BIOSURFACTANT PRODUCTION BY *PSEUDOMONAS* SP. AND *BACILLUS* SP. USING PEANUT SHELL AS A RENEWABLE SUBSTRATE AND ITS APPLICATIONS

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Abstract: Biosurfactants are amphiphilic compounds produced by various bacteria and fungi which reduce surface and interfacial tension. Their low toxicity, environmental friendly nature and the wide range of potential industrial applications in bioremediation, health care, oil and food processing industries make them a very sought after group of compounds. However, their large scale production and applications are currently restricted due to high–cost production. The objective of the present study is the production of biosurfactants from wastes as a cost effective renewable alternative substrate. *Pseudomonas* sp. and *Bacillus* sp. were isolated from petrol contaminated area and were screened for biosurfactant production using primary screening techniques. *Pseudomonas* sp. and *Bacillus* sp. could reduce surface tension from 29.91mN/m to 24.40mN/m and 34.32mN/m to 30.87mN/m respectively. Biosurfactant was produced using Peanut shell as a renewable substrate. Characterization of the biosurfactant was done by TLC method. The optimization conditions like pH, temperature, substrate concentration and volume of inoculum were found to be 7.5, 37°C, 2% and 10ml respectively. These conditions gave biosurfactant production of 1.835g/L by *Pseudomonas* sp. and 0.816g/L by *Bacillus* sp. The extracted biosurfactants were used in preparation of detergent, in the removal of heavy metals and bioremediation of petrol contaminated soil.

Keywords: Biosurfactants, *Pseudomonas* sp., *Bacillus* sp., peanut shell, detergent,heavy metal, bioremediation.

Introduction: Naturally occurring surface-active compounds derived from microorganisms are called biosurfactants. Biosurfactants are amphiphilic biological compounds produced extracellularly or as part of the cell membrane by a variety of yeast, bacteria and filamentous fungi. The ability to reduce surface tension is a major characteristic of surfactant [1]. Due to their amphiphilic structure, biosurfactants increase the surface area of hydrophobic water-insoluble substances, increase the water bioavailability of such substances and change the properties of the bacterial cell surface. Surface activity makes surfactants excellent emulsifiers, foaming and dispersing agents [2].

Biosurfactants can be synthesized by many different microorganisms and are grouped into six major classes based on their composition. These classes are glycolipids, phospholipids, polysaccharide–lipid complexes, lipoproteins–lipopetides, hydroxylated and cross-linked fatty acids, and the complete cell surface. 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. These molecules have attracted considerable scientific attention due to lower toxicity, higher biodegradability, activity at extreme temperatures, pH and salinity and

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 [3].

The enormous market demands for surfactants are currently met by numerous synthetic, mainly, petroleum-based chemical surfactants. These compounds are usually toxic to the environment and are non-degradable. Tightening environmental regulations and increasing awareness for the need to protect the ecosystem have effectively resulted in an increasing interest in biosurfactants as possible alternative to chemical surfactants. They have advantages over their chemical rivals in bioavailability, biodegradability, activity under extreme condition, lower toxicity, ecological acceptability, structural diversity, productivity on cheap and renewable substrates, capacity for modification and mass production through biotechnology and genetic engineering [4].

Interest in microbial surfactants has been progressively escalating in recent years. These molecules have a potential to be used in a variety of industries like cosmetics, pharmaceuticals, humectants, food preservatives and detergents [5]. Most widely distributed environmental pollution is the oil contamination caused by tanker accidents, storage tank ruptures, pipeline leaks and transport accidents. This contamination causes significant environmental impacts and presents substantial hazards to human health. Bioremediation involves the acceleration of natural biodegradation processes in contaminated environments. Biosurfactants have been recommended for environmental applications because they are cost-effective and readily biodegradable with low or no environmental toxicity. Bioremediation of petroleum hydrocarbon contaminated soils and removal of heavy metals has been recognized as an efficient, economic, versatile, and environmentally sound treatment [6].

Presently the production of biosurfactants is highly expensive due to the use of synthetic culture media. Therefore, greater emphasis is being laid on procurement of various cheap agro-industrial substrates including vegetable oils, distillery and dairy wastes, soya molasses, animal fat, waste, and starchy waste as raw materials. These wastes can be used as substrates for large-scale production of biosurfactants with advanced technology [5].

The current study is based on biosurfactant production from bacteria isolated from petroleum contaminated soil and their effects in soil bioremediation.

