction

Biosurfactants are amphiphiles which has a unique structure that allows them to gather at the interfacial surface between liquid phases with different polarities (e.g., oil and water or air and water) and in so doing reduce surface tension as well as interfacial tensions(Gudiña, Rangarajan, Sen, & Rodrigues, 2013). They have a variety of domestic and industrial applications and are a group of very essential and important products(Franzetti, Tamburini, & Banat, 2010). Nowadays, due to a hike in environmental awareness and advancements in technology in biological sciences, different types of biosurfactants are produced from bio related wealth such as microorganisms(Gudiña, Fernandes, Rodrigues, Teixeira, & Rodrigues, 2015; Lotfabad et al., 2009). Biosurfactants consequently are the obvious alternative to such processes as they possess innumerous advantages over chemical surfactants, viz. lower amounts of toxicity, more effective biodegradability, and efficacy over a extensive range of pH and temperature values(Banat et al., 2010; Cameotra, Makkar, Kaur, & Mehta, 2010). Bacterial biosurfactants were originally anticipated to play a role as emulsifiers of biodegradable hydrocarbons(Neu, 1996). However, a large range of roles for biosurfactants are found such as their antimicrobial(Augustin, Majesté, Hippolyte, & Léopold, 2015; Machado, Mohideen, Saravanakumari, & Prabhavathi, 2013; Mbawala, Mouafo, & Kom, 2013; Nitschke, Costa, & Contiero, 2010), antiadhesive (Gudiña, Rocha, Teixeira, & Rodrigues, 2010), emulsifying(Augustin et al., 2015; Gudiña et al., 2015), and antioxidant properties(Yalcin & Cavusoglu, 2010).

Biosurfactants are extracted as extracellular compounds or contained on the microorganisms' cell surface, where the microbial cell itself is a biosurfactant(Atlas,

1981). They are primarily comprised of glycolipids, hydroxylated and cross-linked fatty acids, polysaccharides lipid complexes, lipoprotein-lipopeptides and phospholipids(Banat, Makkar, & Cameotra, 2000). Biosurfactants are believed to be used by microorganisms for transportation across the membrane, protection against other organisms and in host-microbe interaction. In addition, they have the properties of wetting penetrating actions, spreading, the hydrophobicity of surfaces, metal sequestration, microbial growth enhancement, and antimicrobial activity(Banat et al., 2000). They are specific in activity, and the specificity depends on the structure and functional group of a particular molecular. Currently, biosurfactants have a wide range of applications in different industries. They are used as emulsifying agents for drugs transport to infection sites, supplementary agents to pulmonary surfactant and adjuvants for vaccines(Steel, Barrow, & Feltham, 1993).The composition of culture media and chemophysical parameters strongly influence kinetic production, biosurfactant congeners and properties(Abouseoud, Maachi, Amrane, Boudergua, & Nabi, 2008; Lotfabad et al., 2009).

The purpose of this study is the production of biosurfactant from previously isolated *Bacillus oceanisediminis* H2 and complete identification of the produced biosurfactant based on purification, preliminary characterization, FTIR and LC-MS results.

4.2 Materials and Methods

4.2.1 Sample

Bacillus oceanisediminis H2 was collected and maintained as mentioned in chapter 1 (1.2.1).

4.2.2 Culture media

Nutrient Broth (NB) and Mineral salts media (MSM) was prepared as mentioned in chapter 1 (1.2.2). Mineral salts media (MSM) was prepared and supplemented as mentioned in chapter 2 (2.2.2).

4.2.3 Production of biosurfactant

The production media was inoculated with 24hrs grown culture of *B. oceanisediminis* H2, which was maintained in nutrient agar. Two such culture slants were provided for the inoculation. The production was carried out through fermentation in 1L erlenmeyer flask maintained at 37°C and 180 rpm. The process was carried out for 5 consecutive days.

After completion of fermentation, the aliquot was centrifuged at 6000 rpm to make it cell free and its pH of the suspended solution was lowered to 2 and kept overnight at 4°C. This was a step for the extraction of the biosurfactant though acid precipitation (Das, Mukherjee, & Sen, 2008). Later it was centrifuged at 15000 rpm at 4°C whose pellet was resuspended in equal volume of deionized water with pH adjusted to 7.0.

The now crude biosurfactant was vacuum evaporated by a rotatory vacuum evaporator (Eyela Rotary Evaporator N-1200BV-W) and lyophilized to obtain solid crude biosurfactant, and weighed.

4.2.4 Measurement of Surface tension

The surface tension measurements were done as mentioned in chapter 1 (1.2.8).

4.2.5 Purfication of the crude biosurfactant

The crude biosurfactant was subjected for thin layer chromatography (TLC) analysis to verify the produce of biosurfactant followed by post development. The developing solution for the TLC was a solvent system comprising of chloroform-methanol-water-acetic acid in the ratio 65:25:2:1.5 (Das et al., 2008; Macala, Yu, & Ando, 1983). The biosurfactant obtained as a fermentation product was dissolved in methanol on aluminum TLC silica gel 60 F_{254} plates (Merck, Darmstadt, Germany) (Kim, Lim, Lee, Lee, & Lee, 2000).

The plate was then developed with the solvent system and then seen under a short-wavelength (254 nm) ultraviolet (UV)-emitting mercury lamp and the spots thus formed were distinct to view in white light and then was developed with 0.02% ninhydrin solution prepared in absolute ethanol. The spraying of the ninhydrin solution was followed by its chromogenic development through heating the ninhydrin sprayed TLC plates at 110°C for detection of peptides, free amino acid groups as well as both acidic and non-acidic lipids (Das et al., 2008; Elgubbi, 2015; Kim et al., 2000).

Crude biosurfactant was separated on a silica gel column (60 Mesh) (Merck, Darmstadt, Germany). Elution was carried out with the mobile phase: chloroform-methanol-water (65:25:4) and fractions of 1 ml were collected and tested by thin layer chromatography again for comparing it to the TLC of the crude biosurfactant (Jemil et al., 2017).

The fractions collected were further analyzed by fourier transform infrared spectroscopy (FTIR) (Carrillo, Teruel, Aranda, & Ortiz, 2003) and liquid chromatography – mass spectroscopy (LC-MS) (Grover, Nain, Singh, & Saxena, 2010).

