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Bioremediation of oil contaminated soil using biosurfactant produced by *Pseudomonas aeruginosa*

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ABSTRACT:

Biosurfactants are surfactants that are biologically produced from bacteria, yeasts and fungi. Examples include *Pseudomonas aeruginosa* which produce biosurfactants from various substrates including sugars, oil and wastes. Biosurfactants have the property of reducing surface and interfacial tension. These are biodegradable and nontoxic. In the present study saw dust and rice husk were used as substrates for biosurfactant production. *Pseudomonas aeruginosa* was isolated from contaminated soil sample collected from diesel contaminated site for the production of Biosurfactant. The fermentative production of biosurfactant from *Pseudomonas aeruginosa* was carried out by solid state fermentation (SSF) using two low cost substrates (rice husk and saw dust). These substrates were inoculated with *P. aeruginosa* and surface tension was measured on 1st, 3rd, 5th, 7th and 9th day. The highest reduction of surface tension (22.9 mN/m) was obtained using saw dust on 7th day. Produced biosurfactant have been tested for remediation of oil contaminated soil. The contaminated soil was prepared in the laboratory by mixing oil, sand and seed (diesel contaminated soil) in the ratio of 10:2:1. Contaminated soil was further transferred to containers and was allowed for acclimatization. Three containers were used in this study 1) Containing contaminated soil without biosurfactant (C1). 2) Container containing contaminated soil with 4 g of biosurfactant per kg of soil (C2). 3) Containing contaminated soil with 8 g biosurfactant per kg of soil (C3). The oil remaining in soil was determined by solvent extraction method using n-hexane. Oil removal was high in the C2 container which contained 4 g of biosurfactant per kg of soil.

Keywords:
Biosurfactant, Solid state fermentation, Bioremediation, *Pseudomonas aeruginosa,* Surface tension.

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INTRODUCTION

Biosurfactants are diverse group of surface-active chemical compounds that are produced by a wide variety of microorganisms. Some biosurfactants are known to have therapeutic applications as antibiotics, antifungal or antiviral compounds. Biosurfactants can also be used in the bioremediation of soil (Youssef *et al.,* 2004).

Biosurfactants can be synthesized by many different microorganisms and are grouped into six major classes based on the producing microorganism. These classes are glycolipids, phospholipids, polysaccharide–lipid complexes, lipoproteins–lipopetides, hydroxylated and cross-linked fatty acids, and the complete cell surface (Urum & Pekdemir., 2004). The two major classes of biosurfactants include lipopeptides and glycolipids, lipopeptides being synthesized by many bacilli and other species, and the latter being synthesized by *Pseudomonas species* (Youssef *et al.,* 2004). These molecules have attracted considerable scientific attention due to lower toxicity, higher biodegradability, activity at extreme temperatures, pH and salinity and possibility of their production through fermentation using low cost agro-based substrates. Carbon substrate is an important limiting factor affecting the production of microbial surfactants. The type of carbon substrate used for production has been reported to influence both the quality and quantity of biosurfactants (Das *et al.,* 2009).

The most widely distributed environmental pollution can be attributed to oil contamination, caused by tanker accidents, storage tank ruptures, pipeline leaks and transport accidents (Margesin, 2000). This contamination causes significant environmental impacts and presents substantial hazards to human health (Lu *et al.,* 2009). Bioremediation involves the acceleration of natural biodegradation processes in contaminated environments (Calvo *et al.,* 2009). Biosurfactants have been recommended and classified for environmental applications because they are cost-effective and readily biodegradable with low or no environmental toxicity and are more effective than the synthetic surfactants (Urum *et al*., 2003). Bioremediation of petroleum hydrocarbon contaminated soils has been recognized as an efficient, economic, versatile, and environmentally sound treatment (Liu *et al.,* 2008).

Pseudomonas aeruginosa is a typical strain for rhamnolipid production and can utilize vegetable oil or glycerol as the sole carbon source (Zhang *et al*., 2005). A group of biosurfactants that has been studied extensively is the rhamnolipids from *Pseudomonas aeruginosa* (Mulligan, 2000).

The success of bioremediation is dependent upon the microbial ability to degrade these complex mixtures and their rate limiting kinetics. Higher rates of degradation of hydrocarbons are often achieved with a bacterial enrichment consortium isolated from the environment that needs bio restoration. Bacterial consortia display a wide array of metabolic mechanisms in the breakdown of diesel oil components, including production of surface-active agents and emulsifiers (Bento *et al.,* 2005). The combined behaviour of two immiscible liquids such as oil and water that result in the formation of the emulsions is important in the application of surfactants in oil-contaminated soil treatment (Urum & Pekdemir., 2004).

