Application of Bio-surfactantProduced by *Pseudomonas aeruginosa* MTCC 16036 for Remediation of Petroleum Sludge

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ABSTRACT

Present work is to investigate the possible application of produced biosurfactants for biodegradation of motor oil contaminated sand in laboratory. *Pseudomonas aeruginosa* was procured from Microbial Type Culture Collection Centre and Gene Bank for current investigation. The biosurfactant were produced using sludge as source of carbon, whichis low-cost substrates. Biosurfactant has the potential to treat and remediate petroleum sludge. Sludge is a solid or semisolid mass that settles down in the course of storage tanks. Oil field sludge consists of hydrocarbons,which includes mixtures along with asphaltenes and waxes. Moreover,sand, clays and water were also found in the sludge. Sludge generated by oil refining industry are hazardous waste and proper management and safe disposal is a major challenge for

refiners which get accumulated over time in storage pit as well as in the drilling site while extracting crude oil. The results obtained shows that biosurfactants has the capability for removal of toxic elements of sludge by biodegradation. Bioremediation process under aerobic condition is proposed to reduce the oil content to permissible limit. The retention time for the degradation process is to be ascertain on trial and error basis.

Keywords:*Pseudomonas aeruginosa*, petroleum sludge, degradation, biosurfactant.

1. INTRODUCTION

The current scenario, more attention has been given towards biosurfactants due to its major advantages, which are not limited to lower toxicity, higher biodegradability, high selectivity, foaming and specific activity at extreme temperatures, pH and salinity, and the ability to be synthesized from agricultural wastes [17]. Biosurfactant are extracellular compound produced by bacteria, which has the capability to reduce interfacial tension[21]. The foremost advantages of biosurfactants in comparison to chemical surfactants is better biodegradability, eco-friendly, good foaming activity, zero toxicity at intense temperatures, pH and salinity [12]. Biosurfactants are classified into two categories such as low-molecular-massand high molecular-mass polymers. Biosurfactantscan be either anionic or nonionic in nature, where the hydrophobic moiety attracted to fatty acids[6].Well known biosurfactants are synthesized by microbes grown on water immiscible hydrocarbons but few biosurfactants are produced on water-soluble substrates namely sucrose, glucose and ethanol [9]. Eventually, high product titers with vegetable oil as sole carbon source in combination with Pseudomonas strains [10]. Crude oil behaves sluggish during measurement of IFT (interfacial tension) against aqueous phases as though they were a homogeneous hydro- carbon with a particular ACN (alkaline carbon number). Currently, research on biosurfactants has increased globally to enhance the present production rate of

microbial surfactants. Potential usage of biosurfactant in oil industries includes cleaning oil sludge, mobilizing heavy crude oil and managing oil spillage. In addition to this, biosurfactants being used in food industries as additives and emulsifiers, which applied in agriculture and cosmetics [8]. Enhanced soil treatment performed using surfactants consist of anionic, nonionic, cationic and mixed surfactants, and great washing capabilities for hydrophobic organic compounds (HOCs) was achieved from contaminated soil [20]. Biosurfactant has the potential to retrieve unrecoverable oil from the trapped zone, which are held by high capillary pressure. This is achieved by reducing the interfacial tension between oil and water and improving the recovery of oil [5]. Application of biosurfactants in microbial enhanced oil recovery depends on their stability at higher temperature and pH conditions [2]. Certain types of bacteria produce low molecular weight molecules that efficiently reduce surface and interfacial tension such as glycolipids and lipopeptides [15]. The negative effects of the synthetic biosurfactant can be overcome by the microbial biosurfactants. Mostly all the microbes are capable of producing surfactants among which Yeast are readily grown and are easy to cultivate in large-scale level [11]. Surfactin and rhamnolipid are the most effective biosurfactants, which are capable of reducing interfacial tension between water/oil [19]. The major disadvantage faced by oil industries is about the recovery of oil very economically. *Pseudomonas aeruginosa*, *Bacillus cereus, Bacillus thuringiensis* are most widely used for biosurfactant production. Due to the spontaneous increasing demand for petroleum over recent years, application of biosurfactant in oil recovery plays a key role. The major problem facing by oil industries is to recover oil to the maximum possible extent using economical methods. In this regard, microbial enhanced oil recovery with the aid of biosurfactants is promising. Hence, extensive identification and characterization of new suitable strain for biosurfactant production and degradation during oil