4.2.6 Structural analysis

4.2.6.1 Fourier transform infrared spectroscopy (FTIR)

Fourier transform infrared spectroscopy (FTIR) was performed to find out the functional groups and the chemical bonds present in the active fraction of the biosurfactant and thus resolve its chemistry. The FTIR analysis was performed by using a Shimadzu IR Prestige-21FT-IR spectrometer (Shimadzu, Europe) with sample dispersed in the pellets of KBr. The active fraction was lyophilized which were of biosurfactant samples (0.3–0.5 mg) were made into a powder in approximately 80 mg of spectral-grade KBr (Merck, Germany). The spectrometer was supplied with dry nitrogen for an hour so as to remove carbon dioxide traces beforehand the spectral measurements were taken. The measurements were done in the absorbance approach. 4 cm^{-1} resolution was conventionally performed which gave IR traces over the range of 400–4000 cm^{-1}. The work was carried out at Dept. of Metallurgical Engineering, Jadavpur university, Kolkata, India.

4.2.6.2 Liquid chromatography - mass spectroscopy (LC-MS)

The purified samples were analyzed with a liquid chromatography mass spectrometer (Xevo G2-XS QTof Quadrupole Time-of-Flight Mass Spectrometry, Waters India Pvt. Ltd.) in positive mode at Indian Institute of Chemical Biology, Kolkata, India.

4.3 Results and Discussions

4.3.1 Production of biosurfactant

In this study, biosurfactant production was determined by biosurfactant activities in terms of surface tension reduction. The surface tension measurement was done by drop count method with a Traube's stalagmometer. The lowering of surface tension was indicative of the face that biosurfactant was produced from the biosurfactant production media that was carried out for 5 days with *Bacillus oceanisediminis* H2. From 300mL production media (Fig 4.1A) 0.110g of solid crude biosurfactant (Fig 4.1B) was obtained after successful lyophilisation. The methanolic extract after resuspension measured 80mL from which the lyophilisation was carried out. The surface tension of the methanolic extract was found to be 46.909 mN/m at a concentration of 1.375 µM, which is sufficiently lower than 72 mN/m of that of water. This concluded that there was an efficient biosurfactant produced from the fermentation of the biosurfactant production media carried out for 5 days incubated at 37°C and 180 rpm.

(A)

(B)

Fig 4.1: (A) Fermentation broth after 5 days of fermentation showing frothing indicative of biosurfactant production, (B) Lyophilized sample of produced crude biosurfactant.

4.3.2 *Purification of the crude biosurfactant*

The crude biosurfactant which was obtained from the fermentative process was extracted via chemical segregation method by acidifying the cell-free broth, which were then extracted in methanol to obtain moderately purified sample of the biosurfactant. This solvent extracted partially purified biosurfactant was subjected to a silica gel G column chromatographic separation. Fractionation of the crude extract was monitored using TLC by comparing the R_f values of each of the fraction with the TLC obtained from the crude extract.

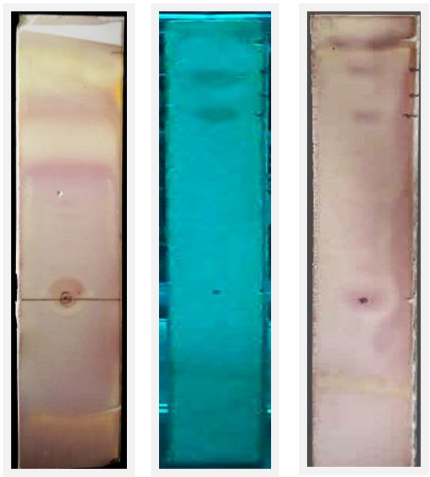


Fig 4.2: TLC plates post development; (A) Ninhydrin sprayed crude biosurfactant after heating it to 110°C; (B) Partially purified biosurfactant in UV light of wavelength 254nm; (C) Ninhydrin sprayed partially purified biosurfactant after heating it to 110°C.

The R_f values for the two spots of the TLC plate with the methanolic extract of the crude biosurfactant was calculated to be 0.678 and 0.78 respectively. The partially purified biosurfactant methanolic aliquot was seen to be spotted at two places with R_f values similar to the crude extract TLC plate in both cases of exposure to short wave UV light and 0.02% ninhydrin reagent, representing existence of peptide and lipid moieties in the molecule (Fig 4.2).

Fractionation by the silica gel G column chromatography produced 50 1mL fractions withonly 6 lanes providing a single spot, out of which the 33rd fraction had a R_f value of 0.614 which was similar to the R_f value of the partially purified TLC plate (Fig 4.3).

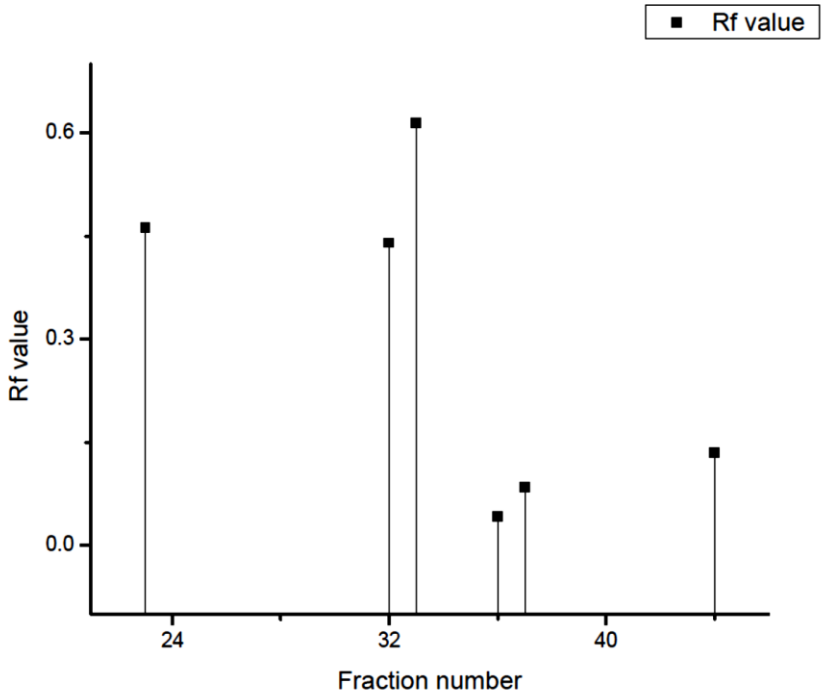


Fig 4.3: R_f values of the fractions obtained from the silica gel column chromatography after post development with 0.02% ninhydrin reagent.

