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Full Length Research Paper

Stenotrophomonas koreensis a novel biosurfactant producer for abatement of heavy metals from the environment

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The removal of heavy metal contaminants from the environment is one of the potential areas in which the usefulness of biosurfactants has not been thoroughly explored. The molecular nature of biosurfactants offers the possibility of interaction with the metals in solution, aiding in their subsequent removal and/or recovery. In the present research work, a systematic isolation and screening program was undertaken for obtaining biosurfactant-producing bacteria. A total of 129 isolates were screened and three bacterial isolates were selected for high surface tension reducing ability*. Pseudomonas aeruginosa, Stenotrophomonas koreensis* (Strain DX1 16S ribosomal RNA gene, partial sequence NCBI Acc. No. GQ 493998 BankIt 1255714) and *Rhodococcus* spp isolates were identified by routine microbiological tests, API-32 and 16s rRNA profiling. The surface tension reduction of MS medium for the three isolates was: *P. aeruginosa*, 62.3 to 31.6 dynes/cm; *S. koreensis*, 62.4 to 27.8 dynes/cm; in 24 to 30 h for both organisms and *Rhodococcus* spp, 64.4 to 43.7 dynes/cm in a period of 48 h. The emulsification index for all three isolates was 100% in diesel, petrol, toluene and sunflower oil. The ability of *S. koreensis* to remove heavy metal ions from solutions was explored. More than 30% of lead and cadmium ions were removed from 200 ppm metal solutions.

Key words: Biosurfactant, *Stenotrophomonas koreensis*, surface tension, heavy metals, lead, cadmium.

INTRODUCTION

Surfactants are amphipathic molecules consisting of both hydrophilic and hydrophobic moieties that partition preferentially at the interface between fluid phases having different degrees of polarities and hydrogen bonding eg. oil and water or air and water interfaces (Benincasa et al., 2001; Bodour et al., 2003). Synthetic surfactants used to increase contaminant solubility are often toxic, representing an additional source of contamination (Bodour and Miller-Maier, 1998). Microbially produced surface active compounds that is, biosurfactants have similar properties as that of chemical surfactants, but are less toxic, biodegradable and can be produced *in situ* at the contaminated site (Bonglo, 1998; Bordoloi and Konwar, 2007). These molecules reduce surface tension, critical micelle concentration and interfacial tension in both aqueous solutions and hydrocarbon mixtures (Bosch et al., 1988). Biosurfactants have gained increased attention because of their ability to be produced from cheap raw materials and effectiveness in extreme conditions of temperature, pH and salinity (Cha, 2000; Das and Mukherjee, 2007). The properties of the various biosurfactants have been extensively reviewed (Desai and Banat, 1997; Muthusamy et al., 2008).

Most microbial surfactants are complex molecules comprising a wide variety of chemical structures such as glycolipids, lipopeptides, fatty acids, polysaccharide-protein complexes, phospholipids and neutral lipids. Rhamnolipidsproduced by *Pseudomonas aeruginosa* is a

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major class of biosurfactants and extensively studied by investigators (Desai et al., 1994; Harman and Artiola,1995; Kosaric, 2000). Due to the diverse synthetic capabilities of microorganisms, it is not surprising that a large variety of biosurfactants and novel compounds are produced by them, providing new possibilities for industrial applications (Lang and Wullbbrandt, 1999; Banat et al., 2010). Thus, there is an increasing interest in the possible use of biosurfactant in mobilizing heavy crude oil, transporting petroleum in pipe lines, managing oil spills, biodegradation of hydrocarbons in the soil, removal of heavy metals, production of detergents, agro-industries and in the manufacture of pharmaceutical products (Banat et al., 2010; Franzetti et al., 2010; Plociniczac et al., 2011). A lot of research efforts are directed towards the isolation of organisms that produce biosurfactants, since the biosurfactants offer the possibility of large scale manufacture at low operating costs. Hence, in the present investigations, efforts were undertaken in order to employ a systematic isolation and screening program for biosurfactant producers and attempts were made for the isolation of novel organisms that could offer better solutions to the current industrial problems. In earlier investigations, although biologically produced surfactants have been projected as useful chemicals for many applications, there are few reports about the uses in heavy metal removal. Therefore, attempts were made to explore if biosurfactant producing organisms could be used for heavy metal removal/recovery.