spills is necessary. In our study, biosurfactant is produced by *Pseudomonas putida* MTCC 2467 and potentially applied to reduce the surface and interfacial tension, which influence the enhancement of oil. The strain produce biosurfactant can be suitably used in oil fields, biomedical and environmental applications. This is the first report describing biosurfactant production using strain *Pseudomonas putida* MTCC 2467.Recent report on biosurfactant was production using *Candida lipolytica* UCP 0988 with cost effective medium formulation along with 2% of waste frying oil, 2% corn steep liquor and 5% molasses at 120 h, 30° C maintained at 180 rpm. Surface and IFT was reduced until 24 mN/m and 11 mN/m respectively [4]. *Bacillus subtilis* and *Pseudomonas aeruginosa* are the well-known bacteria for producing biosurfactant named surfactin and rhamnolipid, whichwere applied in microbial enhanced oil recovery process [1]. MEOR found to be relatively low because of the following factors: (a) understanding the mechanism on in-situ geo-environmental aspects of bacteria (b) stability of key parameters such as pH and water saturation on fundamental processes of MEOR process [18].*Pseudomonas aeruginosa* (ATCC 9027) has the potential of producing biosurfactant which in turn helped in reduction of IFT from 73 mN/m to 33 mN/m at 30° C and pH 8.0 [3]. *Pseudomonas putida* MTCC 2467 produced biosurfactant (2.7 g/L) when glucose was used as carbon source (2% w/v). Further, the stability of the biosurfactant was unaffected at high temperature and pH conditions [5]. The prime most difficulty for bioremediation of oil-contaminated soil is mass transfer of the oil pollutants in the soil that leads to poor nutrient and microbe interaction, which accounts for efficient biodegradation[13]. Method to enhance the bioavailability of the oil contamination is to transport the pollutant to bulk phase of aqueous [7].

2. MATERIALS AND METHODS

2.1. Microbe and Maintenance conditions

Pseudomonas aeruginosa MTCC 16036 was procured from Microbial Type Culture Collection (MTCC), Institute of Microbial Technology, India for the present research work. Culture maintained in nutrient agar plates with the following composition (g/L): beef extract, 1.0; peptone, 5.0; yeast extract, 2.0; NaCl, 5.0; agar, 15.0; pH 6.0 ± 0.2, storage temperature −2° C to −9° C.Storage tank bottom sludge at Chennai Petroleum Corporation Limited was used as substrates for biosurfactant production.The tank bottom sludge along with sand was used for experimental analysis. The sludge was dark brown in colour mixed with soil.

2.2. Conditions for Media cultivation

Nutrient broth was used for media cultivating with the following composition (g/L) was used for preparation of inoculum. Beef extract, 1.0; yeast extract, 2.0; peptone, 5.0; NaCl, 5.0. *Pseudomonas aeruginosa*(MTCC 16063) was grown in Nutrient broth for 8 – 12 h at 30 $^{\circ}$C (A_{600nm}0.7) and 2% (v/v) inoculum was used forbiosurfactant production using mineral salt medium with the following composition (g/L) KNO_3, 0.3; Na_2HPO_4, 0.2; KH_2PO_4, 0.013; NaCl, 0.001; $MgSO_4$, 0.05; $CaCl_2$, 0.003; $FeSO_4$, 0.001.

To above mentioned mineral salt media, 0.1 ml of trace elements are added with following composition (g/L) $MnSO_4.4H_2O$, 1.78; $Na_2MoO_4.2H_2O$, 0.39; $CoCl_2.6H_2O$, 0.42; EDTA, 0.5; $NiCl_2.6H_2O$, 0.004; KI, 0.66; $ZnSO_4.7H_2O$, 2.32; H_3BO_3, 0.56; $CuSO_4.5H_2O$, 1.0.

Hydrocarbon sludge was sole carbon source utilized for biosurfactant production.

2.3. Analytical methods

2.3.1 Sludge analysis

10gm of sludge is pre-weighted and transferred in to a petri plate. The sludge is distilled and closed with cotton along with petroleum cluster (40 – 60^{O}C) until the colour of the top becomes colourless. Transfer the material remarks in the round bottom flask to a pre-weighted beaker. The weight of the material is to be noted. The total oily material present in the wax is determined. Further distillation carried out in the presence of chloroform. The weight of the material is the total weight (%) of the asphaltene content present in the sludge. Total weight (oily matter + asphaltene weight) is the oil content % in the sludge. Now find the weight of the beaker. Dilute in five different concentration so that the residue is left over. The loss in weight will provide the water (%) present in the sludge.

