INTERNATIONAL JOURNAL OF ADVANCES IN PHARMACY, BIOLOGY AND CHEMISTRY

Research Article

Isolation of Biosurfactant Producing *Exiguobacterium* sp. from Lonar Lake

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ABSTRACT

Naturally occurring surface active compounds derived from micro-organism are called biosurfectant which reduces surface tension. Lonar Lake is unique due to its salinity, alkalinity and biodiversity. The Lonar lake water is alkaline having average pH 10.5. Lonar Lake harbors diverse microorganisms having potential to produce variety of biotechnological products. Present study focused on biosurfectant producing bacteria from Lonar Lake. From four different sites of Lonar Lake 12 sediment, matt and water samples were collected and screened on mineral salt medium containing 2% soybean oil as a carbon source. Two bacterial strains were isolated having ability to produce biosurfactant. Surface tension measurement, oil displacement test and emulsification index methods were used to screen the capability of isolates for biosurfactant production. The bacterial isolates were identified by standard biochemical test and 16S rRNA sequencing. Both strains were identified as *Exiguobacterium* sp. these isolates were further characterized for their ability to produce biosurfactant on different carbon sources and at different pH, temperature and salt concentration. The study revealed that maximum biosurfactant production was found in coconut oil and break oil. Also these bacteria were able to grow and produce biosurfactant over a wide range of pH, salinity, and temperature. The results of present study suggested that these isolate might be helpful for remediation oil at polluted site of marine environment.

Keywords: Biosurfactant, Lonar Lake, *Exiguobacterium* species, Bioremediation.

INTRODUCTION

In India, the Lonar crater, popularly called as the Lonar soda lake is situated in the Buldhana district of Maharashtra state. It is one of the largest craters in the world and the only crater which is formed due to high velocity meteoritic impact on basaltic rock, about 50,000 year's ago [1]. The uniqueness of the lake water is its salinity and high alkalinity. The lake water is alkaline having an average pH of 9.5-10. Lonar Lake is a closed one without any outlet and evoked much scientific value for biodiversity among the researcher. Due to the uniqueness of this lake, remains unexplored regarding the potential biosurfactant producers. Alkaliphilic microorganisms have attracted much interest because of their ability to produce different industrially important extracellular enzymes and substances that are active and stable at high pH values [2]. Biosurfactant are amphiphilic compounds produced on living surface mostly on microbial cell surface or excreted extracellularly and contain hydrophobic and hydrophilic moieties that reduce surface tension and interfacial tension between individual molecules at the surface and interface respectively [3]. Surfactants are key ingredients used in detergents, shampoos, toothpaste, oil additives and a number of other consumer and industrial products. The biosurfactants are complex molecules covering a wide range of chemical types including peptides, fatty acids, phospholipids, glycolipids, antibiotics, lipopeptides, etc. [4].

As biosurfactant are potential candidates for many applications in industries the development of this line of research is of paramount importance, mainly in view of the present concern with protection of the environment. The most significant advantage of a microbial surfactant over chemical surfactant is its ecological acceptance as it is biodegradable and nontoxic to natural environment [5]. Tambekar *et al,* [6] studied the biosurfactant production from Lonar lake bacterium *Achromobacter xylosoxidans* using soybean oil. Satpute *et al,* [7] studied the

biosurfactant and bioemulsifier producing ability of marine bacterial isolates including *Acinetobacter, Arthrobacter, Pseudomonas, Halomonas, Myroides, Corynebacteria, Bacillus* and *Alteromonas* sp. the result revealed that the isolates are with great ability to produce surface active agent. Tambekar and Gadakh, [8] had also studied the biosurfactant producing bacteria isolated from hydrocarbon contaminated soil. 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 add toxic intermediates in the environment. Biosurfactant have special advantage over their commercially manufactured counterparts because of its low toxicity, biodegradable nature, and effectiveness at extreme temperature, pH, salinity and ease of synthesis [9]. Therefore, present study focused on the isolation of biosurfactant producing bacteria from Lonar Lake and stability of produced biosurfectant at different pH, temperature and salt concentration. There capability to produce biosurfactant on different carbon sources was determined.

