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# Isolation and characterization of biosurfactant producing bacteria from alkaline Lonar Lake

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**Abstract**
Biosurfactant are the surface active agent produced by the microorganisms and is amphiphilic in nature. They are considered to be a strong alternative to chemical surfactant as low toxicity and biodegradability. Alkaline Lonar Lake is a unique environment and rich in diversity of extremophiles with a source of industrially important microorganisms. In present study 20 sediment samples collected from Lonar lake six different isolates having ability to produce biosurfactant were isolated by enrichment process and continuous sub culturing. Out of them an isolate L2 showed a great potential to produce biosurfactant with respect to reduction in surface tension, oil displacement test, drop collapse test and emulsification index when coconut oil was used as carbon source. The highest reduction in surface tension 20.30 mN/M with 37.58% water oil emulsion formation was recorded when coconut oil was used as carbon source. The result of biochemical characterization and 16S rRNA sequencing showed that the isolate L2 was identified as *Bacillus circulans.* The isolate showed a characteristic feature for potential to produce biosurfactant and required a further investigation.

**Keywords:** biosurfactants, bacillus circulans, 16s rRNA, surface tension

## Introduction

Surface active agent that allow or promotes 'wetting' of a soiled surface and dispersion or suspension (emulsification) of greasy soil in a solution are known as surfactants. Surfactants achieve this by reducing the surface tension of the solvent (such as water) or the interfacial tension between two non-miscible liquids such as water and oil. They are amphiphilic in nature which makes them bifunctional (Naveen kumar *et al*., 2010) [14]. Surfactants and emulsifiers are widely used for food, cosmetic and pharmaceutical applications. In recent years great emphasis has been given to the environmental impacts caused by chemical surfactants due to their toxicity and difficulty in being degraded in the environment (Van *et al.,* 2006) [24]. Increasing environmental concerns, the advance in biotechnology led to biosurfactant being a potential alternative to the chemical surfactants available on the market (Banat *et al.,* 2000) [1]. Although biosurfactant have promising use in bioremediation processes, their industrial scale production is currently difficult due to high raw material costs, high processing costs and low manufacturing output (Henkel *et al.*, 2012) [8]. As a result, the current research challenges are to increase the yield and to reduce the cost of raw materials (Mukherjee *et al.,* 2006) [13]. The large majority of the currently used surfactants is petroleum-based and is produced by chemical means. These compounds are often toxic to the environment and their use may lead to significant environmental problems. Biosurfactants can be as effective synthetic chemical surfactants and for certain applications they have advantages such as high specificity. Most of the biosurfactants and many chemical surfactants employed for bioremediation purposes are biodegradable (Liu *et al*., 2004)**.**

One of the unique habitat with saline and hyper alkaline ecosystem formed entirely on basalt rock by meteor impact in Maharashtra state (India) is Lonar Lake. The crater located in India ~550 km east of Mumbai and Arabian Sea is 150 m deep and 1830 m across (Fredriksson *et al.,* 1973) [6]. A raised him up to 100 m in width and 20 m to 30 m high forms an almost continuous ring and acts as a divide for the run-off of rain water flowing into the Lake. The Lake is an extreme and unique environment with alkalinity ranging from 2.1-3.2 g/L $CaCO_3$ in water; 6.85-14.9 g/L $CaCO_3$ in sediment; salinity in terms of NaCl ranging from 8.3- 9.3 g/L in water: 8.8-15.6 g/L in sediment; pH ranging from 9.5-10 for water and 9.5-10.5 for sediment (Tambekar *et al.,* 2010; Surakashi *et al.,* 2010) [21, 19]. Both culture based studies may be employed to reveal the diversity of bacterial and archaeal communities in lake these studies may yield a number of novel bacteria (Joshi *et al.,* 2008) [9]. Bushnell and Hass, (1941) were among the first to demonstrate bacterial production of biosurfactants by isolating *Corynebacterium simplex* and a strain of *Pseudomonas* in a mineral media containing either kerosene, mineral oil or paraffin. Bicca *et al.,* (1999) [2] documented biosurfactant production by *Rhodococcus rubber* 239 with an emulsification index of 63%. More recently (Kim *et al.,* 2000) [10], purified two types of bio surfactants from *Nocardia* sp. L-417 that were stable over a broad range of pH and temperatures.

