Studies on biosurfactant from Exiguobacterium sp

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ABSTRACT

Biosurfactants are naturally occurring surface active compounds produced by micro-organism, which reduces surface tension. Lonar Lake is unique with alkaline water having average pH 10.5 harbors diverse microorganisms having potential to produce variety of biotechnological products. The study is focused on production of biosurfactant by using bacteria Exiguobacterium sp. isolated from Lonar Lake. Twelve samples of sediment, matt and water were screened on mineral salt medium containing 2% soybean oil as a carbon source. The bacterial strain *Exiguobacterium* sp having potential to produce biosurfactant is isolated and identified by standard biochemical tests and 16S rRNA sequencing. Surface tension measurement, oil displacement test and emulsification index methods were used to screen the capability of isolates for biosurfactant production. The study revealed that maximum biosurfactant production was found in coconut oil and break oil. Also the bacterium is able to grow and produce biosurfactant over a wide range of pH, salinity, and temperature. The results of present study suggested that the isolate might be helpful for remediation of oil at polluted site of marine environment.

Key words: Biosurfactant, Lonar Lake, *Exiguobacterium* species, Bioremediation

INTRODUCTION

Lonar Soda Lake is situated in Buldhana District of Maharashtra State, in India. Lonar Lake was formed due to meteoroid impact on basaltic rock about 50,000 years ago. It is one of the largest craters in the world (Deshmukh *et al*, 2011). The Lonar Lake is unique due to its high salinity and alkalinity. The lake water is alkaline in nature with pH ranges from 9.5 to 10. It is a blocked type lake with no outlet or tributary. It induces researchers to investigate its biodiversity values due to its unique nature. Because of much stress given on its biodiversity properties, it remains uncharted pertaining to its potential of biosurfactant producers. In recent years the presence of alkaliphilic microorganisms in slime, matt and water of the lake it attracts much attention of investigators because they have ability to produce different industrially important extra cellular enzymes and substances that are active and stable at high pH values (Tambekar and Dhundale, 2012).

Biosurfactant are amphiphilic compounds produced on living surface mostly such as microbial cell surfaces, excreted extracellularly and contain hydrophobic and hydrophilic moieties that decrease surface tension and interfacial tension between individual molecules at the surface and interface respectively (Priya and Usharani, 2009). Surfactants are basic precursors, which are used in detergents, shampoos, toothpaste, oil additives and a number of other consumer and industrial products. Biosurfactants are complex molecules covering a wide range of chemical types including peptides, fatty acids, phospholipids, glycolipids, antibiotics, lipopeptides, etc. (Anandaraj and Thivakaran, 2010).

As biosurfactant are potential precursors for many applications in industries the development of such trend of research is of principal importance, mainly due to the environmental concern. The ecological acceptance is most important advantage of a biosurfactant over chemical surfactant as it is biodegradable and nontoxic to environment (Sekar *et al*., 2010). Tambekar *et al.,* (2012) reported to produce biosurfactant from Lonar lake bacterium *Achromobacter xylosoxidans* using soybean oil.

Satpute *et al.* (2010), reported the biosurfactant and bioemulsifier producing ability of marine bacterial isolates including *Acinetobacter, Arthrobacter, Pseudomonas, Halomonas, Myroides, Corynebacteria, Bacillus* and *Alteromonas* sp. the result indicated that the isolates have immense ability to produce surface active agent. Tambekar and Gadakh, (2012) had isolated and studied the biosurfactant producing bacteria from hydrocarbon contaminated soil. Synthetic surfactants have been used in the oil industry as a medium to clean up oil spills, as well as to increase oil recovery from oil reservoirs. Such synthetic surface active agents are non biodegradable and add toxic compounds in the environment. Biosurfactant have special advantage over their commercial chemically (synthetically) manufactured counterparts because of its low toxicity, biodegradable nature, and effectiveness at higher temperature, pH, salinity and simplicity of synthesis (Tabatabee *et al.,* 2005). In the backdrop of above information, present study focused on the isolation of biosurfactant producing bacteria from Lonar Lake and to study stability of produced biosurfactant at different pH, temperature and salt concentration. There potential of producing biosurfactant on different carbon sources was also determined in this study.

MATERIALS AND METHODS

Collection of samples: Samples of water, matt and sediment were collected from different locations of the Lonar Lake during August, 2012. Water samples were collected in sterile plastic tight (screw) caped bottle whereas sediment and matt samples were collected in pre autoclaved sterile zip lock bag and were stored at 4^0C prior to analysis.