MATERIALS AND METHODS

Collection of samples: Water, matt and sediment samples were collected from different sites of the Lonar Lake during August, 2012. The water samples were collected in sterile plastic tight cap bottle while sediment and matt samples were collected in zip lock bag respectively and were stored at 4^0C till analysis.

Isolation, Enrichment and biochemical characterization: Sediment, matt (1g) and water (10mL) samples from Lonar Lake were separately inoculated in 250mL Erlenmeyer's conical flask containing 100mL mineral salt medium having composition (g/l): $NaNO_3$ 2.5g; KCl 0.1g; KH_2PO_4 3.0g; K_2HPO_4 7.0g; $CaCl_2$ 0.01g; $MgSO_4.7H_2O$ 0.5g and 5mL of trace element solution contains: $FeSO_4.7H_2O$ 0.116g/l; H_3BO_3 0.232g/l; $CoCl_2.6H_2O$ 0.41g/l; $CuSO_4.5H_2O$ 0.008g/l; $MnSO_4.H_2O$ 0.008g/l; $[NH_4]_6Mo_7O_{24}$ 0.022g/l; $ZnSO_4$ 0.174g/l with 2% soybean oil as a sole source of carbon and were incubated at 37^0C at 200 rpm on rotary shaker for 7 days and same procedure was successively repeated 5 time for enrichment of bacterial culture [10]. After enrichment of culture, the broth were inoculated on solid nutrient agar plate and well isolated and morphologically different colonies were selected and stock culture were prepared. All these isolates were further characterized by standard biochemical test according to Bergey's manual of systematic bacteriology.

Preliminary screening for biosurfactant production

Surface tension measurement: Five mL of inoculums of the bacterial culture were added to 250 mL Erlenmeyer flask containing 100 mL mineral salt medium with 2% soybean oil as a carbon source. The experimental flasks were incubated at 37^0C on rotary shaker at 200 rpm. After 5 days of incubation broth were centrifuged at 8000 rpm for 20 min for cell removal and cell free supernatant was collected in sterile flask. The reduction in surface tension of cell free broth was determined by using stalagmometer by drop counting method [11].

Oil spreading method: A 50 mL of distilled water added to the Petri dish followed by addition of 20 µL of soybean oil. A thin layer was allowed to form on water surface. Then 10 µL of cell free culture supernatant was dropped on oil surface. The diameter of zone of clearing of oil surface was measured immediately [12].

Emulsification index (E_{24}): Emulsification index of culture supernatant was determined by adding 2 mL of soybean oil to 3 mL of culture supernatant and vortex vigorously for 2 min and leave to stand for 24 h. An emulsification index is given as percentage of height of emulsified layer (mm) divided by total height of the liquid column (mm)[13].

Identification of bacteria on the basis of 16S rRNA sequencing: Biosurfactant producing bacterial cultures were identified by using 16S rRNA of bacterial small subunit rRNA genes were amplified by PCR using primers corresponding to *Escherichia coli* positions 27F and 1492 R (8F, 5'-AGA GTT TGA TYM TGG CTC AG-3'; 1492 r, 5'-CGG TTA CCT TGT TAC GAC TT-3') (27-28). The plasmid DNA was isolated from positive clones. The rRNA gene inserts were sequenced on an automated ABI 377 sequencer (NCCS, Pune) using M13 universal sequencing primer. The resulting sequences (approximately 15,000 bp) were compared with sequences in the Gene bank database of NCBI using the BLAST network service[14].

Biosurfactant production on different carbon source: The effect of different carbon source on biosurfactant production from *Exiguobacterium* sp. by providing different vegetable oil and petroleum oil as a substrate and surface activity and emulsification index was determined.

Effect of different salt concentrations on biosurfactant production: The effect of salinity on biosurfactant production was determined by adding different concentrations (1-4%) of NaCl in

mineral salt medium. The broths were incubated at 37^0C on shaker at 200 rpm for 5 days and the reduction in surface tension and emulsification index of culture supernatant was determined [9].