Materials and methods:

Sampling and Screening:

The sample collected was petrol contaminated soil near a petrol pump located in Vasai (w). The soil sample was collected in a sterile polythene bag. The soil sample was weighed to 10g and added to 100ml of st. nutrient broth (NB) and was incubated at 37°C for 1 week on shaker. One ml of the above solution was then transferred into NB. Isolation was performed on st. nutrient agar plates. The plates were incubated at 37°C for 24-48 hrs. Isolates obtained were streaked onto specific slants for maintenance and further characterization.

The bacterial isolates were tested for their biosurfactant producing properties. The supernatant was subsequently subjected to the preliminary screening methods using oil spreading technique, drop collapse technique and hemolytic activity [7].

The screened biosurfactant producing organisms were then identified by morphological and biochemical characterization.

The peanut shells were washed, dried and

grinded into a fine powder and were used for further determination. The cultures were inoculated in 250ml flasks containing 100 ml of Mineral Salt Medium with peanut shell as a substrate. The flasks were incubated at 37°C for 7 days with shaking conditions. After incubation, the bacterial cells were removed by centrifugation at 5000 rpm for 20 minutes. The supernatant was taken and the pH of the supernatant was adjusted to 2 using 1N HCl. Equal volume of Chloroform: Methanol (2:1) was added to the solution. The mixture was shaken well for mixing and left overnight for evaporation. White colored sediment obtained was the desired biosurfactant [8].

The process parameters were optimized to obtain higher productivity of the biosurfactant. Optimization was carried out with glucose and peanut shell as substrates. The observed variables were inoculum volume, hydrocarbon (C-source) percentage, temperature and pH. The ranges of various parameters were studied. Optical density (O.D) was measured at 620nm with variation of the parameter under study, keeping other parameters constant at a time [9].

Characterization of the biosurfactant was done by Thin Layer Chromatography. The biosurfactant was separated on the silica gel plate using chloroform: methanol: water (70:10:0.5). Ninhydrin reagent was sprayed to detect lipopeptide biosurfactant as red spots. Anthrone reagent was sprayed to detect glycolipid biosurfactant as yellow spots [1].

The dry weight technique was used to quantify microbial growth [1]. The dry weight of the biosurfactants was calculated by the following formula:

Dry weight of biosurfactant = (weight of the plate after drying – weight of the empty plate).

Surface tension was calculated by measuring the height reached by the liquid when freely ascended trough a capillary tube. The surface tension was calculated according to the following formula:

$$\gamma = \frac{rh\delta g}{2}$$

γ= Surface tension (mN/m); δ= Density (g/mL); g = gravity (980 cm/s^2); r = capillary radius (0.05 cm); h = height of the liquid column (cm) [10].

2 ml of broth supernatant and 3 ml of a selected hydrocarbon (petrol, kerosene, engine and coconut oil) were mixed in a test tube and vortexed for 2 min. The height of emulsion layer was measured after 24 h to determine the emulsification index. The equation used to determine the emulsification index (E_{24}) is as follows:

E_{24} = height of emulsion layer × 100%
height of total solution

The protein and carbohydrate content of the biosurfactant extracted from *Pseudomonas* sp. and *Bacillus* sp. was estimated by Folin-lowry and Anthrone method respectively.

Detergent was prepared using the extracted biosurfactant. The following ingredients were used for the preparation of the detergent:

Sodium Carbonate: 50%, Sodium Percarbonate: 17%, NaCl: 8%, Biosurfactant: 15%, Perfume: Cologne

Sodium carbonate was dissolved in hydrogen peroxide and kept in hot air oven at 100°C for recrystallization. NaCl and biosurfactant were added to the mixture of sodium carbonate and sodium percarbonate. Perfume was added for fragrance. The foaming activity of the detergent prepared was checked by the stability of the foam formed for 2 hrs [10].

The emulsification activity of the prepared detergent was compared with commercially available detergents and SDS. 3 ml of the detergents and 2 ml of oil and petrol were mixed in a test tube and vortexed for 2 min. The height of emulsion layer was measured after 24 h to determine the emulsification index. The

emulsification activity of the detergent containing biosurfactant, commercially available detergents and SDS was compared [11].

Nutrient broth tubes containing Tween 80 and detergent of concentrations 0.1% - 1% were inoculated with test organisms *E. coli, S. aureus and B. subtilis*. Nutrient broth tubes with culture and without Tween 80 and detergent were kept as control. The tubes were incubated at 37°C for 24 hrs. After incubation, the tubes were observed for growth compared with control tubes.