In the present study, diesel contaminated soil was collected and *Pseudomonas aeruginosa* was isolated for the production of biosurfactant. The produced biosurfactant was used for the bioremediation of contaminated soil.

MATERIALS AND METHODS

Microorganism, media composition

Diesel contaminated soil was used for the isolation of Biosurfactant producing microorganism. Serial dilution followed by streaking technique was carried out for the isolation of the organism. Cetrimide agar was used for culture maintenance and preparation of the inocula.

Gram staining and Biochemical characterization of *Pseudomonas aeruginosa*

The isolated strain was characterized by conventional methods like gram staining and biochemical tests such as gelatin hydrolysis, citrate utilization, nitrate reduction, indole, starch, MRVP and catalase test.

Inoculum and Substrates

Petri plates were inoculated with 1ml of inoculum and incubated at controlled temperature for the production of biosurfactant. Rice husk and saw dust were collected from rice mill and carpenter shop respectively and were used as substrates for the production of biosurfactant. Substrates were sterilized in autoclavable petri plates prior to use. Minimum water content was added to maintain the moisture content required for the growth of *Pseudomonas aeruginosa*.

Biosurfactant production from saw dust and rice husk

Five petri plates numbered 1 to 5 were prepared for each substrate and inoculated with *P. aeruginosa*. Plates with samples were analysed on every alternative days viz., 1st, 3rd, 5th, 7th and 9th day of fermentation. Fermented substrates were transferred to conical flask and 100 ml of water was added to it. Flasks were kept in rotary shaker for 2 hrs. Substrates were filtered using filter cloth, filtrate was centrifuged at 4000 rpm for 15 min and supernatant (crude biosurfactant) was collected.

Measurement of surface tension

Stalagmometer was used to measure surface tension of samples. Surface tension (S.T) was calculated using the formula (Turkovskaya *et al*., 2001 and Veenanadig *et al.,* 2000).

$$\text{S.T. of sample} = \frac{(\text{density of sample}) * (\text{drops of water}) * (\text{S.T. of water})}{(\text{Density of water}) * (\text{drop of sample})}$$

Where surface tension of water =72 mN/m

Density of water = 1g/ml

Preparation of contaminated soil

The soil (sand) was collected and was sieved using sieve shaker machine. Soil particle size of 4.75 µm was used for this study. Three containers were used for contamination of soil. A fixed mass of 4 kg sand was measured and placed in each container. Further, soil was contaminated with oil (used two wheeler oil) and seed (diesel contaminated soil) in the ratio of 10:2:1 at room temperature and it was allowed to contaminate for 10-12 days.

Addition of Biosurfactant

After contamination of soil, 4 g and 8 g of biosurfactant per kg of soil were added to containers C2 & C3 respectively and C1 was kept as a blank (no biosurfactant was added).

Determination of Total Petroleum Hydrocarbon (TPH) using n-hexane extraction

The soil samples present in the containers were analyzed using TPH method by n-hexane extraction to determine the oil content remaining in the soil samples. Four grams of soil sample was taken and mixed with 20 ml of distilled water and stirred for 5 mins. The sample was acidified using HCl and 10 ml of n-hexane was added and mixed for 10 mins. The sample was transferred to separating funnel and was allowed to stand for 10 mins. The topmost layer contained n-hexane and oil. Bottom layer containing water was drained; the top layer was transferred to evaporating dish and was placed in hot air oven for one hour to evaporate the n-hexane. The TPH was measured using the formulae:

TPH in the contaminated soil% = (W2-W1)*100/ Wt of soil in g

Where, W1 = weight of the empty dish.

W2 = weight of the dish with separated oil after dried in oven at 103ºC for 2 hours.

RESULTS AND DISCUSSION

Gram staining and Biochemical characterization

The results of Gram staining and biochemical

tests are reported in Table 1. The growth of microorganism on specific media, results of gram staining and biochemical tests indicates that the organism is *Pseudomonas aeruginosa.*

Production of Biosurfactant

In order to economize the biosurfactant production, the low cost carbon sources, saw dust and rice husk were used. Biosurfactant was produced using *Pseudomonas aeruginosa* with rice husk and saw dust as substrates. Microbial molecules which exhibit high surface activity and emulsifying activity are classified as biosurfactants. These molecules reduce surface and interfacial tensions in both aqueous solutions and hydrocarbon mixtures making them potential agents for bioremediation (Bento *et al.,* 2005). Biosurfactant activity can be measured by changes in surface and interfacial tensions and emulsification/emulsion stabilization. In this work, a reduction in surface tension to 22.9 mN/m was achieved with monoculture isolates, a defined consortium and saw dust as substrate on 7th day of SSF.