Recent day's pollution of the water and soil environment occurs due the uncontrolled and excessive use of petroleum product which causes a serious damage to the environment. Some organisms have an ability to produce such extracellular compounds while growing in such water immiscible substrate. Deshmukh *et al.,* (2011) isolated seventy four bacteria from this site, out of these *Alcanivorax sp*. was reported first time which is well known genus for its oil degradation capacity,

indicating the probable existence of oil reservoir in vicinity of Lonar Lake. Biosurfactant producing bacteria has potential application for remediation of hydrocarbon contaminated sites and also helpful in many industrial applications including petroleum, food, agricultural, pharmaceutical, cosmetic, oil and pulp industry. Therefore, culture dependent approach was used to isolates oil degrading organisms from Lonar Lake which depends completely on hydrocarbons as a sole source of carbon and probably proved a milestone in this regards. As they are potential candidates for many commercial applications in the petroleum and food processing industries. The potentials of biosurfactants with antimicrobial properties in combating diseases in a world plagued with an increasing prevalence of antibiotics resistance are certainly enormous. Thus, microbial surfactants are expected to be among the most versatile process chemicals for use in the near future. In keeping with this objective, we investigated abilities of bio surfactants producing bacterial strains isolated from Lonar Lake.

## Materials and Methods

The sediment samples were collected with the help of a scooper in sterile polyethylene bags, while water samples were collected directly into sterile bottles. About 20 different samples were collected from the sampling site.

### Isolation of Bacteria

The biosurfactant producing bacteria were isolated using mineral salt agar having composition as described by Haddad *et al.,* (2008) only the addition of 2% oil as carbon source to confirm the ability of isolate to utilize oil as carbon source. From the sample 1 gm sediment sample was inoculated to 250 ml flask containing 100 mL media. The flask was shaken well at $37^0$C and samples were allowed to incubate on rotary shaker for 7 days at 120 rpm for enrichment. After the enrichment the incubated sample were inoculated on solid agar plate and well isolated colonies were selected for further process

### Preliminary screening of biosurfactant /bioemulsifier producing bacteria

The screening of isolate for ability to produce biosurfactant was done using surface tension measurement of cell free broth, oil displacement test, drop collapse test and emulsification index measurement as suggested by Soudi *et al*., (2009) [18].

**Biochemical characterization and 16S rRNA sequencing** the isolates showed the ability to produce biosurfactant were further confirmed by biochemical characterisation and were identified by 16S rRNA sequencing and phylogenetic analysis was done using software MEGA version 4.0 by neighbor joining algorithm (Tamura *et al*., 2007) [22] and the sequence were deposited in the gene bank and accession number was taken.

### Effect of Temperature, pH and salt concentration

The effect of different parameters like, temperature, pH and salt concentration was studied in laboratory set up. The selected variations for temperature $20^0$C, $30^0$C, $40^0$C and $50^0$C., for pH 5, 6, 7, 8 & 9 while different salt concentration are 1%, 2%, 3%, 4% & 5% were used for the characterization of biosurfactant.