Bioremediation of petrol contaminated soil: The soil (sand) was collected and was sieved using sieve. Two containers were filled with 10 grams of soil each. Further, petrol was added in each of the container in the ratio of 10:2 at room temperature and allowed to incubate for 10-12 days. After contamination of soil with petrol, 0.5 g of biosurfactant was added to one container and other was kept as a control (no biosurfactant was added). The soil samples present in the containers were analyzed using TPH method by dichloromethane extraction to determine the petrol 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 dichloromethane 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 dichloromethane and petrol. 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 dichloromethane [12].

The Total Petrol Hydrocarbon (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 petrol after dried in oven at 103ºC for 2 hours.

Results & discussion:

Biosurfactants are amphiphilic molecules with great diversity, environmental acceptability and broad spectrum of functions and industrial applications which make them interesting bio-products. Soil is a known habitat and source of versatile microorganisms and since the microorganisms capable of emulsifying and solubilizing hydrophobic agents have an apparent advantage over their competitors, sampling of this nature provides a source rich in microorganisms with desired characteristics [4]. In this study, organisms were isolated from petroleum contaminated soil. Among the five isolates obtained, isolate 2 and isolate 4 were found to produce biosurfactants.

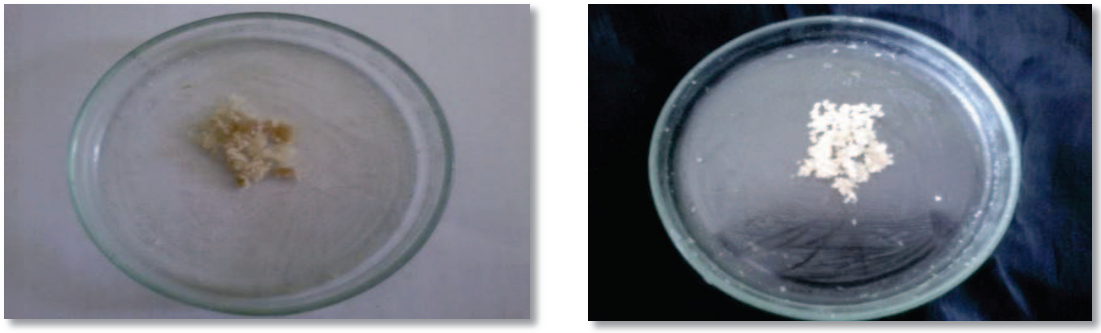
The primary screening of biosurfactant producing bacteria was carried out using haemolytic activity, drop collapse and oil spreading techniques. Selection of these methods was due to their strong advantages including simplicity, low cost, quick implementation and use of relatively common equipment that is accessible in almost every microbiological laboratory. The drop collapse method depends on the principle that a drop of liquid containing a biosurfactant collapses and spreads over the oily surface. There is a direct relationship between the diameter of the sample and concentration of the biosurfactant and in contrast, the drop lacking biosurfactant remains beaded due to the hydrophobicity of the oil surface that cause aggregation of droplets. They were also screened by oil displacement method. The culture supernatant was able to displace oil and spread in water forming a zone of clearance [1].

A qualitative assay to determine biosurfactant producers was also developed based on their

ability to cause hemolysis of red blood cells. Screening of biosurfactant producers via this method was previously outlined that only those isolates which showed hemolysis were considered to be the potential biosurfatant producing microbes. The estimation of this test was based on the fact that surfactants interact strongly with cellular membranes and proteins. Exotoxins called hemolysins cause lysis of the red blood cells. Blood agar lysis was used in this study since it is widely used to screen for biosurfactant production. It correlates with the studies of [13] which used blood haemolysis test for screening biosurfactant producing organisms. In addition the haemolytic assay was a simple, fast and low cost method for the screening of biosurfactant producers on solid medium.

The biosurfactant producing organisms were found to be *Pseudomonas* sp. and *Bacillus* sp. based on morphological, cultural and biochemical characterizations.

Pseudomonas sp. and *Bacillus* sp. inoculated in mineral salt medium with glucose and peanut shell as a substrate produced biosurfactants. Reference [8] used the similar method for the extraction. The biosurfactant was extracted by centrifugation and sedimentation. White collared sediment obtained was the biosurfactant as shown in Fig. 1.