Isolation, Enrichment and biochemical characterization: Sediment, matt (1g) and water (10ml) samples from Lonar Lake were individually inoculated in 250ml Erlenmeyer's flask previously containing 100ml mineral salt medium. The mineral salt medium was composed of $NaNO_3$ 2.5g/l; KCl 0.1g; KH_2PO_4 3.0g/l; K_2HPO_4 7.0g/l; $CaCl_2$ 0.01g/l; $MgSO_4.7H_2O$ 0.5g/l and 5ml/l trace element solution containing $FeSO_4.7H_2O$ 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. These flasks were incubated at 37^0C at 200 rpm on rotary shaker for 7 days and same procedure was consecutively repeated 5 times for enrichment of bacterial culture (Namir *et al.,* 2008). After enrichment of culture, the broth was inoculated on solid nutrient agar plate. The well isolated and morphologically distinct colonies were selected and stock culture was prepared. All these isolates were further characterized by following standard biochemical test as per Bergey's manual of systematic bacteriology.

Preliminary screening for biosurfactant production

Surface tension measurement: 5 ml inoculum of the bacterial cultures were added to 250 ml Erlenmeyer flask containing 100 ml mineral salt medium with 2% soybean oil as a sole source of carbon. The experimental flasks were incubated at 37^0C on rotary shaker at 200 rpm. After 5 days incubation broths were centrifuged at 8000 rpm for 20 min for cell removal. The cell free supernatant was collected in sterile flask. The reduction in surface tension of cell free broth was determined by Stalagmometer using drop counting (collapse) method (Morikawa *et al*., 1993).

Oil spreading method: 50 ml of distilled water was added to the Petri dish followed by addition of 20 µl of soybean oil. A thin layer was allowed to form on water surface. Later 10 µl of cell free culture supernatant was trickled on oil surface. The diameter of zone of clearance of oil surface was measured immediately (Maneerat and Phetrong, 2007).

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 it vigorously for 2 min, it was then allowed 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) (Cooper and Gondenberg, 1987).

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. These 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 (Altschul *et al*., 1997).

Biosurfactant production on different carbon source: The effect of different carbon source on biosurfactant production by *Exiguobacterium* sp. was observed by providing different vegetable oil and petroleum oil as a substrate. Their 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. The reduction in surface tension and emulsification index of culture supernatant was determined (Tabatabee *et al*., 2005).

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 (Tabatabee *et al*., 2005).

Effect of different temperature on biosurfactant production: The effect of temperature on biosurfactant production was investigated by incubating the broths at 200 rpm for 5 days under controlled temperature conditions (20-50^0C). The reduction in surface tension and emulsification index of culture supernatant was determined (Tabatabee *et al*., 2005).

RESULTS AND DISCUSSION:

Out of total 12 (sediment, matt and water) samples collected from Lonar Lake two bacterial strains (AMS1) and (AMS2) having ability to produce biosurfactant were isolated and further characterized on different carbon sources. The isolates were characterized biochemically. The result of biochemical characterization showed that, both isolates were gram positive short rods, sluggishly motile and ferment glucose, galactose, fructose, mannitol, dextrose, trehalose and sucrose with production of acid. The strains were unable to ferment raffinose and lactose, however the difference in sugar fermentation between the isolates was observed only in case of arabinose and sorbitol (Table 1).

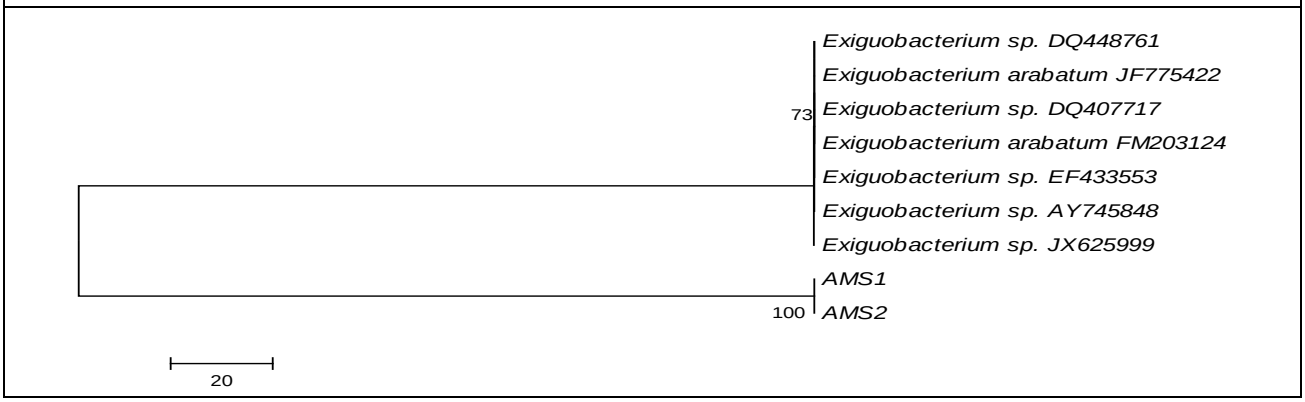
Table 1: Morphological and biochemical characteristics of *Exiguobacterium* species

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	+	+

Even if the bacterial isolates differs in some biochemical characteristics the result of the 16S rRNA sequencing result showed that both isolates belong to genus *Exiguobacterium*. Therefore further characterization for biosurfactant production was performed with strain AMS1.