The present work was aimed at the isolation and screening for biosurfactant producing microorganisms from hydrocarbon contaminated soil and crude oil samples. The development of a systematic screening program and screening methodology was also one of the primary aims of the work, since some screening tests are prone to errors in the selection. Another objective was to indentify and characterize organisms, especially if novel strains were isolated in the screening work. Finally, the possibility of using biosurfactant producers for remediation of heavy metal contamination was explored.

MATERIALS AND METHODS

Isolation of bacteria

Soil samples contaminated with petroleum and its products were collected from eastern Maharashtra region at Jawaharlal Nehru Port Trust, Navi Mumbai and oil contaminated sites in north Maharashtra region. Samples were inoculated in 100 ml of mineral salts medium (MSM). [glucose: 10 g; $(NH_4)_2SO_4$:1 g; Na_2HPO_4: 4 g; yeast extract: 5 g; KH_2PO_4: 3 g; NaCl: 2.7 g; $MgSO_4$: 0.6 g and 5 ml/L trace element solution containing $FeSO_4.7H_2O$: 5 mg; $ZnSO_4.7H_2O$:3.34 mg; $MnSO_4.7H_2O$: 1.56 mg; $CoCl_2.2H_2O$: 2 mg (1 L distilled water)]. For enrichment of biosurfactant producers instead of glucose, 5% of diesel, petrol and dodecane were added in three flasks respectively. Then the flasks were incubated on a rotary shaker at 37°C for up to 4 weeks at 120 rpm. Samples were withdrawn from the flasks at weekly intervals to test for growth of bacteria.

Screening for biosurfactant production

The isolated cultures were grown in 150 ml of MSM in 500 ml Erlenmeyer flasks and samples were withdrawn from the medium at definite time intervals (one week) for screening. Medium without inoculation of bacteria served as the negative control. Two approaches were used for screening the biosurfactant producers:

(i) Qualitative screening: Blood agar lysis method-cultures were spread onto blood agar plates to observe for any hemolysis which was indicative of biosurfactant production (Bodour and Miller-Maier, 1998; Bordoloi and Konwar, 2007). Colonies showing hemolysis were tested further by oil spread technique, drop collapse method and blue agar plate method. For oil spread technique-50 ml of distilled water was added to large Petri dish (25 cm diameter) followed by addition of 100 µl of crude oil to the surface of water. 10 µl of culture were then added to the surface of oil. A clear zone on oil surface indicated biosurfactant activity of the culture and diameter of the clear zone was proportional to the production of biosurfactant by the bacteria. The diameter of the clear zone on the surface of oil was determined. The experiment was carried out in triplicates. The drop collapse technique and blue agar plate method was carried out as described by Bodour and Miller-Maier (1998).
ii) Quantitative screening: All isolates that tested positive in the qualitative tests were subjected to quantitative assays viz. determination of emulsification index by the method of Bosch et al. (1988) and determination of surface tension by the du Nouy ring method (Benincasa et al., 2001; Bodour et al., 2003; Mukherjee et al., 2009). All samples for quantitative assays were analyzed in triplicates.

Optimization of the growth parameters for production of biosurfactant

Different carbon (mannitol, sucrose, starch, glycerol, olive oil, dodecane) and nitrogen sources (ammonium sulphate, potassium nitrate, ammonium nitrate, ammonium dihydrogen orthophosphate) were tested in order to find out the best combination of nutrients for biosurfactant production using MSM as basal medium (Das and Mukherjee, 2007). The effect of medium pH (4-10) and growth temperature (25, 37, 45 and 50°C) were also tested in order to determine the optimal conditions for maximum biosurfactant production. Each factor was varied by keeping all other factors constant and all samples were taken in triplicates. Subsequently, the Placket-Burman design was used to find out the critical media components.