4.3.3 Chemical analysis of the bioactive fraction

The 33rd biosurfactant fraction obtained from the TLC plate showed only one spot. This fraction was being spotted in presence of ninhydrin reagent as well as short wave UV light suggesting the occurrence of both peptide and lipid domains in the extracted biosurfactant. This was additionally confirmed with FTIR spectra of the compound (Fig 4.4). A wide-ranging absorbance with wave numbers at 3450 $cm^{-1}$to 3220 cm^{-1} having its maximum at 3194 cm^{-1} was observed. Absorbance in this area is the characteristic of N-H stretching vibrations and is common in amine containing groups. Sharp peaks at 1651 cm^{-1} and 1402 cm^{-1} was suggestive of C=O stretching vibrations and C-H bending vibrational absorbance. This further suggests it is a peptide group and is common in

compounds with alkyl groups. Other peaks were observed at 1229 cm^{-1} and 1123 cm^{-1} depictive of C-N stretch vibrations common in peptide bonds. Thus it can be said that the biosurfactant is of lipopeptide nature and can be suggestive of the fact that it is similar to the structure of surfactin.

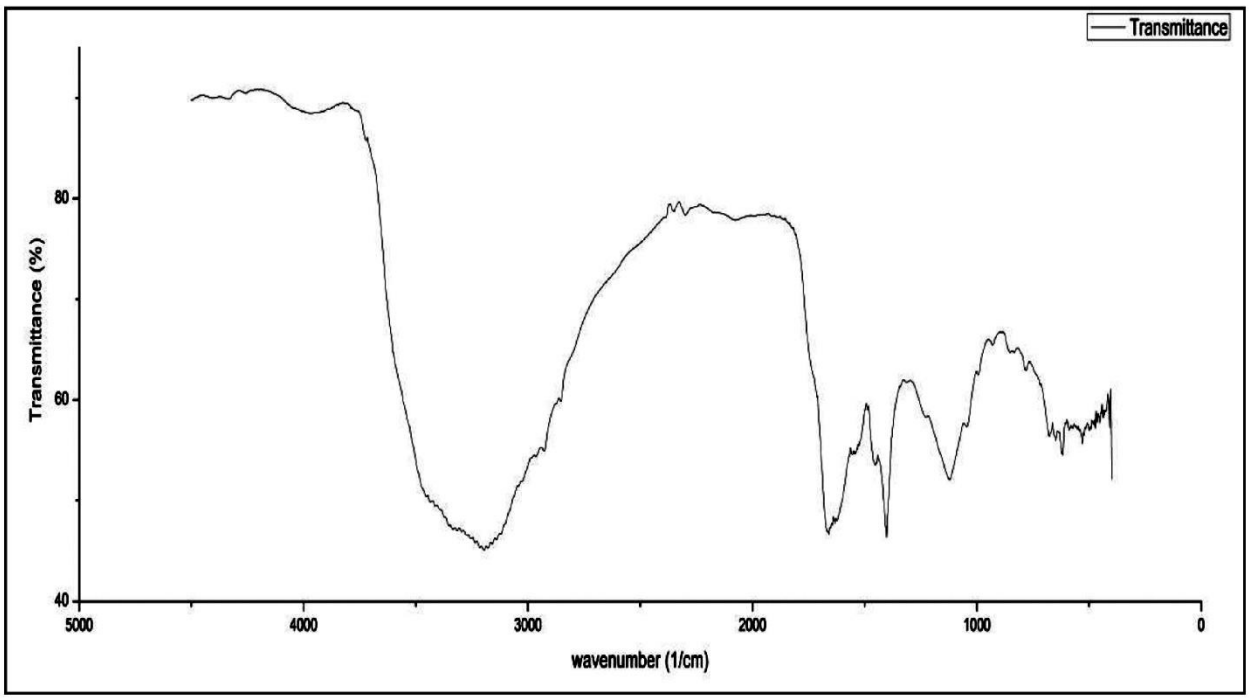
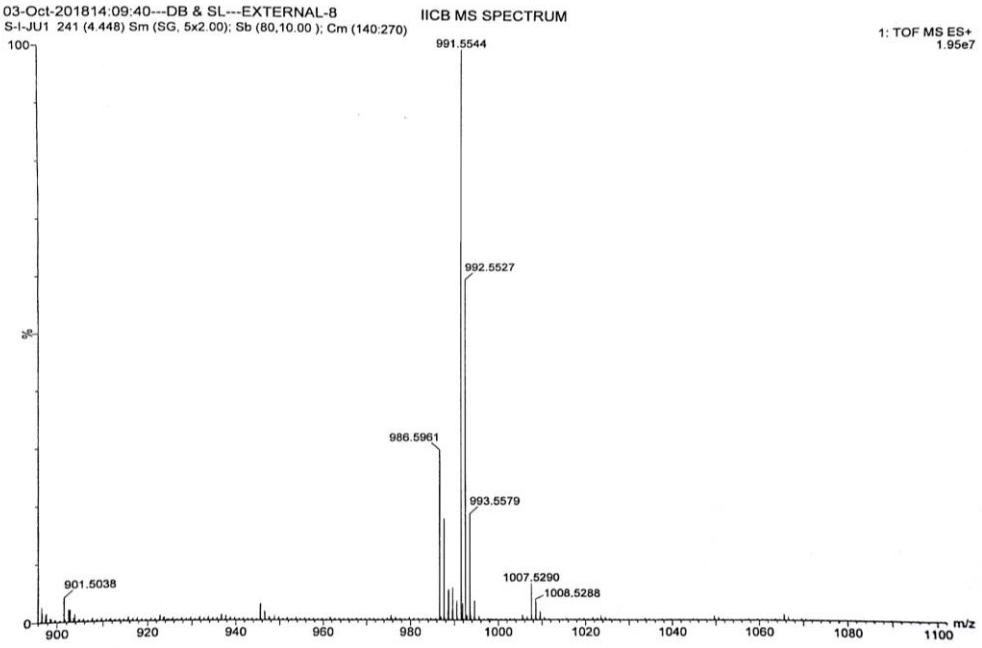
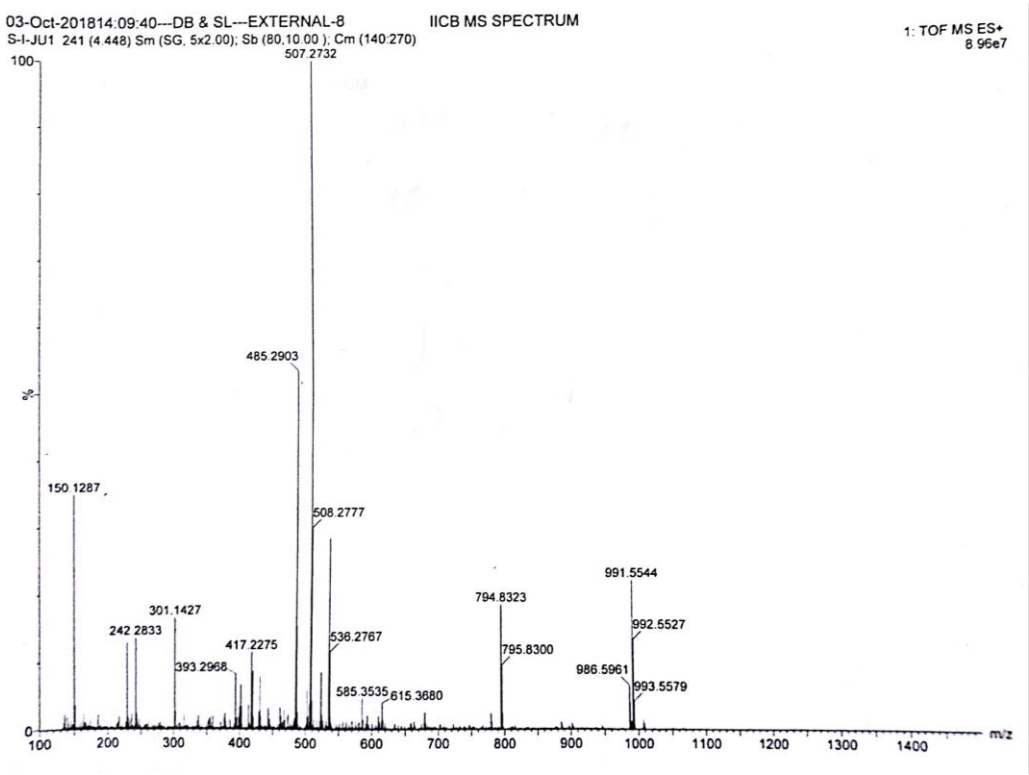