(a) (b)

Fig. 1: White precipitate obtained (biosurfactant) after evaporation and drying (a) Biosurfactant from *Pseudomonas* sp. (b) Biosurfactant from *Bacillus* sp.

The cell growth and accumulation of metabolic products were strongly influenced by growth factors viz., temperature, pH, substrate concentration and volume of inoculums. Optimization was carried out and the optimum conditions were found to be 7.5, 37°C, 2% and 10 ml for pH, temperature, substrate concentration and volume of inoculum respectively as shown in Fig. 2 (a)-(d). Thus optimization process can give high yield of metabolites.

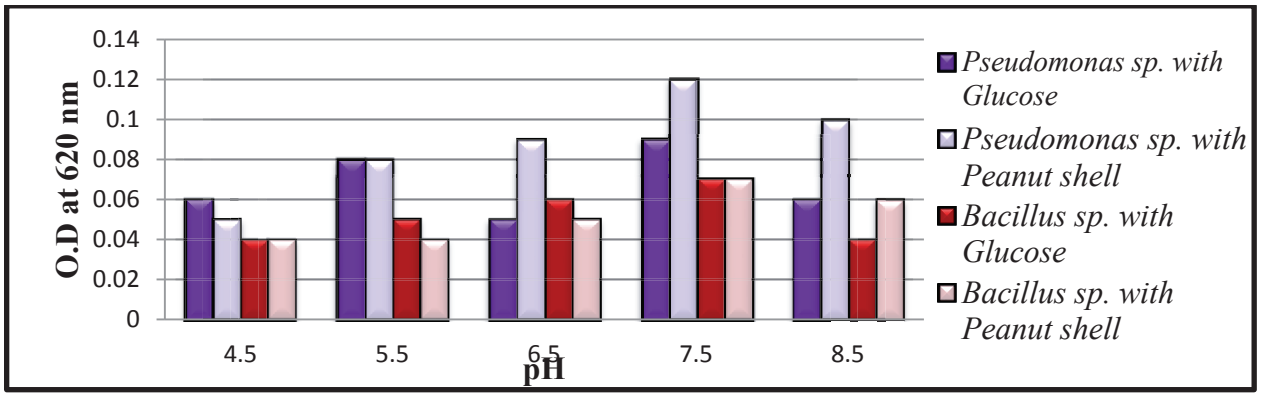


Fig 2 (a)

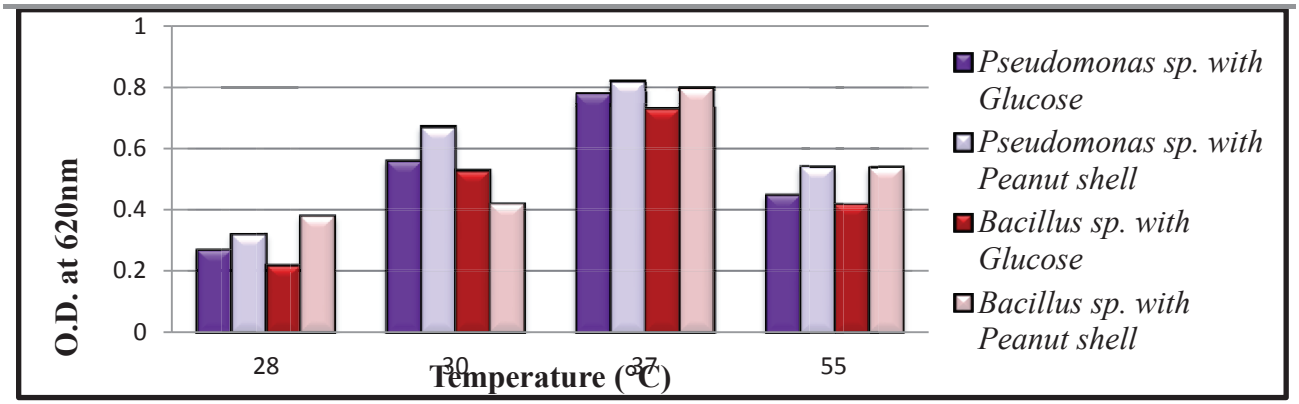


Fig 2 (b)