Determination of lead and cadmium tolerance for *S. koreensis* by Kirby-Bauer method and metal removal studies

S. koreensis was grown in Nutrient broth (pH 6.8) in the presence of varying concentrations (25-800 ppm) of the metals viz. lead and cadmium. Once the tolerance level was determined, the organisms were grown in presence of 200 ppm of each metal and incubated for up to 6 days (37°C, 120 rpm) in order to allow the interaction of organisms with the metal ions. Samples were withdrawn at 12 h intervals and the concentration of metal ions remaining in solution was determined by AAS (Shimadzu AA-6300, Japan). Un-inoculated flasks containing the metals were used as controls.

RESULTS AND DISCUSSION

Isolation of bacteria

Most studies on biosurfactant producing organisms have

Table 1. Biosurfactant production profile of selected isolates with qualitative and quantitative tests.

Name of isolate	*Blood hemolysis	*Blue agar	*Oil spread	*Drop collapse	EI (%)	Surface tension dyne/cm
S. koreensis	++++	++	++++	++++	100% (24 h)	62.4 to 27.8
P.aeruginosa	+++	++	+++	+++	100% (24 h)	62.3 to 31.6
Rhodococcus	++	-	++	++	100% (48 h)	64.4 to 43.7
R_{72}	+++	-	-	-	-	-

*Number of '+' signs indicates the degree of biosurfactant activity in qualitative tests, '-' sign indicates the absence of the desired activity.

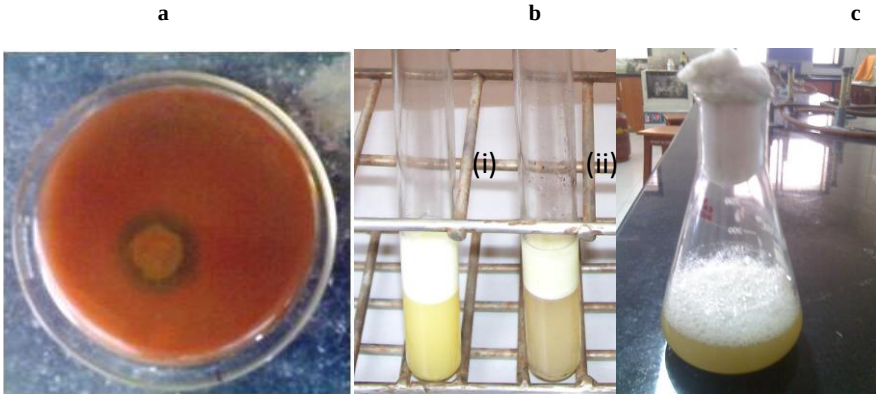

Figure 1. Biosurfactant activity of *S. koreensis* in (a) blood agar, (b) EI-24 in (i) petrol and (ii) diesel, (c) foam formation in production medium.

been carried out on previously isolated strains of bacteria and fungi. According to many published reports, investigators successfully isolated biosurfactant-producing organisms from soils contaminated with hydrocarbons or mineral oils (Mukherjee et al., 2009; Moran et al., 2001; Mulligan, 2005). Taking this as a guideline, in the present investigation, samples of soil contaminated with petroleum and its products were collected for isolation of unique and acclimatized bacteria that might possess superior biosurfactant activity. From the enriched samples, a total of 129 bacterial strains were isolated and preserved on nutrient agar slants until use.

Screening for biosurfactant production

The screening program yielded three bacterial isolates that could reduce the surface tension with high efficiencies (Table 1 and Figure 1). The isolates were identified by routine microbiological tests, API32 and 16s rRNA profiling as *Stenotrophomonas koreensis* (NCBI Acc. No. GQ 493998), *Pseudomonas aeruginosa* and *Rhodococcus* sp. Among the three isolates, *S. koreensis* showed highest levels of biosurfactant activity with the qualitative and quantitative assays, hence it was chosen for further experimentation. It was found that the quantitative du Nouy ring method was the most reliable and sensitive method for determination of surface tension reducing ability followed by the emulsification index method. Moreover, it was also observed that isolates that showed blood haemolysis (in this case the isolate designated R_{72}) were not necessarily efficient producers of biosurfactants as evidenced from their inability to reduce the surface tension of the medium. Similar observations were also made by Plaza et al. (2005) where blood agar haemolysis method yielded many false positive isolates. It could be seen that among the qualitative tests, the oil spread and drop collapse methods were the most reliable methods for screening, since organisms testing positive with these tests were also strongly positive with the quantitative tests. In general, it could be concluded that the qualitative oil spread method may be used as a standard screening method on account of its simplicity, rapidity and reliability. For further work on optimization of biosurfactant yield and its quantification, the highly sensitive quantitative de Nouy ring method may be used.