Effect of different pH on biosurfactant production: The effect of pH on biosurfactant production was determined by changing pH (6-10) of mineral salt medium with HCl and NaOH. The broths were incubated at 37^0C on shaker at 200 rpm for 5days and the reduction in surface tension and emulsification index of culture supernatant was determined [9].

Effect of different temperature on biosurfactant production: The effect of temperature on biosurfactant production was also investigated. The broths were incubated at 200 rpm for 5 days in selected temperature (20-50^0C) and the reduction in surface tension and emulsification index of culture supernatant was determined [9].

RESULTS AND DISCUSSION

From 12 (sediment, matt and water) samples collected from Lonar Lake two bacterial strains (AMS1) and (AMS2) with ability to produce biosurfactant were isolated and further characterized on different carbon sources. The isolates were characterized biochemically the result of the biochemical characterization showed that, both the isolates were gram positive short rods, sluggishly motile and ferment glucose, galactose, fructose, mannitol, dextrose, trehalose and sucrose with acid production. Both the strains were unable to ferment raffinose and lactose, while the difference in sugar fermentation between the isolates was found only in case of arabinose and sorbitol (Table 1). Though the bacterial isolates differs in few biochemical test the result of the 16S rRNA sequencing result showed that both the isolates belong to genus *Exiguobacterium*. So, further characterization for biosurfactant production was performed with only strain AMS1.

Table 1: Morphological and biochemical characteristics of *Exiguobacterium* species

Characters	Test	(AMS1)	(AMS2)	Characters	Test	(AMS1)	(AMS2)
Colony characters	Color	Pale orange	Pale orange	Sugar fermentation	Fructose	A	A
	Shape	Circular	Circular		Raffinose	-	-
	Elevation	Flat	Flat		Dextrose	A	A
Morphology of bacteria	Gram character	+	+		Sorbitol	-	A
	Shape	Rod	Rod		Sucrose	A	A
	Arrangement	Single	Single	Growth at pH	pH 6	+	+
	Motility	+	+		pH 7	+	+
Biochemical test	Catalase	+	+		pH 8	+	+
	Oxidase	-	-		pH 9	+	+
	Indol	-	-		pH 10	+	+
	MR	+	+	Growth at NaCl	1 %	+	+
	VP	-	-		2 %	+	+
	Citrate utilization	-	-		3 %	+	+
	Nitrate reduction	+	+		4 %	+	+
Sugar fermentation	Trehalose	A	A		5 %	+	+
	Glucose	A	A		6 %	+	+
	Arabinose	A	-	Growth at Temperature	20^0C	+	+
	Mannitol	A	A		30^0C	+	+
	Lactose	-	-		40^0C	+	+
	Galactose	A	A		50^0C	+	+

Bootstrap analysis was used to evaluate phylogenetic tree stability according to a consensus tree from the neighbor-joining based on 1,000 replicates for each. Phylogenetic analysis based on 16S rRNA gene sequences indicated that strain ASM1 and ASM2 were affiliated to phylum Firmicutes with genera *Exiguobacterium* (Fig 1). The highest similarity values with the sequences of obligately alkaliphilic and alkalitolerant, estuarine bacterium *Exiguobacterium* sp. JX625999 forming bacteria.