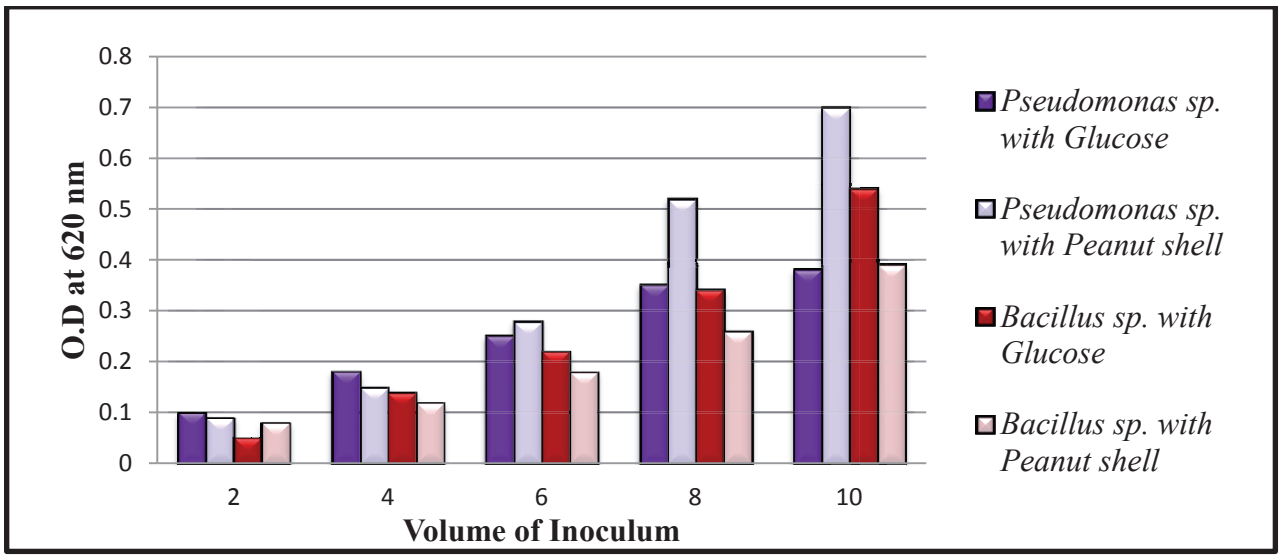


Fig 2 (c)

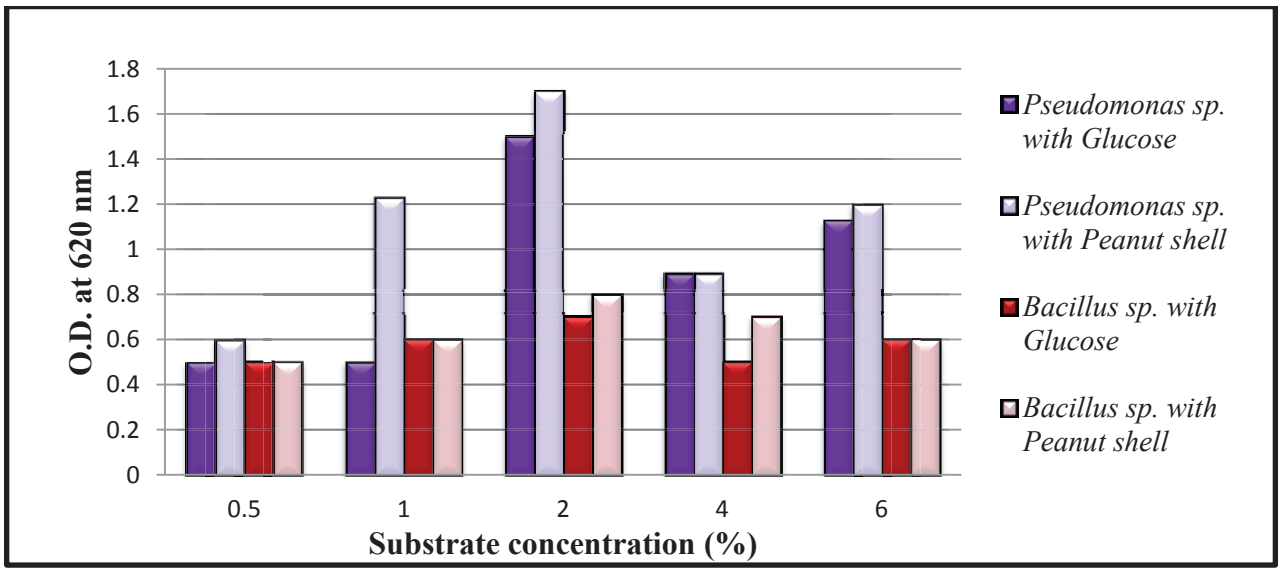


Fig 2 (d)

Fig. 2: Optimization of growth conditions. (a) Optimization of pH (b) Optimization of temperature (c) Optimization of volume of inoculums (d) Optimization of substrate concentration.