2.3.2Paraffin content analysis

In order to determine the percentage of paraffins in petroleum fractions, the sludge is mixed with equal volume of alcohol along with methyl ethyl ketone. Take about 3 –5 gm of the sample. 0.5–1gm of the sample is taken at toom temperature. Ensure that paraffin content of precipitatenot exceed 100 mg. Prepare a mixture of 330 gm of acetone and solid CO_2. Dissolve the oil in equal volume of alcohol and ether or (Methyl ethyl ketone), the amount of the solvent should be sufficient to keep the fraction in solution at – 15°C. The solution is prepared at the atmospheric temperature followed by cooled to –20°C and placed in the funnel. Kept in the cooling bath. The time required to cool the mixture for complete separation of paraffins is about 45 minutes. It order to get better result the sample should be subjected to agitation. The suspension of paraffin with a rod during the course of cooling. For an oil rich in paraffin, it is recommended to dissolve the oil in ether and then add alcohol. About the end of the cooling, prepare the cooling bath for

filtration. The filter bed is quite cold for about 10 mins filter the paraffin thus separated and aspirate slowly while washing and at the end when traces of solvent are left. Wash the precipitate with minimum amount of diethyl ether mixture at –20^{O}C. Washing at 5 cc of the filtrate on evaporation does not leave any liquid paraffin. This residue is considered verylow and is dissolved in benzeneand filtrated. Repeated washing about 4 – 5 time with 5 – 10 cc of solvent is preferred. Once washing is done take traces of, ether that remains on the filter paper is dried. The filtrate sill contains small quantity of paraffin. Transfer the filtrate in 350 cc beaker. Solvent under fumes chamber is evaporated. Now dissolve the residue in alcohol ether, effect the second precipitation of the paraffin, and filter through second filter paper. The precipitates dissolved in warm benzene. The volume of benzene employed is of the order of 20 cc. Collect the solutions in a 100 cc conical flask. Evaporate the benzene with precaution. If the paraffin that remains after evaporation is hard, heat to 120 ^{O}C for 30 mins. If the paraffin is soft, keep in a desiccator for overnight in vacuum at 50^{O}C.

2.3.3Biosurfactant analysis

Sample subjected to centrifuge at 10000 rpm to removecells present. The supernatant was subjected to acid precipitation test by adding 6 N HCl at 4 °C and pH 2.0. Precipitate formed was further pelletedto centrifugation at 10000 rpm for 25 min and re-suspending with double distilled water and pH was adjusted to 7.0. It is freeze dried and weighed. Dichloromethane used to separate the biosurfactant using rotary evaporator under vacuum. This concentrated liquid obtained was considered pure form of biosurfactant.

2.3.4. Interfacial tension measurement

Interfacial tension (IFT) measurement of the cell free broth along with 10 mL crude oil was mixed and these mixtureswere subjected to IFT analysis digital tensiometer K6 (Kruss GmbH, Germany), by plate method. Sample (10 mL broth + 10 crude oil) placed into the container provided. Analytical results were doneusing automatic controller that pull the plate in downward and contacted sample liquid placed in the glass. The force acting on the rectangular plate with known length were measured and converted into surface tension digitally.

2.3.5. Biosurfactant stability analysis

Identifying the effect on pH and stability of biosurfactant, pH of the biosurfactant solution was adjusted to various pH (1.0 - 12.0) by adding 3 N NaOH also 3 N HCl. Surface tension had been determined to check the stability of biosurfactant. In the similar manner the effect of temperature stability on biosurfactant, the samples were heated at different temperature conditions ranging from 40, 50, 60, 70, 80, 90,100, 110 and 120 °C for 2 hours and analyzed for surface tension measurements subjected before and after the heat treatment.

2.4. STATISTICAL ANALYSIS

All experiments were performed three times and reported values are mean of three individual experiments with $p<0.005$.