Fig 4.4: FTIR spectra of the silica gel G column chromatography-purified bioactive fraction of the biosurfactant produced by *Bacillus oceanisediminis* H2.



(A)



(B)

Fig 4.5: Liquid chromatography – Mass spectroscopy (LC-MS) analysis of biosurfactant molecular masses produced from *Bacillus oceanisediminis* H2; (A) Mass spectra of *m/z* 991.5544. (B) Mass spectra of *m/z* 991.5544 showing specific fragment *m/z* 507.2732.

LC-MS analysis of the bioactive fraction of the fractionations from the silica gel G column chromatography showed two distinct well resolved clusters of peaks within the mass range *m/z* 991.5544 ($2M + Na^+$) and 507.2732 ($M + Na^+$). Presence of this mass confirmed the presence of surfactin in the extract (Fig 4.5, Table 4.1).

In the case of surfactin (991.5544), the comprehensive LC-MS analysis of *m/z* 991.5544 showed the occurrence of a fragmented mass 507.2732 which was a confirmatory to its identity as surfactin.

Table 4.1: $[M + H]^+$, $[M + Na]^+$, and $[2M + Na]^+$ of LC-MS for peaks from biosurfactant production medium.

Sample	$[M + H]^+$	$[M + Na]^+$	$[2M + Na]^+$	MW
Biosurfactant from fermentation	485.2903	507.2732	991.5544	484 (Fragment) 990 (Surfactin)

Several strains of *B. subtilis* have been previously reported for their production of antimicrobial cyclic lipopeptides which includes fengycins and surfactins that has wide antimicrobial properties(Arrebola, Jacobs, & Korsten, 2010). *B. mojavensis* which is isolated from varied environmental conditions have been found out to have activity against phytopathogens which are highly significant(Ozyilmaz & Benlioglu, 2013).Bacillus sp. have been previously found to generate mixture of narrowly associated cyclic lipopeptide isoforms of the biosurfactant surfactin A(Snook, Mitchell, Hinton, & Bacon, 2009). Surfactins are considered to be a factor for the effective inhibition of fungi, bacteria, mycoplasmas, and viruses(Bacon, Hinton, Mitchell, Snook, & Olubajo, 2012). Surfactin is also highly remarkable for its expansive variety in antibacterial activity.

In this study the biosurfactant producer *Bacillus oceanisediminis* H2 was able to produce an efficient biosurfactant which was able to lower the surface tension to a

greater extent. Further studies could be an alternative to the production of biosurfactant though a low-cost method and with ease of production.

4.4 Conclusion

Bacillus oceanisediminis H2 produced 0.37 g/L of biosurfactant during the 5 days of fermentation in the biosurfactant production media with glycerol and yeast extract as sole carbon and nitrogen sources respectively. FTIR and LC-MS analysis pointed out that the biosurfactant produced was of lipopeptide nature and specifically confirmed that it was surfactin with a molecular weight of 990 Da, respectively. This is the first report of efficient biosurfactant produced by *Bacillus oceanisediminis* H2 which was previously isolated from tannery processing industrial wasteland.

4.5 References

Abouseoud, M., Maachi, R., Amrane, A., Boudergua, S., & Nabi, A. (2008). Evaluation of different carbon and nitrogen sources in production of biosurfactant by Pseudomonas fluorescens. *Desalination*, *223*(1–3), 143–151.

Amrhein, S., Suhm, S., & Hubbuch, J. (2016). Surface tension determination by means of liquid handling stations. *Engineering in Life Sciences*, *16*(6), 532–537.

Arrebola, E., Jacobs, R., & Korsten, L. (2010). Iturin A is the principal inhibitor in the biocontrol activity of Bacillus amyloliquefaciens PPCB004 against postharvest fungal pathogens. *Journal of Applied Microbiology*, *108*(2), 386–395. https://doi.org/10.1111/j.1365-2672.2009.04438.x

Atlas, R. M. (1981). Microbial degradation of petroleum hydrocarbons: An environmental perspective. *Microbiological Reviews*, *45*(1), 180.

Augustin, M., Majesté, P. M., Hippolyte, M. T., & Léopold, T. N. (2015). Effect of biosurfactants extracted from a locally fermented milk (Pendidam) on its shelf life. *Journal of Advances in Biology & Biotechnology*, *3*(1), 12–22.

Bacon, C. W., Hinton, D. M., Mitchell, T. R., Snook, M. E., & Olubajo, B. (2012). Characterization of endophytic strains of Bacillus mojavensis and their production of surfactin isomers. *Biological Control*, *62*(1), 1–9. https://doi.org/10.1016/j.biocontrol.2012.03.006

Banat, I. M., Franzetti, A., Gandolfi, I., Bestetti, G., Martinotti, M. G., Fracchia, L., … Marchant, R. (2010). Microbial biosurfactants production, applications and future potential. *Applied Microbiology and Biotechnology*, *87*(2), 427–444.