The biosurfactant produced were characterized by using TLC. The sediment obtained was placed in the TLC plate and the plates when sprayed with Ninhydrin which is a lipopeptide and yellow color spot confirmed the presence of rhamnolipid**.** Reference [13] also reported rhamnolipid from *Pseudomonas* sp. and surfactin from *Bacillus* sp. by TLC. reagent showed red color spots and with Anthrone reagent showed yellow color spots. Red color spot on the TLC plate confirmed the presence of surfactin The dry weight of the biosurfactants was estimated and it was observed that *Pseudomonas* sp. gave higher yield than *Bacillus* sp using peanut shell as a substrate than that of with glucose as shown in Table I.

Table I: Dry weight of biosurfactants

	Organism	**Dry weight of the biosurfactant (g/L)**			
		Set I	**Set II**	**Set III**	**Mean ± SD**
With glucose as substrate	***Pseudomonas* sp.**	1.161	1.143	1.139	1.471±0.011
	***Bacillus* sp.**	0.495	0.521	0.506	0.507±0.013
With Peanut shell as substrate	***Pseudomonas* sp.**	1.835	1.846	1.792	1.824±0.12
	***Bacillus* sp.**	0.816	0.821	0.793	0.81±0.012

The surface tension was determined by capillary tube method. The organisms were able to reduce the surface tension below 40mN/m which is the standard criterion for biosurfactant producing organisms. Table II shows that the surface tension reduction was found to be greater by *Pseudomonas* sp. than that of *Bacillus* sp. The findings of the current study correspond with those obtained by other researchers [9]. It was also observed that the reduction of surface tension was greater with the use of biosurfactant from peanut shell than of glucose.

Table II: Surface tension Determination

Biosurfactant produced from organism	**Surface Tension (mN/m)**	
	With glucose as substrate	With peanut shell as substrate
Bacillus sp.	33.32	30.87
Pseudomonas sp.	28.91	25.4

The second factor that has been studied in complementary stage was emulsion activity using four different hydrocarbons including petrol, kerosene, engine oil and coconut oil. Reference [14] also tested the efficacy of biosurfactant with four different types of oil. The emulsification activity was found to be greater with engine oil as compared with others as shown in Fig. 3.

