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Production and characterization of the biosurfactant obtained by Bacillus subtilis

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ABSTRACT

Biosurfactants are surface active amphiphilic compounds with effective surface-active and biological properties applicable to several industries and processes. In this study, isolation and identification of biosurfactant producing strain were assessed. Soil samples from petrol pumps in Kalyan and Ulhasnagar area was collected and 38 strains were isolated. To confirm the ability of isolates to produce biosurfactant, emulsification assay, E_{24} test and surface tension measurement tests were performed. Bacillus subtilis isolated from petrol pump station, was used to produce biosurfactant using a modified mineral salt medium with 2% diesel as sole source of carbon. The chemical composition of the biosurfactant was qualitatively analyzed by thin layer chromatography. The FT-IR analysis revealed the lipopeptide nature of the biosurfactant. The surface tension measurement using Kruss processor tensiometer showed the CMC of 140 mgL^{-1}. The biosurfactant was found to be quite stable under varying conditions of temperature and pH.

Keywords: Biosurfactants, FT-IR, CMC and Bacillus subtilis

I. INTRODUCTION

Biosurfactants are the biologically synthesised surface-active agents (Nitschke and Pastore, 2006). They are amphiphilic compounds consisting of hydrophilic and hydrophobic domains. The hydrophilic domain can be carbohydrate, amino acid, phosphate group or some other compounds whereas the hydrophobic domain usually is a long chain fatty acid (Lang, 2002). The majority of known biosurfactants are synthesized by microorganisms grown on water immisible hydrocarbons,but some have been produced on water soluble substrate such as glucose,glycerol and ethanol (Abu-Ruwaida et al.,1991). Microorganisms have been reported to produce several classes of biosurfactants such as glycolipids, lipopeptides, phospholipids, neutral lipids or fatty acids and polymeric biosurfactants (Franzetti et al., 2010; Banat et al., 2010). Chemically synthesized surfactants have been used in the oil industry to aid clean up of oil spills as well as to enhance oil recovery from oil reservoirs. These compounds are not biodegradable and can be toxic to environment. Biosurfactant have special advantage over their commercially manufactured chemical surfactants because of their lower toxicity, biodegradable nature and effectiveness at extreme temperature, pH, salinity and ease of synthesis (Ilori and Amund, 2001; Ilori et al., 2005). This study describes the screening and isolation of a potent biosurfactant producing microorganism, the biochemical characterization and emulsification ability of the biosurfactant.

II. MATERIALS AND METHODS

Screening of biosurfactant producing bacteria

Biosurfactant producing bacteria were isolated by successive enrichment culture technique from the petroleum contaminated soil using Minimal Salt

medium containing diesel oil (2%) as a sole source of carbon. The minimal salt media used consist of (g/L^{-1}):$MgSO_4$:0.2, $CaCl_2$:0.02, KH_2PO_4:1, K_2HPO_4:1, NH_4NO_3:1, $FeCl_3.6H_2O$:0.05 pH adjusted to 7.0 (Patel and Desai, 1997).The isolation was done on solidified minimal salt medium where diesel oil was introduced in vapour phase transfer technique as described by Raymond et al.,(1976). Incubation was carried out at room temperature for 5 days.

Emulsification Index ($E_{24\%}$) - Emulsification index of cell free broth was determined by adding 2 ml of fuel oil to 2 ml cell free broth, mixing with a vortex for 2 min, and leaving it undisturbed for 24 h. The E_{24} index is given as percentage of height of emulsified layer (mm) divided by total height of the liquid column (mm) (Cooper and Goldenberg, 1987).

Identification of biosurfactant producer-The biosurfactant producers were identified on the basis of their morphological, cultural and biochemical characteristics as described by Holt et al., (1994).

Selection of best biosurfactant producer- The best biosurfactant producer was selected based upon the emulsification activity, biomass and biosurfactant yield and by measuring reduction in surface tension of the culture media.

Emulsification activity assay- 2.0 ml cell free broth sample obtained from each of the isolate were added in a tube containing 15 ml of 0.2 M Tris buffer pH 8.0 and 0.2 ml of fuel oil. The mixture was then vortexed for 10 min, allowed to rest for 1 min. Then extinction was read at 540 nm against blank containing buffer and fuel oil (Banat et al.,1990).

Biomass determination-The culture media was centrifuged at 10,000 rpm for 30 min to obtain pellet. Six volume of a mixture of petroleum ether and acetone (1:3 ratio) was mixed thoroughly with the pellet and centrifuged at 3000 rpm for 20 min. This was repeated till all the unutilised oil sample was removed leaving the solvent layer clear. Such cell mass free of oil settled down even at low speed centrifugation. The upper solvent layer was removed and cell mass was further treated with acetone. The cell mass was then washed with distilled water and dried at 60^{0}C overnight in a preweighed crucible. The dry mass of cells was determined.