Banat, I. M., Makkar, R. S., & Cameotra, S. S. (2000). Potential commercial applications of microbial surfactants. *Applied Microbiology and Biotechnology*, *53*(5), 495–508.

Cameotra, S. S., Makkar, R. S., Kaur, J., & Mehta, S. K. (2010). Synthesis of biosurfactants and their advantages to microorganisms and mankind. In *Biosurfactants* (pp. 261–280). Springer.

Carrillo, C., Teruel, J. A., Aranda, F. J., & Ortiz, A. (2003). Molecular mechanism of membrane permeabilization by the peptide antibiotic surfactin. *Biochimica et Biophysica Acta (BBA) - Biomembranes*, *1611*(1), 91–97. https://doi.org/10.1016/S0005-2736(03)00029-4

Das, P., Mukherjee, S., & Sen, R. (2008). Antimicrobial potential of a lipopeptide biosurfactant derived from a marine Bacillus circulans. *Journal of Applied Microbiology*, *104*(6), 1675–1684.

Elgubbi, H. (2015). Modified Ninhydrin Reagent for the Detection of Amino Acids on TLC Paper. *EC Nutrition*, *1*, 128–131.

Franzetti, A., Tamburini, E., & Banat, I. M. (2010). Applications of biological surface active compounds in remediation technologies. In *Biosurfactants* (pp. 121–134). Springer.

Grover, M., Nain, L., Singh, S. B., & Saxena, A. K. (2010). Molecular and biochemical approaches for characterization of antifungal trait of a potent biocontrol agent Bacillus subtilis RP24. *Current Microbiology*, *60*(2), 99–106.

Gudiña, E. J., Fernandes, E. C., Rodrigues, A. I., Teixeira, J. A., & Rodrigues, L. R. (2015). Biosurfactant production by Bacillus subtilis using corn steep liquor as culture medium. *Frontiers in Microbiology*, *6*, 59.

Gudiña, E. J., Rangarajan, V., Sen, R., & Rodrigues, L. R. (2013). Potential therapeutic applications of biosurfactants. *Trends in Pharmacological Sciences*, *34*(12), 667–675.

Gudiña, E. J., Rocha, V., Teixeira, J. A., & Rodrigues, L. R. (2010). Antimicrobial and antiadhesive properties of a biosurfactant isolated from Lactobacillus paracasei ssp. Paracasei A20. *Letters in Applied Microbiology*, *50*(4), 419–424.

Jemil, N., Manresa, A., Rabanal, F., Ayed, H. B., Hmidet, N., & Nasri, M. (2017). Structural characterization and identification of cyclic lipopeptides produced by Bacillus methylotrophicus DCS1 strain. *Journal of Chromatography B*, *1060*, 374–386.

Kim, S. H., Lim, E. J., Lee, S. O., Lee, J. D., & Lee, T. H. (2000). Purification and characterization of biosurfactants from Nocardia sp. L-417. *Biotechnology and Applied Biochemistry*, *31*(3), 249–253.

Lotfabad, T. B., Shourian, M., Roostaazad, R., Najafabadi, A. R., Adelzadeh, M. R., & Noghabi, K. A. (2009). An efficient biosurfactant-producing bacterium Pseudomonas aeruginosa MR01, isolated from oil excavation areas in south of Iran. *Colloids and Surfaces B: Biointerfaces*, *69*(2), 183–193.

Macala, L. J., Yu, R. K., & Ando, S. (1983). Analysis of brain lipids by high performance thin-layer chromatography and densitometry. *Journal of Lipid Research*, *24*(9), 1243–1250.

Machado, R. T., Mohideen, R. A. H., Saravanakumari, M., & Prabhavathi, P. (2013). Anti-adhesive, antimicrobial and biodegradability assay of a Lipopeptide biosurfactant from Lactococcus lactis. *International Journal of Science Innovations and Discoveries*, *3*(4), 478–483.

Mbawala, A., Mouafo, T. H., & Kom, R. R. (2013). Antibacterial activity of Lactobacillus biosurfactants against Pseudomonas spp. Isolated from fresh beef. *Novus International Journal of Biotechnology and Bioscience*, *2*(1), 7–22.

Najafi, A. R., Rahimpour, M. R., Jahanmiri, A. H., Roostaazad, R., Arabian, D., & Ghobadi, Z. (2010). Enhancing biosurfactant production from an indigenous strain of Bacillus mycoides by optimizing the growth conditions using a response surface methodology. *Chemical Engineering Journal*, *163*(3), 188–194.

Neu, T. R. (1996). Significance of bacterial surface-active compounds in interaction of bacteria with interfaces. *Microbiological Reviews*, *60*(1), 151.

Nitschke, M., Costa, S. G., & Contiero, J. (2010). Structure and applications of a rhamnolipid surfactant produced in soybean oil waste. *Applied Biochemistry and Biotechnology*, *160*(7), 2066–2074.

Ozyilmaz, U., & Benlioglu, K. (2013). Enhanced Biological Control of Phytophthora Blight of Pepper by Biosurfactant-Producing Pseudomonas. *The Plant Pathology Journal*, *29*(4), 418–426. https://doi.org/10.5423/PPJ.OA.11.2012.0176

Rodrigues, L. R., Teixeira, J. A., van der Mei, H. C., & Oliveira, R. (2006). Physicochemical and functional characterization of a biosurfactant produced by Lactococcus lactis 53. *Colloids and Surfaces B: Biointerfaces*, *49*(1), 79–86.

Singh, A., Van Hamme, J. D., & Ward, O. P. (2007). Surfactants in microbiology and biotechnology: Part 2. Application aspects. *Biotechnology Advances*, *25*(1), 99–121.

Snook, M. E., Mitchell, T., Hinton, D. M., & Bacon, C. W. (2009, April 17). Isolation and Characterization of Leu7-Surfactin from the Endophytic Bacterium Bacillus mojavensis RRC 101, a Biocontrol Agent for Fusarium verticillioides [Research-article]. https://doi.org/10.1021/jf900164h

Steel, K. J., Barrow, G. I., & Feltham, R. K. A. (1993). *Cowan and Steel's manual for the identification of medical bacteria*. Cambridge University Press.

Yalcin, E., & Cavusoglu, K. (2010). Structural analysis and antioxidant activity of a biosurfactant obtained from Bacillus subtilis RW-I. *Turkish Journal of Biochemistry-Turk Biyokimya Dergisi*, *35*(3), 243–247.

Chapter 5

Application of biosurfactant for effective removal of maximum xenobiotic compounds.