## Result and Discussion

From the 20 soil sediment samples collected from the Lonar Lake four different isolates were isolated which showed the ability to produce biosurfactant when grown on mineral salt medium. From these four isolates one isolate L2 was Gram positive motile. The isolate also aerobic, short rod and spore former. The organism give all indole, methyl red, citrate utilization test negative while unable to ferment the sugars except salicine. Apart from the isolate most of the isolates were gram negative short rods and ferment different sugars and result are in agreement with Bicca *et al*., (1999) [2], as dominance of gram negative bacteria in biosurfactant production, while are contradictory with respect to the isolate from Lonar lake. Tambekar *et al*., (2012) [20] had isolated gram negative biosurfactant producing bacteria *Achromobacter xylosoxidans* from the Lonar lake. Further the isolate was used for the production of biosurfactant using cheap raw substrate like different oil were used as substrate. Franzetti *et al.,* (2011) [5] studied the bioemulsifier producing bacteria by using different isolation media containing low cost substrate for cost effective production of biosurfactant. Besides, Thavasi *et al.,* (2011) [23] indicates that there is a possibility of biosurfactant production using renewable, relatively inexpensive and easily available resources. Hence, it is imperative that similar initiatives can be taken with the organisms identified in this study. Mukherjee *et al.,* (2006) [13] described some practical approaches that have been adopted to make the biosurfactant production process economically attractive: these include the use of cheaper raw materials, optimized and efficient bioprocesses and overproducing mutant and recombinant strains for obtaining maximum productivity. The result revealed that isolate gives positive drop collapse test for oils used for study except castor oil and hexadecane. The isolate also showed ability to reduce the surface tension of cell free broth with oil sources. The highest reduction in surface tension upto 20.30 mN/m was recorded when coconut oil was used as carbon source as coconut oil is easy source of carbon for microbial growth, while the negligible reduction in surface tension was recorded with paraffin and engine oil. Tambekar *et al*., (2012) [20] studied the biosurfactant production from *A. xylosoxidans* and recorded the similar results for reduction in surface tension when soybean oil was used as carbon source.

**Table 1:** Biosurfactant production using different oil as carbon source

| Carbon source | Drop collapse test | Surface tension (mN/m) | Oil displacement test (mm) | Emulsification index (%) |
|---|---|---|---|---|
| Coconut oil | + | 20.30 | 50 | 37.58 |
| Castor oil | - | 46.96 | 15 | 6.67 |

| | | | | |
|---|---|---|---|---|
| Mustard oil | + | 41.28 | 45 | 22.22 |
| Soya oil | + | 54.5 | 25 | 6.45 |
| Olive oil | + | 44.85 | 15 | 32.7 |
| Hexadecane | - | 48.62 | 20 | 15.30 |
| Kerosene | + | 45.93 | 20 | 24.19 |
| Paraffin | + | 66.63 | 20 | 20 |
| Diesel | + | 48.07 | 15 | 11.61 |
| Engine oil | + | 53.16 | 25 | 16.5 |

The isolate L2 also showed the highest oil spreading/ displacement activity which a measurement of biosurfactant production, the highest oil displacement activity 50 mm was recorded with coconut oil while the oil displacement zone 45 mm was recorded with mustard oil was used as carbon source. Chander *et al*., (2012) [4] had also used the mustard oil as a carbon source for biosurfactant production from *B. subtilis* which showed low oil displacement activity in mustard oil compared to other carbon sources, while similar emulsification activity was reported in present work with mustard oil. The lowest oil displacement activity was recorded 15 mm with castor oil, olive oil and diesel was used as carbon source. Similarly Mukherjee *et al.*, (2009) [12] isolated the biosurfactant producing B. circulans from marine sample using glucose salt medium. The isolate L2 also showed the emulsification activity with the used carbon source. The highest emulsification activity 37.58% was recorded with coconut oil while the lowest emulsification activity was recorded with soybean oil. The isolate also showed the ability of emulsification with mustard oil, olive oil, kerosene and diesel was used as carbon source.

After the confirmation of ability to produce biosurfactant and biochemical characterization the isolate was identified by the 16S rRNA sequencing and phylogenic analysis was done. The phylogenetic trees were constructed using the isolate from our study and there related phylogenetic neighbors identified by Ribosomal Database Project (RDP-II) using the MEGA 4.0 software program (Tamura *et al.*, 2007) [22]. The phylogenetic trees were constructed using the neighbor-joining (NJ) algorithm (Saitou and Nei, 1987) a distance matrix were determined using Kimura's, (1980) model for 16S rRNA gene.