Solid state fermentation

Solid state fermentation was carried out using two substrates viz., rice husk and saw dust. The reduction in surface tension is as shown in Figure 1.

There were significant reduction in the surface tension using saw dust as it can be used as a source for producing biosurfactant due to the significant reduction in surface tension.

George and Jayachandran (2008) reported the production of biosurfactant by *P. aeruginosa* MTCC 2297 using orange peel as the carbon source reduced the surface tension of culture broth and the final surface tension reached from a value of 57 mN/m to a level up to 31.3 mN/m.

Table 1: Results of Gram staining and biochemical tests.

TEST	RESULT
Gram's staining	Gram Negative
Catalase test	Positive
Citrate utilization	Positive
Indole production	Negative
Starch hydrolysis	Negative
Methyl red	Negative
Voges Proskauer	Negative
Gelatin hydrolysis	Positive

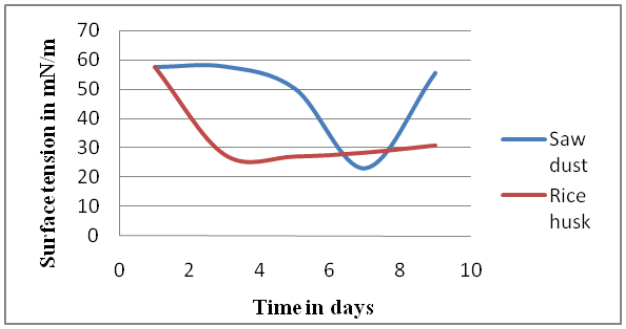

Figure 1: Surface tension of biosurfactant produced from *P. aeruginosa*.

Surface tension decreased rapidly from 72 to 30 mN/m with increase in the Rhamnolipid concentration up to 40 mg/L (Whang *et al.,* 2008).

Dubey and Juwarkar (2001) used glucose, distillery and whey wastes for the production of biosurfactant using *P. aeruginosa* and obtained a reduction in surface tension of 27 mN/m by using the above mentioned substrates.

Bioremediation of Oil Contaminated Soil

Oil contaminated soil was bioremediated in the presence of biosurfactant. Oil content in the contaminated soil was tested using TPH (Total Petroleum hydrocarbon) method using n-hexane extraction. Percentage of TPH in the contaminated soil was measured and is as shown in Figure 2. C1 contained no biosurfactant, C2 and C3 contained 4 g and 8 g of biosurfactant per kg of contaminated soil respectively. TPH in the soil was measured every day; TPH on the first day was 6% in all the containers. The oil percentage in C1, C2 and C3 containers slowly reduced and reached a steady state on 8th day. It was observed that the oil percentages were 2.75, 1.3 and 2 in C1, C2 and C3 respectively. The results indicated that 4 g of biosurfactant per kg of contaminated soil was optimum. The TPH analysis was stopped when there was no considerable difference in two consecutive readings.

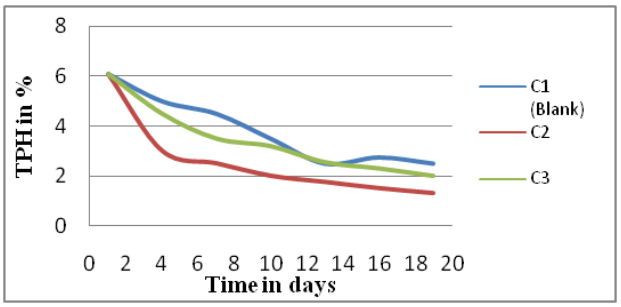

Figure 2: Reduction in Percentage of TPH in C1, C2 and C3 during bioremediation. Scheibenbogen *et al.*, (1994) found that the rhamnolipids from *P. aeruginosa* UG2 were able to effectively remove hydrocarbon mixtures from a sandy loam soil and that the degree of removal was dependent on the type of hydrocarbon removed and the concentration of surfactant used.