5.1 Introduction

Humans have caused a significant problem for generations by polluting ecosystems either directly or indirectly. The fact that the world continues to rely on energy sources that are environmentally harmful makes pollution a foregone conclusion. A number of procedures and regulations have been enacted to limit the rate at which environmental pollution occurs. The detrimental effects of pollutants on living creatures is why humans have created strategies to fight pollution and counteract the impacts of any pollutants if they manage to infiltrate the environment. Pollution clean-up, also known as remediation, is the process of returning the environment to its normal state. Biological, chemical, and physical cleanup procedures are the primary types, depending on the kind of remediation agent (Ghosal et al. 2016). Bioremediation refers to the employment of biological agents to lower the levels of pollution in a contaminated site or an ecosystem (Bharagava et al. 2017a; Saxena and Bharagava 2016). Biological agents, processes, or mechanisms can be used to repair a contaminated environment, and this is called bioremediation (Singh et al. 2011). Any or a combination of degradation, mineralization, and transformation can be used in the process of bioremediation (Saxena and Bharagava 2017). Pollution linked with organic substances is generally accompanied by degradation and mineralization. In the first, pollutants are broken down into smaller and less harmful compounds; in the second, pollutants are transformed into carbon dioxide and water, or methane, under different conditions (Haritash and Kaushik 2009). Unlike transformation, inorganic pollutants are connected with change due to their nature and origin. Additionally, certain

inorganic contaminants do not contain carbon, which is a necessary energy source for many microorganisms. To transform pollutants, change them into another chemically similar form. Sometimes, the modified pollutant is more harmful than the primary pollutant (Ghosal et al. 2016). Microbes use carbon found in organic contaminants to power their metabolic processes, so degradation and mineralization can occur.

Heavy metal pollution is caused by industrial tannery effluent, which is a major source of it in our environment. Industrial use and the environment's greatest pollutants are heavy metals. The harm to living creatures in an environment caused by heavy metal contamination has grown increasingly significant(Siddiquee et al. 2015; Su 2014; Deepa and Suresha 2014; Hrynkiewicz and Baum 2014; Okolo et al. 2016).

Utilizing microbes with advantageous characteristics, such as metabolic capacity and/or enzymes and bio-surfactants, as an alternate method to improving their remediation efficiency is a new strategy (Le et al. 2017; Schenk, Carvalhais, and Kazan 2012).

The most often encountered class of organic pollutants in Europe is polycyclic aromatic hydrocarbons (PAHs) (European Environment Agency, n.d.). Most of them come from the usage of coal-based goods in industrial operations from the past and present (coking plant, gas plant, wood-treating facilities etc). The evaluation of soil that is polluted with polycyclic aromatic hydrocarbons (PAHs) is based on the hazardous, carcinogenic, and mutagenic characteristics of 16 PAHs ("ES&T Special Report: Priority Pollutants: I-a Perspective View | Environmental Science & Technology" n.d.). However, oxygenated and nitrogenated polycyclic aromatic compounds are also observed in conjunction with these PAHs (O-PACs and N-PACs). Compounds like these are known to have hazardous, mutagenic, and carcinogenic characteristics (Chesis et al. 1984; Durant et al. 1996; Staffan Lundstedt et al. 2007; Donnelly et al. 2005) and are also better soluble in water, which results in greater mobility (Lemieux et al. 2009). That's why interest in these substances has begun to rise in recent years, and numerous studies have examined them (Staffan Lundstedt et al. 2007; S. Lundstedt et al. 2014; Bandowe and Wilcke 2010; Arp et al. 2014; Meyer, Cartellieri, and Steinhart 1999; Biache et al. 2008; Ghislain, Faure, and Michels 2012). However, because they're not regulated, the data available on these compounds is quite limited. New plumes of

pollution can be created in the early stages of the contamination process, but remediation methods can lead to more prolonged contamination problems (Staffan Lundstedt, Haglund, and Öberg 2003; Staffan Lundstedt et al. 2007). Research showed the degradation pathway of a specific PAH and the involvement of O-PACs as intermediates or dead-end products. That leads to the accumulation of O-PACs in soils after biodegradation treatments and natural attenuation (B. E. Andersson and Henrysson 1996; B. Erik Andersson et al. 2003; Eriksson, Dalhammar, and Borg-Karlson 2000). Then, to understand their behaviour during bioremediation treatments, it is necessary to examine the microbial community dynamics, including the PAC degradation, when it comes to the soil.

The aim of this study is based upon the bioremediation of tannery effluent wastewater collected from Calcutta Leather Complex, Bantala, Kolkata, India by the application of the produced biosurfactant from the previously isolated strain and identified as *Bacillus oceanisediminis* H2.

5.2 Materials and Methods

5.2.1 Sample

Bacillus oceanisediminis H2 was collected and maintained as mentioned in chapter 1 (1.2.1)

5.2.2 Culture media

Nutrient Broth (NB) and Mineral salts media (MSM) was prepared as mentioned in chapter 1 (1.2.2). Mineral salts media (MSM) was prepared and supplemented as mentioned in chapter 2 (2.2.2).

5.2.3 Production of biosurfactant

Biosurfactant was produced according to the method mentioned in chapter 4 (4.2.2).

5.2.4 Purification of the crude biosurfactant

Purification of the produced biosurfactant was done according to the method that was mentioned in chapter 4 (4.2.5).

5.2.5 Analytical procedures

The physicochemical parameters BOD and COD were measured from the tannery effluent wastewater samples that were collected from Calcutta Leather Complex, Bantala, Kolkata, India. The tannery had a treatment plant thus both the untreated and treated samples were collected for analyses. The produced biosurfactant was applied The BOD was determined using the 5-day BOD test (APHA 5210B) and the COD (APHA 5220), using open reflux oxidation method (Clesceri et al. 2005).

5.2.6 Statistical analyses

The plots were constructed using “Origin 9.0”.

5.3 Results and Discussions

5.3.1 Analytical procedures

A 5 day-BOD analysis was done according to the method mentioned in APHA (section 5210B) (APHA 5210 2018). The COD analysis was done by open reflux oxidation method as mentioned by APHA (section 5220) (APHA 5220 2018). The untreated and treated samples were subjected to treatment by application of the produced and purified biosurfactant in 2% (w/v).

Both the BOD and COD of untreated and treated wastewater samples were lowered by a significant percentage on application of the biosurfactant (Table 1) (Fig 1). This shows that the biosurfactant is capable of remediating the wastewater samples.

Table 5.1: Effects of the untreated and treated wastewater sample on application of produced and purified biosurfactant.