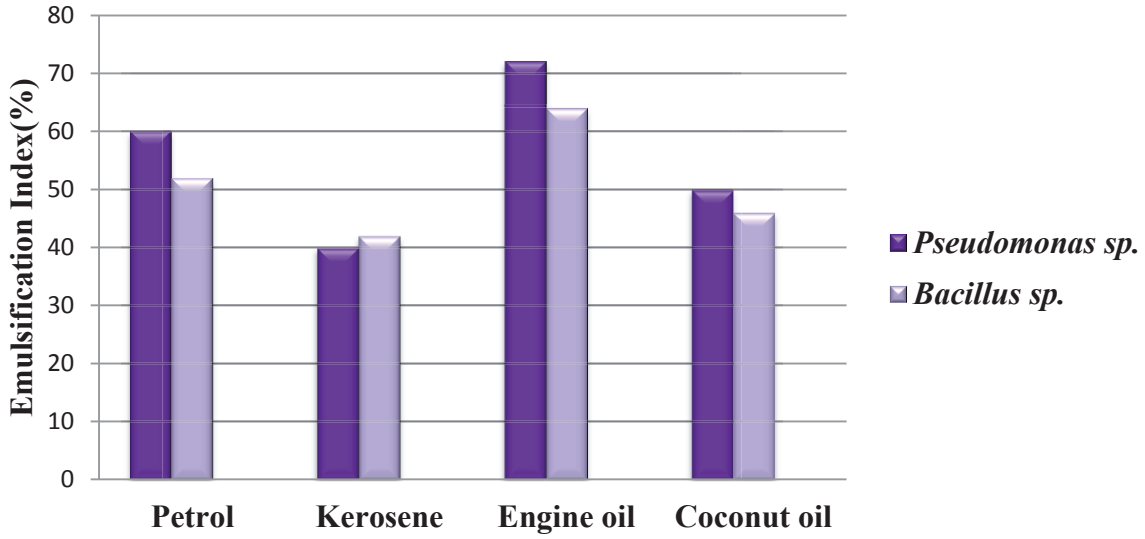


Fig. 3: Emulsification activity with hydrocarbons

The protein content of surfactin produced by *Bacillus* sp. and rhamnolipid produced from *Pseudomonas* sp. was found to be 43 µg/ml and 47 µg/ml respectively. The carbohydrate content in the biosurfactant was estimated by anthrone method. The carbohydrate content of surfactin produced by *Bacillus* sp. and rhamnolipid produced by *Pseudomonas* sp. was found to be 50 µg/ml and 56 µg/ml respectively.

Detergents contain surfactants which lower the surface tension of water and help in removing stains of oil and dirt. Detergent was prepared using the extracted biosurfactants in replacement of the synthetic surfactants. Fig. 5 shows the foaming property of the detergent prepared as shown in Fig. 4.The detergent showed good foaming property with a stability for 2hrs.

Fig. 4: Detergent Powder

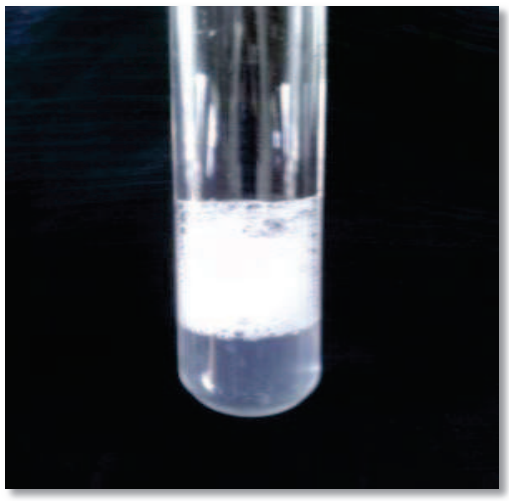


Fig. 5: Foaming stability of Detergent

Detergents are capable of emulsifying or dispersing oils and similar water-insoluble substances. The detergent was checked for its ability to emulsify oil and petrol and compared with commercially available detergents and SDS. The emulsification index was expressed in percentage.

The emulsification index was found to be in

equity with one of the commercially available liquid soap as showed in Fig. 6. Thus, it can be concluded that the biosurfactant can be used in detergent preparation in place of synthetic surfactants from an economic point of view.