3. RESULTS AND DISCUSSION

Bacillus and Pseudomonas sp. considered to be well known bacterial that are potential in producing biosurfactant. These bacteria have the tendency to grow on hydrocarbons that are immiscible with water and other sources where, salt media which are enriched with carbohydrates. The effect of carbon sources such as glucose, sucrose, starch and other few hydrocarbons (Heptadecane, dodecane and Hexadecane) were routinely used for biosurfactant production [8]. Result from conducted experiments clearly shows that increase in cell biomass

was relatively good for the tested carbon source (petroleum hydrocarbon sludge). Presence of 2% hydrocarbon sludge when used as carbon source gave highest biomass (2.6 g/L) as well as biosurfactant (2.8 g/L). The amount of paraffin in 100 gm of the sample but some quantity of paraffin was found to be soluble in the solvent used. Therefore, a correction is applied as follows: 0.2% for the product at 15^{O}C. 0.5% for the product semi-solid at 15^{O}C. 1% for the product solid completely at 15^{O}C. Let 'X' be the increase, for correction, then the amount of paraffin in the oil: 100 P (1+ [X/100]). Bioremediation is the treatment process that uses naturally occurring microorganisms (Yeast, Fungi and Bacteria) to break down or degrade hazardous substances into less toxic or non-toxic substances. Microbes consume and digest organic substances (compounds that contain carbon and hydrogen atoms) for nutrients and energy. To determine potential of biosurfactant in remediation is further confirmed by determining the stability of the surfactant at different pH and temperature. The effect of temperature stability was studied by incubating the biosurfactant at various temperatures between 40 to 120°C for 2 h and measured for surface tension. It has been found that the surface tension of the biosurfactant remained constant between 40 – 120°C suggesting that biosurfactant produced by *P. putida* was highly thermostable.Moreover, the pH of the purified surfactant solution was subjected to various ranging of pH from 1.0 to 12.0, incubated for 1 h and the surface tension was analyzed. Surface tension was reduced withpH 6.0 suggesting that the biosurfactant was not stable below pH 6.0 (acidic conditions) and then the surface tension remained unchanged until pH 12.0. (Figure 4B) which shows clearly that stability of biosurfactant was stable between pH 7.0 to 12.0.Interfacial tension is the critical and crucial parameter in oil recovery techniques. This can only be achieved only due to the production of biosurfactant. Extracted biosurfactant using *Pseudomonas aeruginosa*was able to reduce IFT at a

highest value(10 mN/m). Biosurfactant produced was subjected to different temperature ranges and had no significant effect on reduction of interfacial tension at tested temperature conditions (40 - 120° C).

Conclusions

Present study has proved that the strain *Pseudomonas aeruginosa*MTCC 16037is capable of producing biosurfactant. Further it optimized bysubjecting to different temperature and pH conditions. *P. aeruginosa*has the capability toproduce optimum amount of biosurfactant when grown in mineral salt medium using hydrocarbon sludge carbon source. Moreover, produced biosurfactant can reduce the interfacial tension from 51 mN/m to 11 mN/m as at pH 8.0, which is the key parameter for bioremediation. Hence, *Pseudomonas putida* found to be best suited and can survive at high temperature as well as pH conditions can be used effectively in oil degradation and bioremediation activities. The ability to manipulate all of these parameters will improve current bioremediation efforts by increasing their effectivity while decreasing cost of the treatment.

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Figure 1:

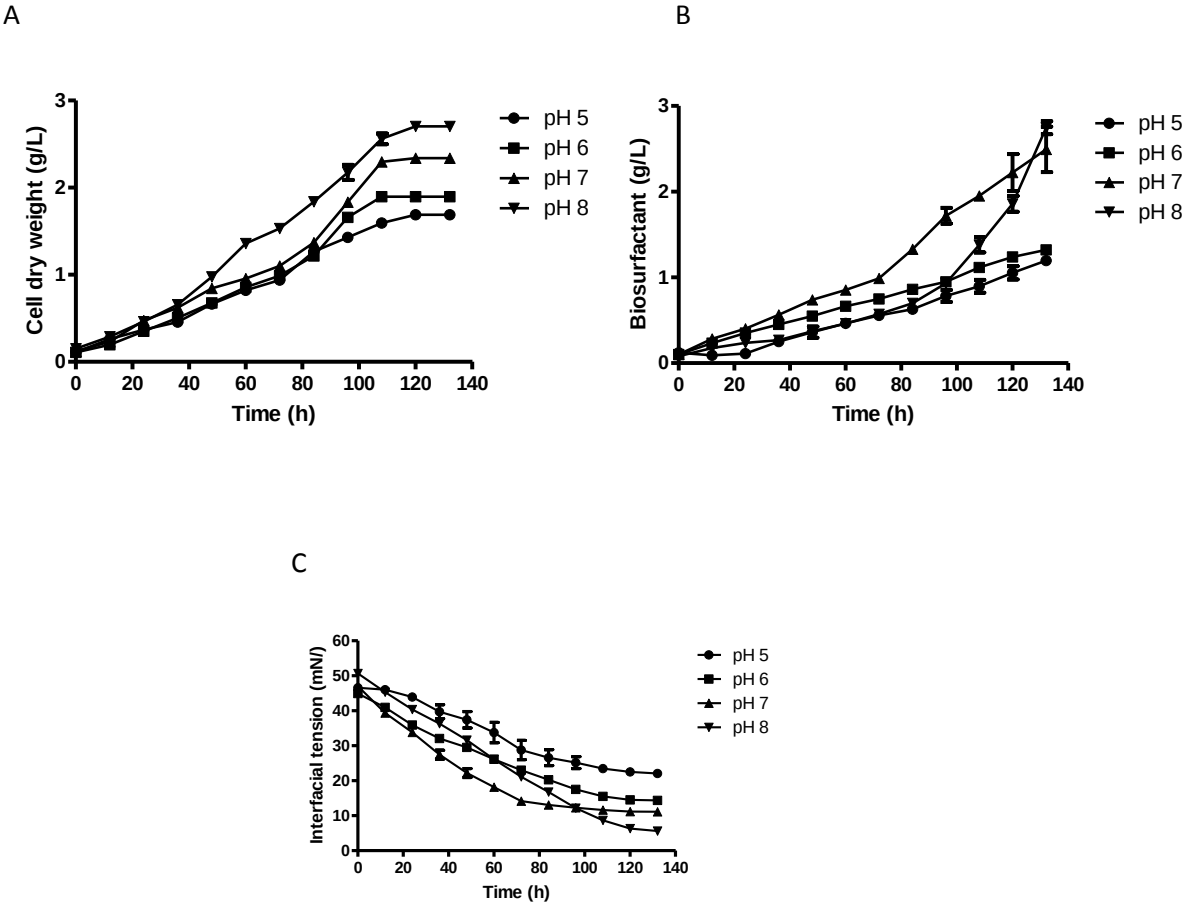

Figure 1: Effect of initial pH on biosurfactant production by *Pseudomonas aeruginosa* MTCC 16036 a) Cell dry weight b) Biosurfactant produced c) Interfacial tension profiles. Each experiment was performed 3 independent times and error bars represent ± SE ($p<0.005$)

Figure 2:

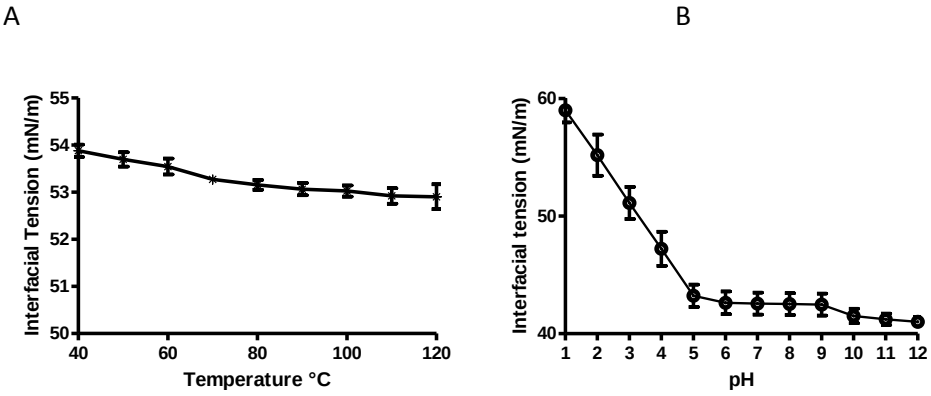

Figure 2: Stability of biosurfactant at a) temperature: produced biosurfactant sample was incubated for 1 h at various temperatures between 40 – 120 °C and analyzed for Interfacial tension. b) pH: samples of produced biosurfactant was adjusted to various pH's ranging from 1.0 to 12.0 and incubated for 1 h and analyzed for Interfacial tension. Each experiment was performed 3 independent times and error bars represent ± SE ($p<0.005$)

Table 1: Biodegradation of petroleum sludge

S.No	Time (h)	Biodegradation (%)
1	0	0
2	12	12.5
3	24	22.5
4	36	31
5	48	37.4
6	60	44.2
7	72	58.7
8	84	62.5
9	96	76.8
10	108	83.6
11	120	86.6
12	132	86.8