	UT (mg/L) *	**UT (mg/L) + BS***	**Percentage reduction (%)**	**T (mg/L) ***	**T (mg/L) + BS***	**Percentage reduction (%)**
BOD	388.13	110	71.65	175	62	64.57
COD	821	323	60.65	402	152	62.18

*BOD = Biological Oxygen Demand; COD = Chemical Oxygen; Demand; UT = untreated (mg/L); BT + BS = Untreated (mg/L) + Biosurfactant; T = Treated (mg/L); AT + BS = Treated (mg/L) + Biosurfactant.

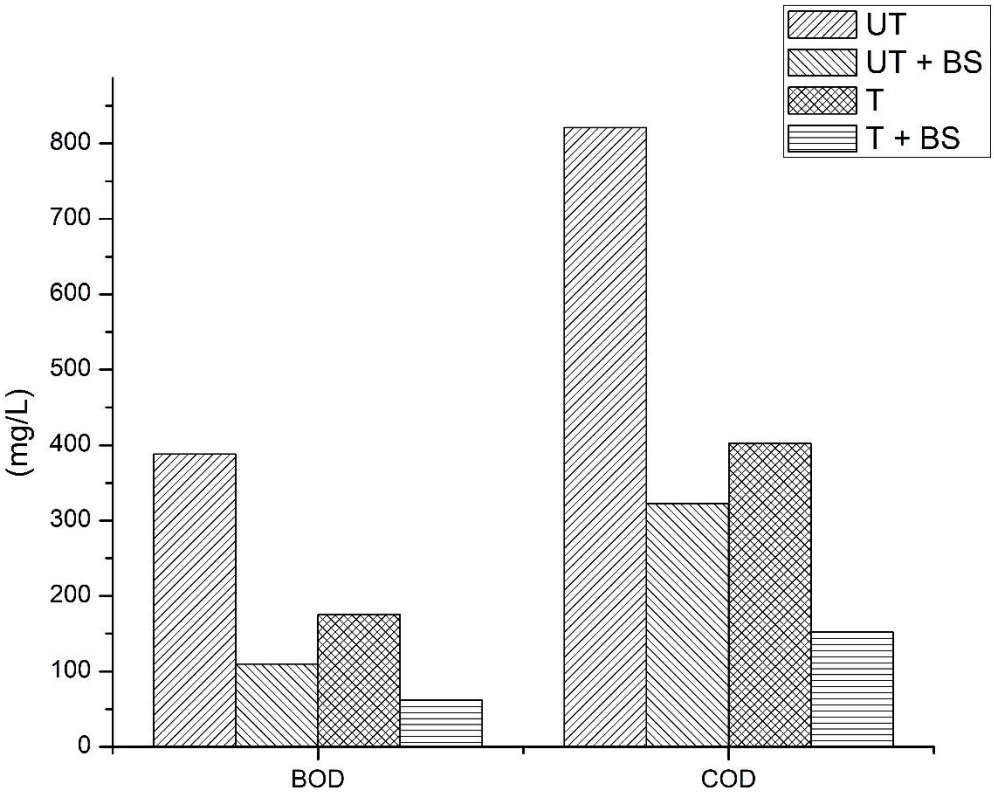


Fig 5.1: Effects of the untreated and treated wastewater sample on application of produced and purified biosurfactant.

5.4 Conclusion

Bacillus oceanisediminis H2 produced a biosurfactant which when applied on tannery effluent wastewater it was notably observed that the BOD and COD levels were significantly reduced. The wastewater samples were collected from Calcutta Leather Complex, Bantala, Kolkata, India which had a treatment plant thus both the untreated and treated wastewater were subjected to application of the purified biosurfactant. It was observed that the BOD of the untreated and treated samples was reduced by a percentage of 71.65 and 64.57 respectively; and the COD was reduced by a percentage of 60.65 and 62.18 respectively. Thus, it shows that the produced biosurfactant by *Bacillus oceanisediminis* H2 is a capable bioremediator.

5.5 References

APHA 5210. 2018. “Biochemical Oxygen Demand (Bod).” In *Standard Methods For the Examination of Water and Wastewater*. Standard Methods for the Examination of Water and Wastewater. American Public Health Association. https://doi.org/10.2105/SMWW.2882.102.

APHA 5220. 2018. “Chemical Oxygen Demand (Cod).” In *Standard Methods For the Examination of Water and Wastewater*. Standard Methods for the Examination of Water and Wastewater. American Public Health Association. https://doi.org/10.2105/SMWW.2882.103.

Clesceri, Lenore S., Arnold E. Greenberg, Andrew D. Eaton, Eugene W. Rice, and Mary Ann H. Franson. 2005. “Standard Methodsfor the Examination of Water and Wastewater.”

Deepa, C. N., and S. Suresha. 2014. “Biosorption of Lead (II) from Aqueous Solution and Industrial Effluent by Using Leaves of Araucaria Cookii: Application of Response Surface Methodology.” *IOSR Journal of Environmental Science, Toxicology and Food Technology* 8 (7): 67–79.

Hrynkiewicz, Katarzyna, and Christel Baum. 2014. “Application of Microorganisms in Bioremediation of Environment from Heavy Metals.” In *Environmental Deterioration and Human Health*, 215–27. Springer.

Le, Thao Thanh, Min-Hui Son, In-Huyn Nam, Hakwon Yoon, Yu-Gyeong Kang, and Yoon-Seok Chang. 2017. “Transformation of Hexabromocyclododecane in Contaminated Soil in Association with Microbial Diversity.” *Journal of Hazardous Materials* 325: 82–89.

Okolo, N. V., E. A. Olowolafe, I. Akawu, and S. I. R. Okoduwa. 2016. “Effects of Industrial Effluents on Soil Resources in Challawa Industrial Area, Kano, Nigeria.” *Journal of Global Ecology and Environment* 5 (1): 1–10.

Schenk, Peer M., Lilia C. Carvalhais, and Kemal Kazan. 2012. “Unraveling Plant–Microbe Interactions: Can Multi-Species Transcriptomics Help?” *Trends in Biotechnology* 30 (3): 177–84.

Siddiquee, S., K. Rovina, S. Al Azad, L. Naher, S. Suryani, and P. Chaikaew. 2015. "Heavy Metal Contaminants Removal from Wastewater Using the Potential Filamentous Fungi Biomass: A Review." *J Microb Biochem Technol* 7 (6): 384–93.

Su, Chao. 2014. "A Review on Heavy Metal Contamination in the Soil Worldwide: Situation, Impact and Remediation Techniques." *Environmental Skeptics and Critics* 3 (2): 24.