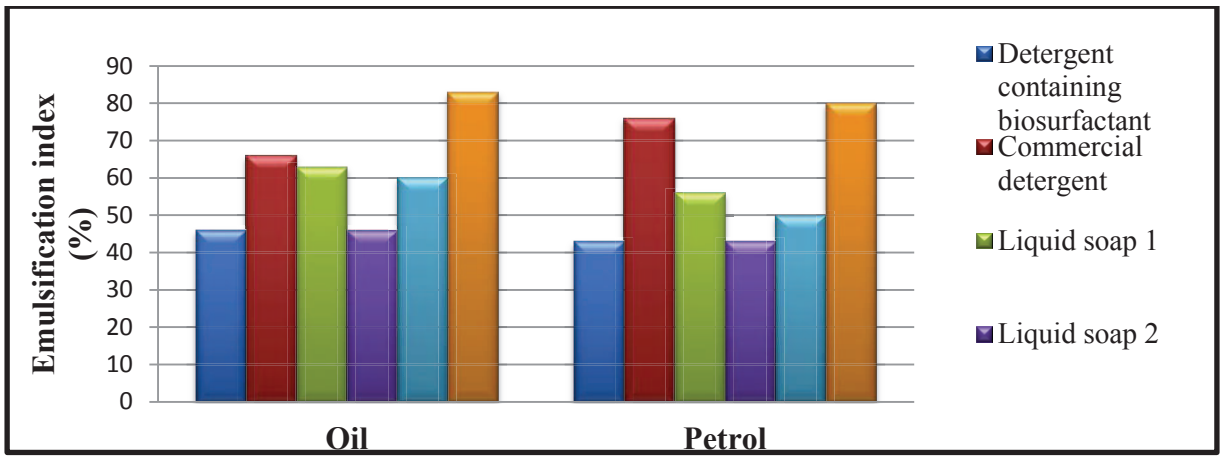


Fig. 6: Emulsification activity of detergent

Biosurfactants disrupt membrane structure through interaction with phospholipids as well as membrane proteins. The antimicrobial effects of biosurfactants can be explained by the structures of biosurfactants resembled to cell membrane. One explanation of the antimicrobial effect of biosurfactants is the adhering property of biosurfactants to cell surfaces caused deterioration in the integrity of cell membrane and also breakdown in the nutrition cyle. The microorganisms were able to grow in medium containing Tween 80 whereas the tubes with the detergent showed no growth. Biosurfactants thus inhibited the growth of bacteria and exhibited antibacterial property which correlates with the studies of [15].The tubes containing Tween 80 showed uniform growth whereas growth was absent in the tubes containing the detergent with biosurfactants as shown in Table III.

Table III: Effect of surface tension on bacterial growth

Organism	Tween 80 (0.1 – 1%)	Detergent (0.1 – 1%)
Escherechia coli	+	–
Staphylococcus aureus	+	–
Bacillus subtilis	+	–

Key: + : Growth – : No Growth

Bioremediation of contaminated soil: Bioremediation involves the acceleration of natural biodegradation processes in contaminated environments. Bioremediation of petrol contaminated soil was done by TPH method. . Percentage of TPH in the contaminated soil was measured as showed in Fig. 7. The soil with biosurfactant showed considerable decrease in the petrol from initial content of 14.9% to 5.2% on 10th day. Reduction in TPH was also observed in soil without biosurfactant to 13% on day 10. This could be due to the oil degrading bacteria present in the soil. The biosurfactant was able to degrade the

hydrocarbon in a period of 12 days. The rhamnolipid degraded petrol and comparison of obtained results with similar studies by [6] revealed that biosurfactant can be used for bioremediation of crude oil pollutant from the environment.

Petrol content in the contaminated soil was tested using TPH (Total Petroleum hydrocarbon) method using dichloromethane

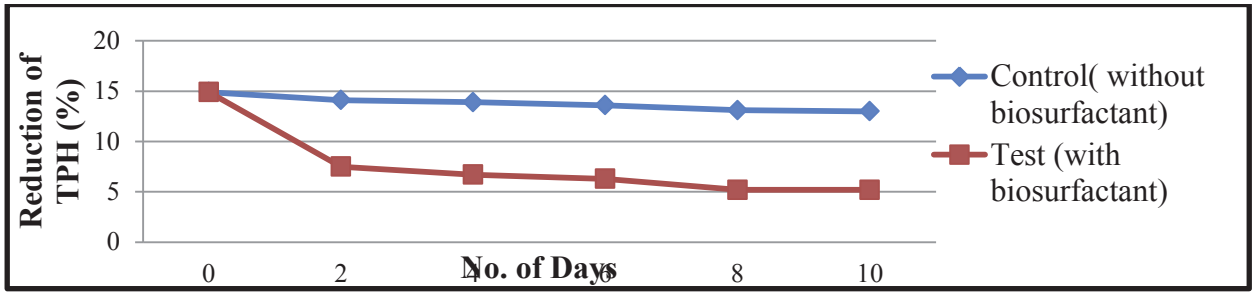


Fig. 7: Reduction in % of TPH in control and test during bioremediation

Conclusion:

The soil sample was sampled successfully from petrol contaminated area. The biosurfactant producing bacteria were isolated, screened and identified as *Pseudomonas sp.* and *Bacillus sp.* on the basis of various physiological, cultural and biochemical characteristics.

The biosurfactant production was carried out with the use of peanut shell as a renewable substrate. The use of peanut shell as a substrate was of beneficial use and also increased the production as compared to the biosurfactant produced with glucose as a substrate. Biosurfactant produced from *Pseudomonas* sp. with peanut shell showed higher reduction in surface tension than of biosurfactant produced from *Bacillus* sp. with glucose as a substrate. The emulsification activity was found to be greater with engine oil than the other oils.

Detergent was prepared successfully using the extracted biosurfactant instead of synthetic surfactant and also showed good emulsifying activities with petrol and oil. The detergent also showed antibacterial properties which can lead to the conclusion that biosurfactant can be used in the inhibition of bacterial growth and could be used in medicine. Bioremediation of petrol contaminated soil with biosurfactant showed significant degration of petrol in the soil.

Thus, biosurfactants can be produced with the use of low cost, waste renewable substrates and can be used instead of synthetic surfactants in various industries.

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