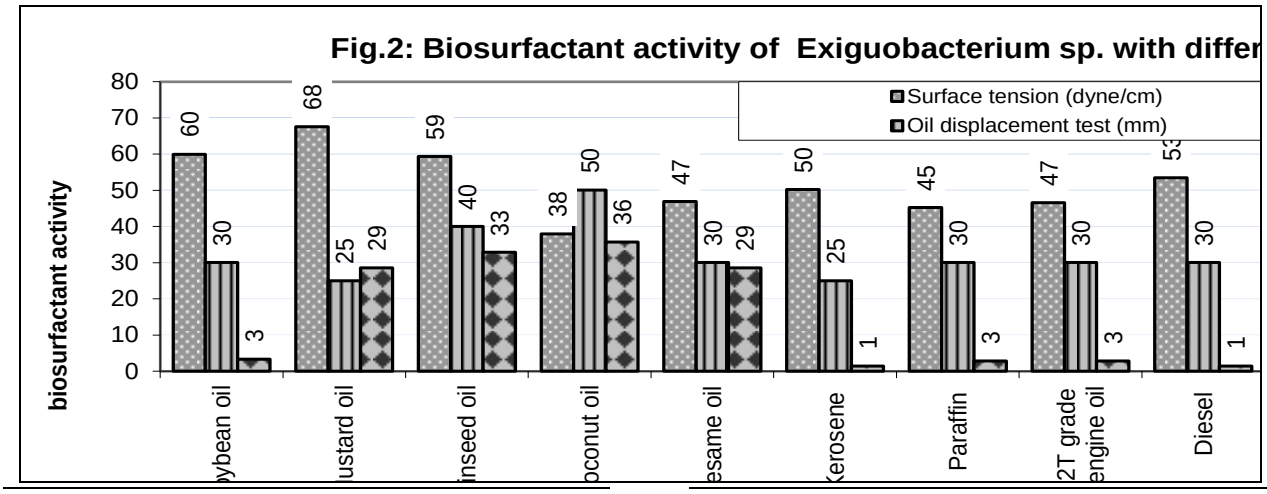
Bootstrap analysis was used to evaluate phylogenetic tree stability according to a consensus tree from the neighbor-joining based on 1,000 replicates for each. Phylogenetic analysis based on 16S rRNA gene sequences indicated that strain ASM1 and ASM2 were affiliated to phylum Firmicutes and genera *Exiguobacterium* (Fig 1). It shows highest similarity values with the sequences of obligate 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 with different carbon sources such as vegetable and mineral oils. For increasing the biosurfactant yield with the isolated bacterial strain, different carbon sources were evaluated for biosurfactant production. All the carbon sources tested favored extracellular production of surface active substance with *Exiguobacterium* sp. which was indicated by the reduction in surface tension of the broth as depicted in (fig 1).

It was observed that bacteria yielded significantly more quantity of biosurfactant with coconut oil as compared to rest of the different vegetable oils. 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*., (2002), studied the influence of vegetable oil on biosurfactant production with bacterium *Serratia marcescences* by using mineral salt medium containing different oil as carbon source. The result was similar as the result of present work; study suggested that fatty acids present in coconut oil stimulate the biosurfactant production.



When comparison of biosurfactant production was done between coconut oil and break oil with the same organism it was observed that more biosurfactant was produced by using break oil than with the coconut oil by the same bacterial strain. 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, (2010) studied the biosurfactant production from yeast *Trichosporon asahii* using diesel as carbon source and the result revealed that the isolated degrade the 95% of diesel after 10 days of incubation.

Tambekar *et al.*, (2012), 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. Tabatabee *et al.*, (2005), 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 substantial effect on surface tension. Maximum surface tension reduction was reported at pH range from 6.2-7.2. Maximum surface tensions reducing salt concentration were 1, 3 and 5%. It was also reported that the optimum 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 uphold 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