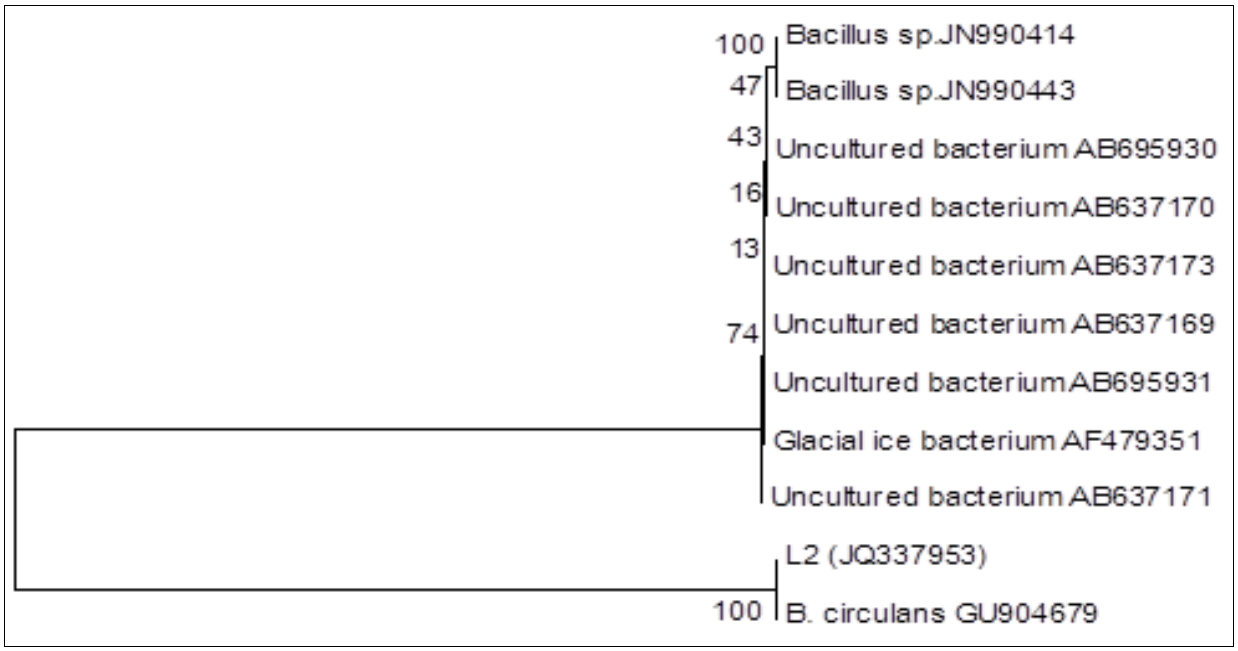


**Fig 1:** Phylogenetic tree of biosurfactant producing bacteria isolated from alkaline Lonar Lake

The result of 16S rRNA sequencing and phylogenetic analysis revels that the isolate L2 was identified as *Bacillus circulans*. The phylogenetic tree showed that, the strain L2 showed a highest degree of similarity with the type strain of genus *Bacillus* and substantial degree of relatedness to reference 16S rRNA sequences of *Bacillus* in the database. The biosurfactant producing strain L2 from our study, showed maximum value of similarity (100%) with reference strain *Bacillus circulans* (GU904679), isolated from the Baobab fermented seeds (Maari) obtained from four different production sites in Burkina Faso Parkouda *et al*., (2010) [15]. The sequence of strain L2 was submitted to NCBI with accession number JQ337953.

The optimization study for the biosurfactant production by the isolate at different pH, salt concentration and temperature was carried out. The result of the optimization study reveals that the isolate L2 (JQ337953) showed the highest biosurfactant activity with respect to reduction in surface tension and emulsification index was recorded at optimum environmental condition that are 7 pH at $37^0$C with 1% salt concentration. Phitnaree *et al.*, (2008) [16] studied the production and application of biosurfactant produced by the isolate *B. subtilis*.

The isolate showed the highest biosurfactant activity at pH 7, temperature $37^0$C and in absence of salt concentration.

## Conclusion

The unique Lonar environment harbour the diversity of different biotechnologically important microorganisms having potential to produce different industrially important enzymes and secondary metabolites. Apart from these the microorganisms are able to utilize the hydrocarbons as source of carbon and produce a surface active compound which will be needed further investigation and may leads to isolation of potential biosurfactant producing bacteria which will be useful in industry as well as in remediation process of polluted sites.

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