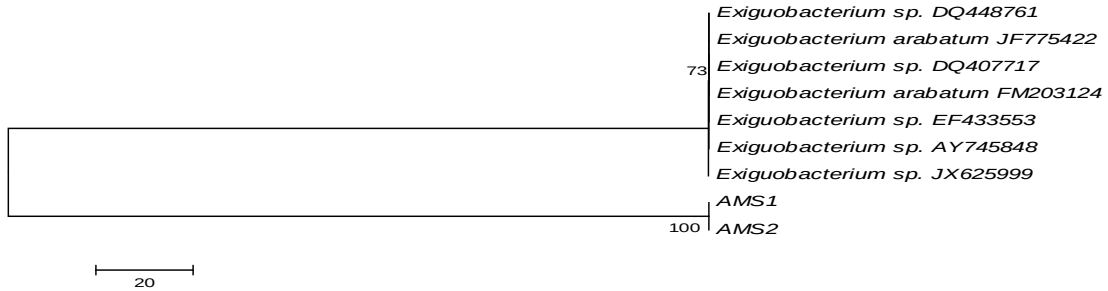
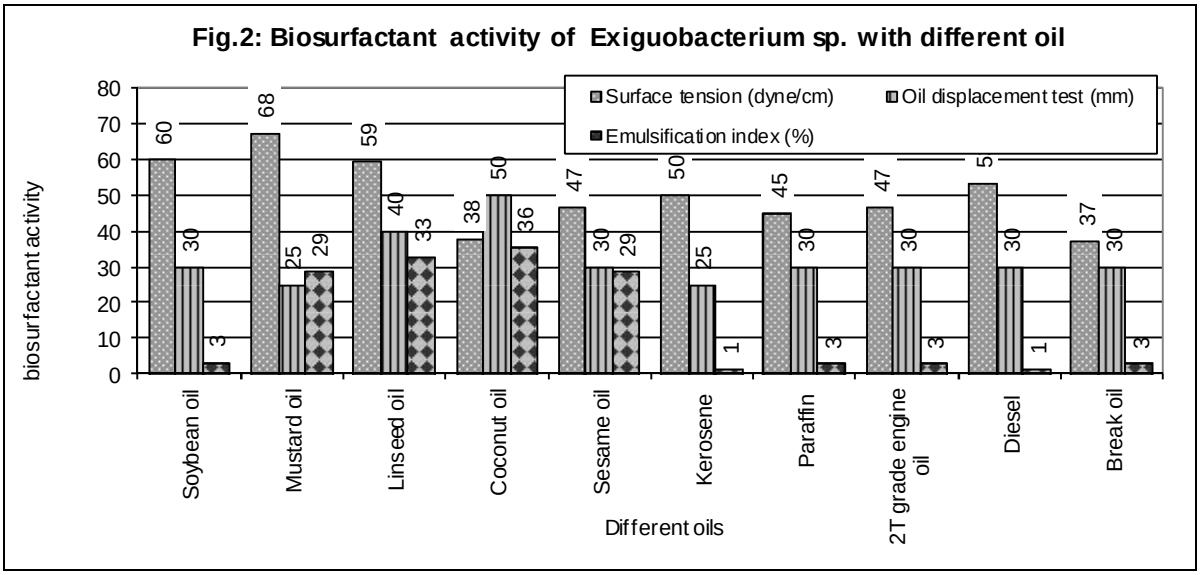


Fig. 1: Phylogenetic tree based on a comparison of the 16S ribosomal DNA sequences of the isolates, the tree was created by the Bootstrap neighbor-joining method by using MEGA 4 Package

The isolates were further characterized for their ability to produce biosurfactant by using different carbon source including vegetable oil and mineral oil. In order to increase the biosurfactant yield by the bacterial strain, different carbon sources were evaluated for biosurfactant production. All the carbon sources tested favored extracellular production of surface active product by *Exiguobacterium* sp. which was indicated by the reduction in surface tension of the broth as depicted in (fig 1).

Carbon substrates screened for the production of biosurfactant by *Exiguobacterium* sp, coconut oil increased the biosurfactant production significantly compared to other carbon sources in case of vegetable oil. The isolate reduces the surface tension of broth to value 37.93mN/m, and zone of oil displacement was 50mm, while the emulsion formed by the culture supernatant with oil was 35.71% (Fig. 2). Ferraz *et al*, [15] studied the influence of vegetable oil on biosurfactant production from bacterium *Serratia marcescences* by using mineral salt medium containing different oil as carbon source and the result were in agreement with our present work, suggesting that the fatty acid present in coconut oil stimulates the biosurfactant production from the organism where as maximum biosurfactant production was observed in break oil. The isolate reduces the surface tension of broth up to value 37.17 mN/m, and 30 mm of zone of oil displacement. Chandran and Das, [16] studied the biosurfactant production from yeast *Trichosporon asahii* using diesel as carbon source and the result reveled that the isolated degrade the 95% of diesel after 10 days of incubation.