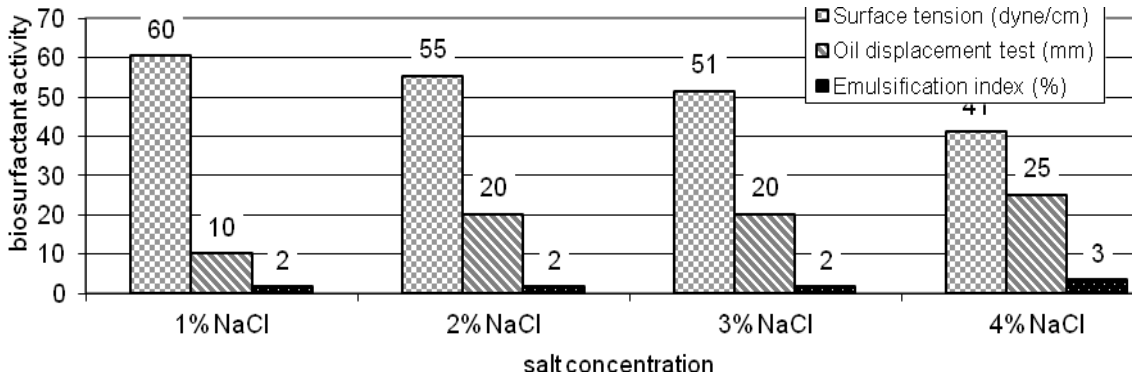


Fig 4: Effect of pH on biosurfactant production

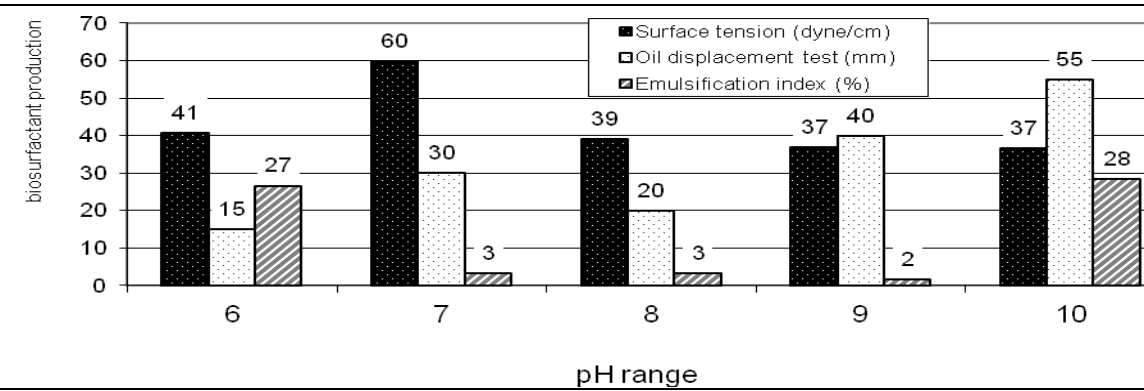
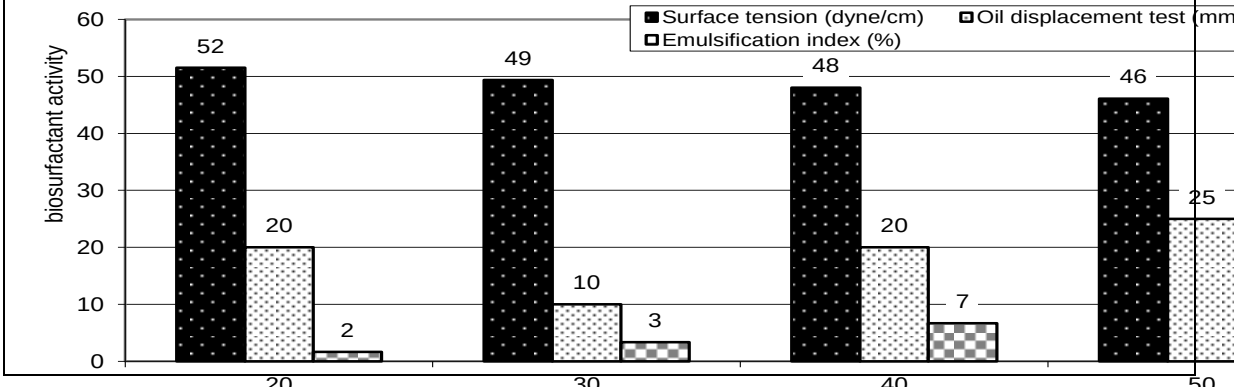


Fig 5: Effect of temperature on biosurfactant production



CONCLUSION

The bacterial strain isolated from Lonar Lake was identified as *Exiguobacterium* sp. It showed potential to utilize different carbon sources, such as vegetable oil and hydrocarbon source, however the maximum biosurfactant production was observed with coconut oil as a sole source of carbon. It was easily degradable source of fatty acid. The study also proved the ability of the *Exiguobacterium* sp. to utilize hydrocarbon source. The biosurfactant produced from isolated organism sustained at high pH, temperature and saline environment, which might be helpful for remediation of the polluted site of marine environment. Present study also opens a promising field to the researchers regarding biosurfactant production from organisms inhabiting in alkaline Lonar Lake and other similar water reservoirs.

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