Extraction of biosurfactant-The pH of the cell free supernatant was adjusted to pH 2 using 6 N HCl and kept at 4^{0}C for 24 hr. The cell free supernatant and chilled mixture of chloroform and methanol (2:1) in equal volume was added and mixed vigorously to obtain the biosurfactant within the organic layer. This layer was separated using a separating funnel and dried at 40^{0}C for 4-5 hours to obtain dry mass. The yield as g/L was recorded (Desai and Banat, 1997).

Surface tension-The surface tension of the cellfree broth was measured by Kruss Processor Tensiometer K-12 (Wilhelmy plate) method as described by (Singh et al., 1989, Tuleva et al., 2005).

Analysis of component of biosurfactant by Thin layer Chromatography-The crude extract was separated by TLC using aluminium sheets silica gel plates with chloroform:methanol:acetic acid and water (25:15:4:2). Ninhydrin reagent was used to detect free amino groups. The lipid components were detected as brown spots after spraying the plate with chromosulphuric acid. The carbohydrate compound were detected as red spots after spraying the plates with α-napthol in concentrated sulphuric acid.

Fourier transform infra red spectroscopy (FT-IR)- 2.0 mg of the biosurfactant was mechanically blended with KBr in the ratio of 1:100 and pressed to form a pellet. Infra-red absorption spectra were recorded on a FT-IR in the 4000-400 1/cm range with a 16-scan speed at a resolution 2 cm^{-1}. KBr pellet was used as the background reference.

CMC determination-In this method, different concentration of biosurfactant was prepared (40 mgL^{-1}- 200 mgL^{-1}) in distilled water. Surface tension of these biosurfactant solutions were determined

using Kruss K-12 tensiometer and the concentration at which the surface tension reduction remains stable i.e. no further reduction in the surface tension is seen is considered as CMC. The CMC was determined by plotting the surface tension versus concentration of bisurfactant in the solution.

Stability study- For determining the stability of the emulsion, the biosurfactant solution and oil mixture were incubated under varying conditions of temperature from 4^0C to 100^0C and pH from 2.0 to 12.0 one hour and then the emulsification activity was recorded at 540 nm. The percent residual emulsification activity was recorded after 24 hr by oil emulsification assay (Banat et al., 1990) and emulsion stability was expressed in percentage.

III. RESULT AND DISCUSSION

Screening of biosurfactant producing bacteria- From petrol pump soil samples of Kalyan and Ulhasnagar area 38 isolates were obtained. 29 isolates showed emulsification index ranging from 10% to 30% and 9 isolates showing emulsification index of more than 30% were selected. Isolate B17 Kalyan petrol pump gave maximum emulsification index of 40%. These 9 isolates exhibited good emulsification activity as shown in Table 1.1.

Table 2. Emulsification index of the isolates

Isolates	Emulsification index (E24%)
U1	30
K1	32
K2	34
K4	34
B17	**40**
B3	36
G6	32
G4	35
B4	38

Identification of biosurfactant producer-The biosurfactant producers were identified on the basis of their morphological, cultural and biochemical characteristics as described by Holt et al., (1994). Among these 9 isolates 5 isolates were Gram positive and 4 were Gram negative. The gram negative isolates belonged to Pseudomonas, Serratia and Azotobacter sp. and among the gram positive isolates Bacillus sp were predominant (Table 1.2)

Table 2. The isolates were identified as follows-

Isolate	Name of the organism
U1	Bacillus megaterium
K1	Bacillus coagulans
K2	Serratia
K4	Bacillus polymyxa
B17	Bacillus subtilis
B3	Pseudomonas aeruginosa
G6	Bacillus licheniformis
G4	Pseudomonas sp
B4	Arthrobacter

Selection of best biosurfactant producer- The isolates gave a biomass yield ranging from 0.74 g/L to 1.85 g/L and the biosurfactant yield from 0.06 g/L to 0.69 g/L. All the isolates lowered the surface tension of the media from 65 mN/m to 42 mN/m and below (Table 1.3).

Table 3. Selection of best biosurfactant producer based on emulsification activity, surface tension, biomass and biosurfactant yield

Isolates	Biomass (gL^{-1})	E.A(O.D-540nm)	Biosurfactant (gL^{-1})	Surface tension(mN/m)
U1	0.85	0.12	0.10	40.3
K1	0.81	0.16	0.06	42.0
K2	0.76	0.15	0.12	42.6
K4	1.3	0.35	0.43	39.2
B17	1.85	0.58	0.69	37.0
B3	0.92	0.18	0.25	40.8
G6	0.95	0.27	0.25	39.9

G4	0.74	0.17	0.12	42
B4	0.81	0.19	0.14	40.4

Thin layer Chromatography of biosurfactant- The biosurfactant showed purple colour spot when sprayed with ninhydrin, indicating the presence of free amino groups. The lipid components were observed as brown spots when sprayed with chromosulphuric acid.