Fig.2: Biosurfactant activity of Exiguobacterium sp. with different oil



Tambekar *et al*, [6] also isolated biosurfactant producing bacteria from Lonar Lake and reported *Achromobacter xylosoxidans* which reduces the surface tension of broth upto 51.60 mN/m. whereas; *Exiguobacterium* sp. isolated from Lonar Lake reduced the surface tension of culture supernatant upto 37.93 mN/m. (Table 2). Biosurfactant is very cost effective hence *Exiguobacterium* sp. can use for the maximum production of biosurfactant using coconut oil as a substrate. Tabatabaee *et al*, [9] studied the effect of different range of pH, salt concentration and temperature on biosurfactant production by *Bacillus* sp. isolated from oil reservoirs.

Study reports that the surface tension of whole broth selected strains maintained nearly constant at all tasted pH (4.2-9.2), indicating that pH variation has no appreciable effect on surface tension. But maximum of surface tension reduction was at pH range from 6.2-7.2.Maximum surface tensions reducing salt concentration were 1, 3 and 5% and also reported that the best temperature for the surface tension reduction for *Bacillus* sp. was between 30^0C-40^0C. In the present study *Exiguobacterium* sp. showed maximum biosurfactant activity at 4% salt concentration (fig. 3), at pH 10 (fig. 4) and at 50^0C temperature (fig. 5) i.e. the biosurfactant produced from the organism can be sustain at high pH, temperature and saline environment and might be helpful for remediation of the polluted site of marine environment.