Optimization of growth parameters

It was observed that glucose as carbon source and potassium nitrate as nitrogen source yielded maximum biosurfactant production as evidenced from the reduction in surface tension by du Nouy ring method (Table 2). A

Table 2. Optimization of carbon and nitrogen sources of MSM for biosurfactant production by *S. koreensis.*

Nitrogen source	Surface tension reduction (dynes/cm)	
	Glucose	Mannitol
$(NH_4)_2SO_4$	31.2	29.4
KNO_3	34.6	29.2
NH_4NO_3	21.4	24.7
$NH_4H_2PO_4$	19.4	20. 2

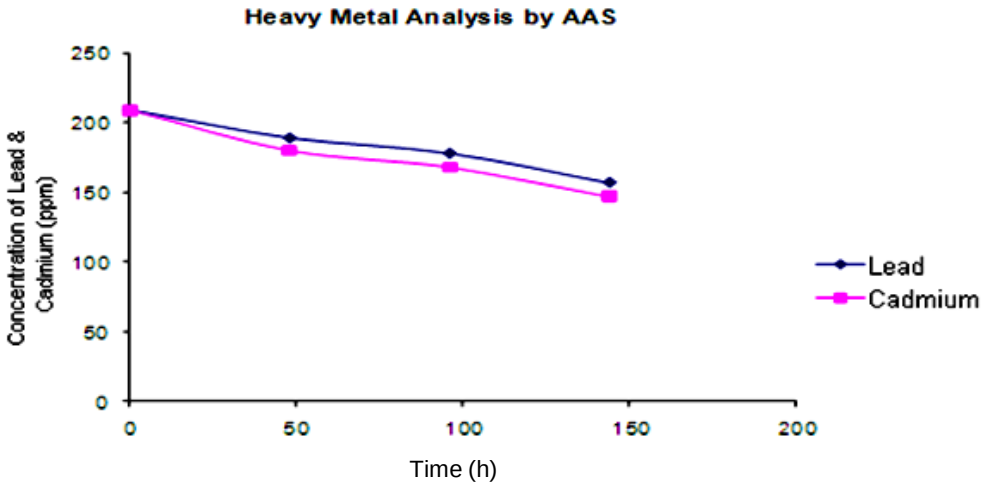

Figure 2. The efficiency of removal of lead and cadmium by *S. koreensis.*

temperature of 37°C and pH 6 were found to yield maximal levels of biosurfactant. The time required for complete emulsification of the added oils and to lower the surface tension of the production medium (MSM) was 24 to 30 h. The surface tension remained more or less the same for several days once the critical micelle concentration is attained. Using the Placket and Burman design for media optimization, it could be concluded that $MgSO_4$ and KH_2PO_4 were the very critical factors that affected biosurfactant production. Thus, the enrichment and screening program on the first place and subsequently the media optimization step have been useful for obtaining more efficient organisms that could be valuable for large-scale industrial applications. Moreover, it was possible to obtain a novel strain of *S. koreensis* that had a high efficiency of reducing the surface tension of liquids (Table 2).