FT IR analysis of biosurfactant- A broad absorbance peak around 3433 cm^{-1} was observed as aresult of C-H stretching vibrations and N-H stretching vibrations. Sharp absorbance peaks are observed at 1463cm^{-1}, 1379 cm^{-1}, 2955 cm^{-1} and 2854 cm^{-1} indicative of aliphatic chains.These peaks reflects the presence of alkyl chains in the compound. Carbonyl group were indicated by bands at 1741 cm^{-1}, 1726 cm^{-1} (fig 1).

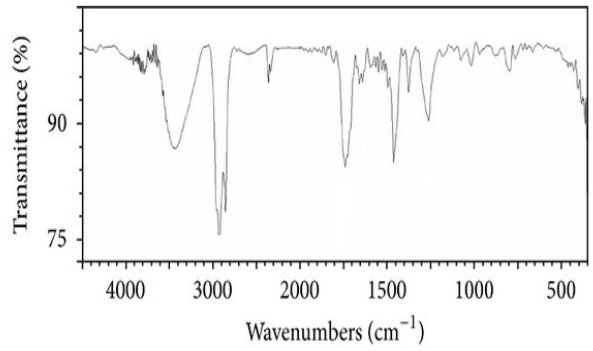

Figure 1. FT-IR spectrum of biosurfactant

CMC determination- The CMC of 140 mgL^{-1} obtained with Bacillus subtilis B17 is very significant, as most of the literature reviewed showed that biosurfactant achieved surface tension 25 to 39 mN/m at higher CMC values. This CMC was much lower when compared with some chemical surfactant, for instance, sodium dodecyl sulphate (SDS) had a CMC value of 2100 mgL^{-1}(Chen et al., 2006) (fig 2).

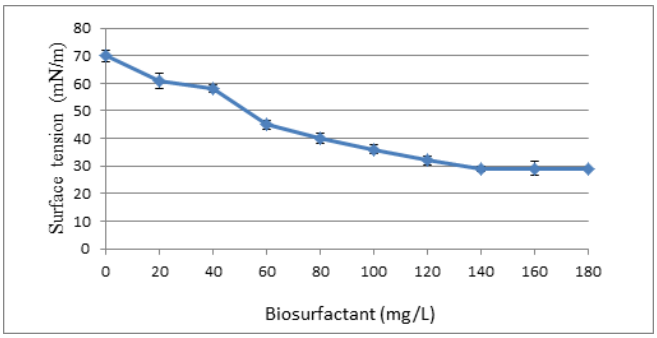

Figure 2. CMC of biosurafctant

Stability studies- Biosurfactant was found quite stable over a temperature range of 4^0C to 100^0C. A slight decrease in the biosurfactant activity was noticed at high temperature. However, biosurfactant was found to retain more than 60% of emulsion activity even at extreme temperatures of 4^0C and 100^0C (fig 3). Similarly, at the acidic pH of 2 and 4 the emulsification activity and the stability was found to be lowest. This loss of activity at pH 2.0 and pH 4.0 may be due to precipitation of the biosurfactant at low pH which hinders the emulsion formation. Increase in pH has a positive effect on emulsification activity and emulsion stability fig 4.

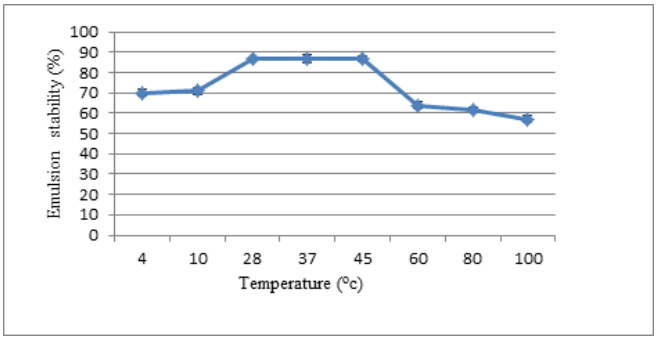

Figure 2. Effect of temperature on stability of emulsion

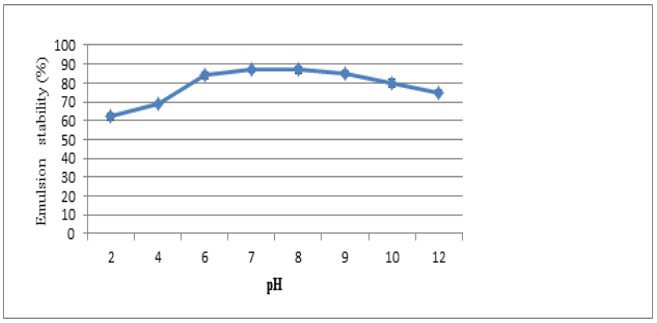

Figure 3. Effect of pH on stability of biosurafactant

IV. CONCLUSION

In the present study, biosurfactant producing organisms were enriched and isolated from petroleum contaminated sites. The potent biosurfactant producer Bacillus subtilis B17 was selected as it showed strong emulsification ability and low CMC. These studies suggest that the biosurfactant obtained from Bacillus sp. can be efficiently used in environmental remediation remediation strategies.

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