Summary

The isolated and screened strain PC1 was screened according to the colony's zonal diameter and maximum surface tension lowering ability when it was incubated in NA plates with precoated anthracene which was 3.4cm and 33.8 mN/m respectively. The growth curve of PC1 shows that it reaches end of log phase in 28hrs and the stationary phase extends up to 72hrs from the time of incubation, suggesting extensive biosurfactant production. PC1 has gram positive, endospore forming, motile, has catalase activity, oxidase activity but cannot coagulase, produce urease or reduce nitrate.

The fermented media was seen to lower maximum surface tension when compared to its uninoculated counterpart at 5 days, pH 8, 37°C with 24hr old culture which was set to be the control. This shows that the isolated strain can be utilized for biosurfactant production. The surface tension measured over time is a minimum of 30.573 mN/m at 120 hrs or 5 days. It further suggests that the isolate strain not only produces biosurfactant optimally at alkaline pH but can also give a nearby efficient production at acidic pH. The response surfaces demonstrate the form and degree of interaction between various variables. The 3D surface shows a curved gradational decrease in surface tension of the fermented GlyMSM media with an increase in time of fermentation and pH of the fermentable media, which gave an optimum of 34.61 mN/m at 40°C and pH 9.0.

In the present analysis, a single variable used to optimize carbon sources and nitrogen sources in conjunction with RSM for optimization of all sources was used and was in near accordance with the RSM optimization data. Glycerol (29.957 mN/m) and Yeast extract (30.23 mN/m), respectively, supplemented the carbon source and nitrogen source with an MSM which measured the lowest surface tension. The final parameters of the fermentation process were used by environmentally optimized variables such as 120 hours, 40°C, pH 9, and with Bacillus oceanisediminis H2 growing for 24 hours. The maximum drop in surface tension was recorded to be 36.428 mN/m, for the fermentation time being 4 days, temperature being 35°C, pH 8.99, with 4 g substrate and 3.99 ml solution. This experiment thus provided the basis for further research of

large-scale fermentation for the development of biosurfactants that reduced surface tension using this isolated strain, Bacillus oceanisediminis H2.

Biosurfactant was generated by Bacillus oceanisediminis H2 in the biosurfactant production media with glycerol and yeast extract as the only carbon and nitrogen sources. The results of the FTIR and LC-MS tests showed that the biosurfactant generated was lipopeptide in origin and particularly verified that it was surfactin with a molecular weight of 990 Da, respectively. For the first time, researchers have discovered an effective biosurfactant made from Bacillus oceanisediminis H2, a bacterium previously found in a tannery waste dump.

When Bacillus oceanisediminis H2's biosurfactant was added to tannery effluent wastewater, the BOD and COD levels were considerably decreased. To test the purification process, wastewater samples were taken from a treatment facility in Calcutta Leather Complex, Bantala in Kolkata, India, and treated with a pure biosurfactant. The BOD was lowered by 71.65 percent in untreated samples and 64.57 percent in treated samples, while the COD was reduced by 60.65 percent in untreated samples and 62.18 percent in treated samples. Bacillus oceanisediminis H2 has been shown to generate a biosurfactant that is an effective bioremediator.

Conclusion and future prospects

PC1 was screened which can lower the surface tension to 33.8 mN/m. It is gram positive, endospore forming, motile, has catalase activity, oxidase activity but cannot coagulase, produce urease or reduce nitrate. The PC1 strain was identified as *Bacillus oceanisediminis* H2. The fermented media was seen to lower maximum surface tension at 5 days, pH 8, 37°C with 24hr old culture at a minimum of 30.573 mN/m, the RSM shows 34.61 mN/m at 40°C and pH 9.0 to be the optimum. RSM for nutritional parameters showed surface tension lowering with Glycerol (29.957 mN/m) and Yeast extract (30.23 mN/m). Surface tension was recorded to be 36.428 mn /m, for the fermentation time being 4 days, temperature being 35°C, pH 8.99, with 4 g substrate and 3.99 ml solution as optimized by RSM in SSF with PP as substrate. *Bacillus oceanisediminis* H2 produced 0.37 g/L of biosurfactant. FTIR and LC-MS analysis pointed out that the biosurfactant produced was of lipopeptide nature and specifically confirmed that it was surfactin with a molecular weight of 990 Da, respectively. The BOD was reduced by a percentage of 71.65 & 64.57 for before treatment and before treatment with biosurfactant added respectively; and the COD was reduced by a percentage of 60.65 & 62.18 for after treatment and after treatment with biosurfactant added respectively.

Use of immobilized organism, use of Fe-NPs, solid-state fermentation, directed fermentation, foam fractionation, and fill and draw mode of operation could prove to be other promising processes for the enhanced industrial production of various biosurfactants. Use of unprocessed, fortified waste substrates and biosurfactant coproduction with another industrially economical product needs to be more critically studied especially in large fermentation vessels. These strategies could prove to be most economical in terms of lowering the production cost for biosurfactants.

Publications and Conferences

Publications

1) Banerjee, Soumik, and Uma Ghosh. "Production, purification and characterization of biosurfactant isolated from Bacillus oceanisediminis H2." *Materials Today: Proceedings* (2021).

2) Banerjee, S., & Ghosh, U. ISOLATION AND IDENTIFICATION OF A STRAIN FOR THE PRODUCTION OF BIOSURFACTANT. Journal of the Maharaja Sayajirao University of Baroda, 54 [No.2 (XIV)], pp 165-171, 2020.

3) Soumik Banerjee, Sarmistha Basak, Uma Ghosh. Optimization of Bio-surfactant Production by Solid State Fermentation using Response Surface Methodology. Research & Reviews: Journal of Food Science and Technology. 2019; 8(2): 21–32p.

4) Banerjee, Soumik, and Ghosh, Uma, "Optimisation of the production of Biosurfactant by an isolated strain", IJAR&D, Special Issue, ISSN :2395-1737, pp. 1-8, 2018.

5) Soumik Banerjee and Uma Ghosh, "Potent bioremediators of environmental Polycyclic Aromatic Hydrocarbons: A comparative study", Renewable Energy Sources & Environment Protection (An International Edition), International Research Publication House, ISBN: 978-93-87388-19-2, 2018.

Conferences

1) Banerjee, Soumik, and Ghosh, Uma, (2018) Oral Presentation on "Optimisation of the production of Biosurfactant by an isolated strain" in the National Seminar on "Sustainable Agriculture for Food Security and better Environment", organized by BHU, Varansi.
2) Soumik Banerjee, Sarmistha Basak, Uma Ghosh. (2018) Oral presentation on "Optimization of Bio-surfactant Production by Solid State Fermentation using Response Surface Methodology." In the International conference on Renewable Energy Potential for sustatinable initiatives (REPSI-2018) Organized by Bharati Vidyapeeth's College of Engineering, New Delhi.

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