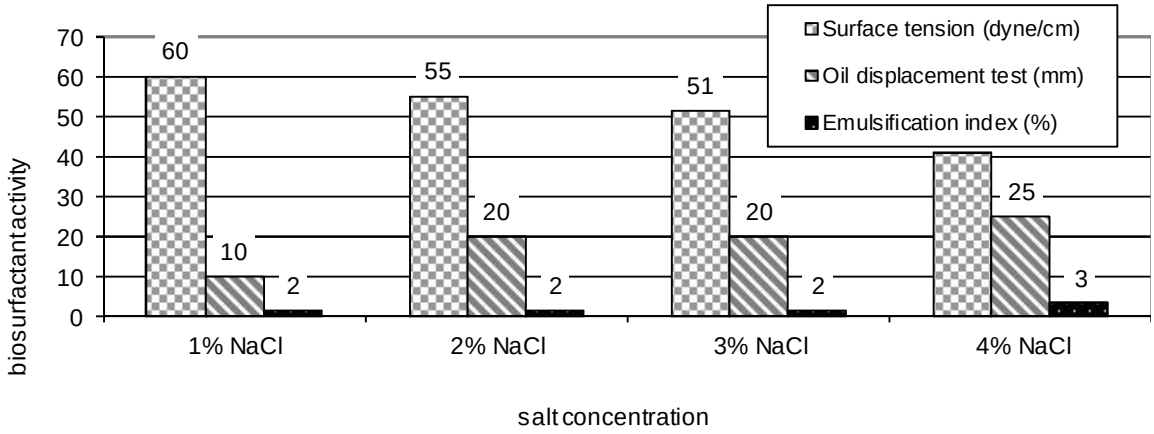


Fig. 3: Effect of salt concentration on biosurfactant production

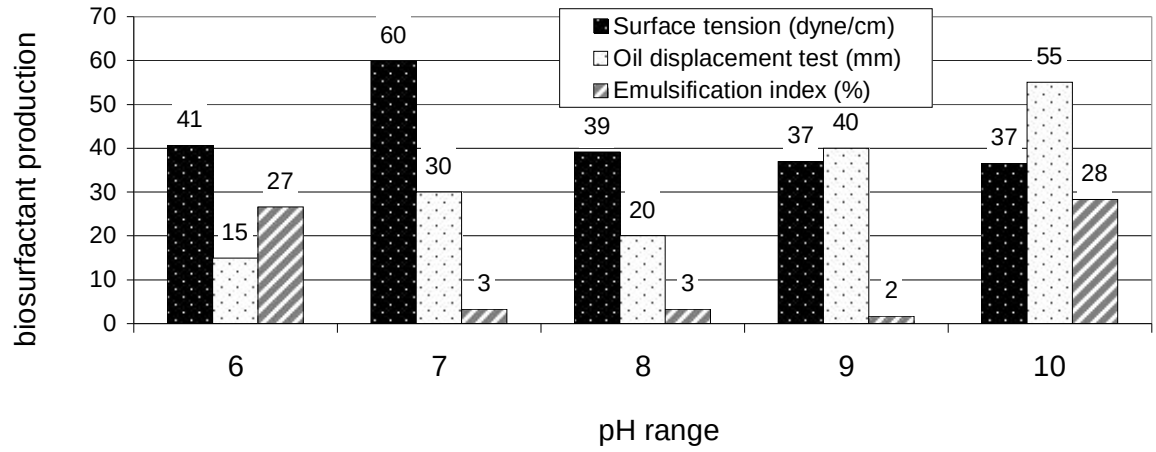


Fig. 4: Effect of pH on biosurfactant production

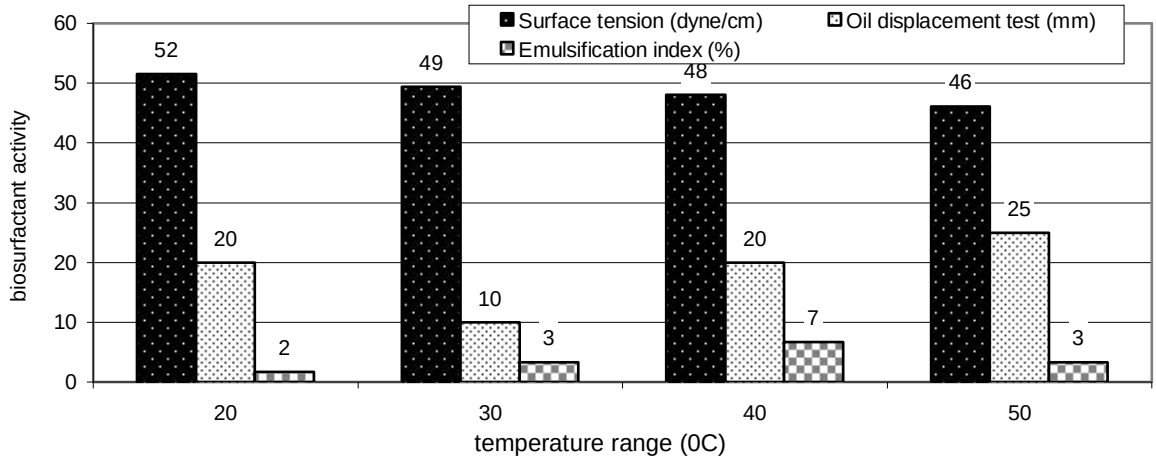


Fig. 5: Effect of temperature on biosurfactant production

CONCLUSION

The strain isolated from Lonar Lake was identified as *Exiguobacterium* sp. showed the ability to utilize the different carbon sources, including vegetable oil and hydrocarbon source, but the maximum biosurfactant production was found in presence of coconut oil suggesting the easily degradable source of fatty acid and also proved the ability of the *Exiguobacterium* sp. to utilize hydrocarbon source. The biosurfactant produced from the organism can

be sustain at high pH, temperature and saline environment and might be helpful for remediation of the polluted site of marine environment. Present study also provides an emerging field to the researchers for the biosurfactant production from alkaline Lonar Lake.

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