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CONCLUSIONS

The following conclusions could be drawn from this study

- *Pseudomonas aeruginosa* was isolated from diesel contaminated soil. Biosurfactant was produced by *Pseudomonas aeruginosa* using rice husk and saw dust as substrates.
- Saw dust proved to be an efficient substrate for the production of biosurfactant when compared with rice husk.
- Biosurfactant of surface tension 22.9 mN/m was produced using saw dust on 7th day.
- Biosurfactant produced using saw dust was used to remediate oil contaminated soil.
- Concentration of 4 g of biosurfactant per kg of contaminated soil proved to be efficient in remediating the soil.

REFERENCES

Bento FM, Camargo FA, Okeke BC, Frankenberger WT. 2005. Diversity of biosurfactant producing microorganisms isolated from soils contaminated with diesel oil. *Microbiological Research*. 160:249-255.

Calvo C, Manzanera M, Silva-Castro GA, Uad I, Gonzalez-Lopez J. 2009. Application of bioemulsifiers in soil oil bioremediation processes, Future prospects. *Science of the total Environment*. 407:3634-3640.

Das P, Mukherjee S, Sen R. 2009. Substrate dependent production of extracellular biosurfactant by a marine bacterium. *Bioresource Technology.* 100:1015-1019.

Dubey K and Juwarkar A. 2001. Distillery and curd whey wastes as viable alternative sources for biosurfactant production. *World Journal of Microbiology & Biotechnology.* 17:61-69.

George S and Jayachandran K. 2008. Analysis of Rhamnolipid Biosurfactants Produced through Submerged Fermentation Using Orange Fruit Peelings as Sole Carbon Source. *Applied Biochemistry and Biotechnology*. 158(3):694-705.

Ligia Rodrigues, Ana Moldes, Jose Teixeira, Rosario Oliveira. 2006. Kinetic study of fermentative biosurfactant production by Lactobacillus strains. *Biochemical Engineering Journal*. 28:109-116.

Liu PWG, Whang LM, Yang MC, Cheng SS. 2008. Biodegradation of diesel-contaminated soil: A soil column study. *Journal of the Chinese Institute of Chemical Engineers.* 39:419-428.

Lu M, Zhang Z, Sun S, Wang Q, Zhong W. 2009. Enhanced degradation of bioremediation residues in petroleum-contaminated soil using a two-liquid-phase bioslurry reactor. *Chemosphere*.77:161-168.

Margesin R. 2000. Potential of cold-adapted microorganisms for bioremediation of oil-polluted

Alpine soils. *International Biodeterioration & Biodegradation*. 46:3-10.

Mulligan CN. 2000. Environmental applications for biosurfactants. *Environmental Pollution.* 133:183-198.

Scheibenbogen K, Zytner RG, Lee H, Trevors JT. 1994. Enhanced removal of selected hydrocarbons from soil by *Pseudomonas aeruginosa* UG2 biosurfactants and some chemical surfactants. *Journal of Chemical Technology & Biotechnology*. 59:53-59.

Turkovskaya OV, Dmitrieva TV and Muratova A, Yu A. 2001. Biosurfactant-Producing *Pseudomonas aeruginosa* Strain. *Applied Biochemistry and Microbiolog.,* 37 (1):71-75.

Urum and Pekdemir T. 2004. Evaluation of biosurfactants for crude oil contaminated soil washing. *Chemosphere*. 57:1139-1150.

Urum K, Pekdemir T and Gopur M. 2003. Optimum conditions for washing of crude oil-contaminated soil with biosurfactant solutions. *Institution of Chemical Engineers Trans IChemE*., 81, Part B.

Veenanadig NK, Gowthaman MK, Karanth NGK. 2000. Scale up studies for the production of biosurfactant in packed column bioreactor. *Bioprocess Engineering*. 22:95-99.

Whang LM, Liu PWG, Maa CC, Cheng SS. 2008. Application of biosurfactants, rhamnolipid, and surfactin, for enhanced biodegradation of diesel-contaminated water and soil. *Journal of Hazardous Materials*. 151:155 -163.

Youssef NH, Duncana KE, Naglea DP, Savagea KN, Knapp RM, McInerneya MJ. 2004. Comparison of methods to detect biosurfactant production by diverse microorganisms. *Journal of Microbiological Methods*.56: 339-347.

Zhang G, Wu Y, Qian X, Meng Q. 2005. Biodegradation of crude oil by *Pseudomonas aeruginosa* in the presence of rhamnolipids. *Journal of Zhejiang University Science*.6 B (8):725-730.