Determination of lead and cadmium tolerance for *S. koreensis* by Kirby-Bauer method and metal removal studies

In the present experiments, lead and cadmium were chosen for heavy metal removal studies due to the following reasons: (i) ease of availability of the metal salts, (ii) availability of sensitive atomic absorption spectrophotometric method for estimations (iii) huge data available on removal and recovery of these metals from solutions, (iv) large scale use of these metals in industries (viz. batteries, cable coverings, plumbing, fuel, paints, PVC plastic, pencils, pesticides, alloys for lead and Ni-Cd batteries, coating, pigments, fertilizers for cadmium) that raises environmental concerns about heavy metal pollution, (v) major toxic effects of these metals on living cells, (vi) reports on rhamnolipid (a type of biosurfactant) interactions specifically with lead and cadmium and use of the interactions to eliminate cadmium toxicity (Desai et al., 1994; Franzetti et al., 2010; Mulligan, 2005). It was found that the isolates obtained in the present studies were tolerant to high metal concentrations of 200 ppm. This resistance could be attributed to the presence of biosurfactant that could effectively complex with the metal ions in solution. The biosurfactant might confer resistance by a complexation mechanism that removes ions from solution and thus keeping the toxic metal away from the bacterial cells (Banat et al., 2010; Mulligan, 2005; Sandrin et al., 2000; Zosim et al., 1983). It was observed that more than 30% of the metal ions were removed from the media (Figure 2). It is evident from Figure 3, that there was a huge reduction in the surface tension of the medium in 24 to 30

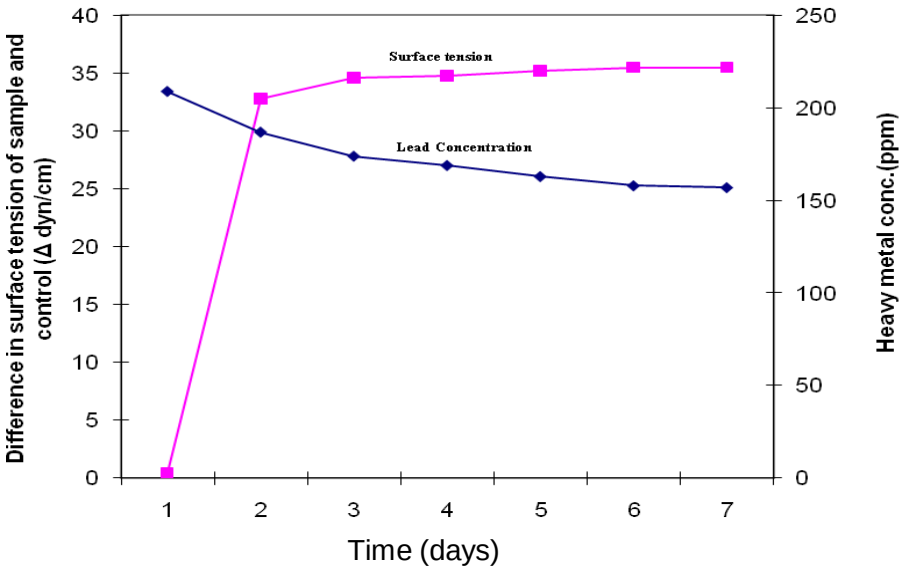

Figure 3. The correlation of reduction in surface tension and metal removal as a result of biosurfactant productionby*S. Koreensis.*

h after inoculation of the culture. This reduction is seen as a spurt of increase in the difference in surface tension of sample and control that is, Δ dyne/cm. The reduction in the surface tension was due to production of biosurfactant that also reduced metal concentration of the medium. Although the metal removal efficiency appears to be low, it must be mentioned that percentage figures are often misleading, since at lower metal concentrations the observed percent removal values could be greater (Paknikar et al., 1999). Moreover, at low metal concentrations, better growth of bacteria would result in higher biomass and biosurfactant yields, and hence better metal removal efficiencies. The concentrations of heavy metals in industrial effluents are often in the range of 10 to 50 ppm. At these lower concentrations, conventional physical and chemical methods of treatment do not work efficiently; whereas, biosurfactant-based method would work efficiently.

Conclusion

A novel metal resistant bacterial culture capable of high biosurfactant activity was isolated from petroleum contaminated soil through a systematic screening program. The bacterial culture produced a biosurfactant rhamnolipid that proffers resistance to metals and was also responsible for removing metals from solutions. Due to the biodegradability and low toxicity, biosurfactants may have a promising future for use in remediation of metalliferous wastes. Considering these aspects, further research on purification and detailed characterization of the material using chromatographic techniques and instrumental analysis is being pursued in the laboratory.

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