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Research Article

Studies on Biosurfactant Production from Lonar Lake's *Achromobacter xylosoxidans* Bacterium

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ABSTRACT

The alkaline Lonar Lake is situated in Buldhana district of Maharashtra is the only meteoritic impact crater in basaltic rock with high alkalinity and salinity. Biodiversity of this lake regarding the industrially important enzymes was studied, but biosurfactant producing bacteria from this was not studied so far. In this study, four sediment and water samples were collected and screened on mineral salt medium containing 2% oil as a carbon source. The isolate gives positive oil displacement test, drop collapse test and reduce surface tension of cell free broth upto 51.60 mN/m. The result of 16S rRNA sequencing showed that the organism was *Achromobacter xylosoxidans* which may be used for the remediation of the petroleum contaminated sites and helps for exploration of this alkaline environment.

Keywords: Lonar Lake, biosurfactant, hydrocarbon degradation, remediation.

INTRODUCTION

In India, the Lonar lake is the world third largest and the only hyper velocity meteoritic impact crater formed about 50,000 years ago situated in Buldhana district (Lat 19°58′, Long 76°36′) of Maharashtra, India. It is a closed system without outlets and regular influents are responsible for its existence. The diameter around the Lake is about 1.75 Km and water enters the Lake through rain, ground water seepage and it does not receive any industrial discharges[1]. The Lonar Lake is unique in the world for its alkalinity and salinity and Lake Water is alkaline (pH 10.5) and highly saline (6391 mg/l). The microbial diversity of saline lakes has been studied primararily by focusing on the isolation and characterization of individual organ-isms with potential industrial application [2,3,4,5].

Joshi *et al* [6], studied the sediment and water samples from Lonar Lake and isolated and identified 196 bacterial strains based on 16S rRNA analysis. These bacterial strains are alkaliphilic, containing Low G+C and produce different types of biotechnologically important enzymes and belong to the phylum Firmicutes. Kanekar *et al* [7] isolated phenol reducing alkaliphilic bacteria from Lonar lake sediments was an interesting finding. Wani *et al* [8], isolated the Cr (VI)-reducing bacterial strain MCMB-821 from the alkaline crater Lonar and identified as *Burkholderia cepacia* which explore and attract many researcher around the country towards this unexplored environment.

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 [9]. Deshmukh *et al* [10], 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 [11]. Therefore, attempt was made 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.

MATERIALS AND METHODS

Sampling Sites and Sample collection

Soil and water samples comprising of two sediment samples and two water samples from different sites of the Lonar Lake were collected during July, 2011 in sterile plastic tight cap bottle and sterile zip lock bag respectively and were stored at 4^0C till analyze [6].

Enrichment of samples

Soil (1g) and water (10ml) samples from Lonar Lake were separately inoculated in 250ml 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 200rpm on rotary shaker for 7days and same procedure was successively repeated for enrichment.

Isolation and biochemical characterization

After enrichment the samples were inoculated on solid nutrient agar plate and well isolated and morphologically different colonies were selected and stock culture were prepared and stored at 4^0C. All these isolates were further characterized by standard biochemical test according to Bergey's manual of systematic bacteriology.

Preliminary screening for biosurfactant production

Biochemically characterized isolates were cultured in mineral salt medium and incubated at 37^0C for 7days at 200rpm on rotary shaker. After incubation broth was centrifuged at 8000rpm for 20min oil layer was discarded and supernatant were subsequently subjected for the preliminary screening.

Surface tension measurements

Surface tension reduction was measured using Krüss Hamburg Nr2215 Tensiometer by submerging the platinum ring in culture supernatant and the force required to pull it through the air–liquid interface was recorded. The results were compared to distilled water and medium composition as negative control[12].

Oil spreading method

Fifty milliliter of distilled water added to the petridish followed by addition of 20µl of soybean oil, thin layer was allowed to form on water surface. Then 10µl of cell free supernatant was dropped on oil surface. The diameter of zone of clearing of oil surface was measured [12].

Drop collapse method

A modified drop collapse method was performed by using 96 well micro titer- plates containing 10µl soybean oil was equilibrated for an hour at room temperature. Then 10µl of culture supernatant was added to the surface of well and observed after 1 minute for collapsing of drop. Drop collapse test was considered positive when the drop diameter more than those produced by distilled water.

Emulsification index (E_{24})

E_{24} of samples was determined by adding 2ml of soybean oil to 3ml of culture supernatant and vortex for 2 min with high speed and leave to stand for 24 hours. The E_{24} index is given as percentage of height of emulsified layer (mm) divided by total height of the liquid column (mm) [13].

$$E_{24} = \frac{\text{Height of emulsion layer}}{\text{Height of total solution}} \times 100\%$$

Identification of bacteria on the basis of 16S rRNA sequencing

The culture showing biosurfactant production was further submitted to NCCS, Pune for 16S rRNA sequencing. The resulting sequences were compared with sequences in the Gene bank database of NCBI[14] using the BLAST network service. Sequences obtained were submitted to RDP and match with known sequence.

Methods for estimation of residual oil

The method for estimating of residual oil in liquid broth was different from that for solid stuffing. The residual oil in broth was extracted with petroleum ether. For extraction of residual oil 10ml petroleum ether was added in the flask, after vigorous shaking flask were allowed to stand for 10min and organic layer was collected by separating funnel. Oil layer containing petroleum ether was evaporated on sand bath and gravimetric estimation was made by weighing the quantity of oil in the beaker[15, 16].

RESULTS AND DISCUSSION

While studying the biosurfactant producing bacteria from alkaline Lonar Lake, a total of four samples comprising of sediment and water samples during rainy season 2011 were collected and processed strictly on oil containing media. After sub-culturing for 4-5 times bacteria were isolated on solid nutrient agar plate. The bacteria were analyzed for standard biochemical test and further identified by 16S rRNA sequencing as *Achromobacter xylosoxidans*.

Table 1: Morphology and biochemical characteristic of bacteria

Test	Result	Test	Result	Test	Result
Colony shape	Circular	VP test	Negative	Celloboise	Negative
Colour	White	Citrate Utilization	positive	Sorbitol	Negative
Gram staining	Negative	Maltose	Negative	Trehalose	Positive
Shape	Short rod	Lactose	Negative	Salicin	Negative
Arrangement	Single	Dextrose	Positive	Sucrose	Negative
Urease	Negative	Mannitol	Negative	Fructose	Negative
Starch hydrolysis	Negative	Xylose	Negative	Growth at 4^0C	Negative
Indole	Negative	Arabinose	Negative	At 42^0C	Positive
Methyl red	Negative	Raffinose	Negative	At 6.5% NaCl	Positive
Bacteria on the basis of 16S rRNA : *Achromobacter xylosoxidans* subsp. xylosoxidans; CBMAI 709					

All selected isolates were gram negative short rod, aerobic and motile and ferment only dextrose and trehalose with acid production and utilized citrate (Table 1).

The isolates when studied for the biosurfactant production gives positive drop collapse test, oil displacement test and emulsify the oil layer upto 58%. Tambekar *et al*, [17] 2012 also isolated the biosurfactant producing bacteria from petroleum contaminated soil, and reported *Pseudomonas aeruginosa* isolate emulsify the oil layer upto 57.14% and reduces the surface tension of broth upto 47.19mN/m whereas, ***Achromobacter xylosoxidans*** isolated from Lonar Lake reduced the surface tension of culture supernatant upto 51.60 mN/m after 3days of incubation which was initially 64.65 mN/m and confirm the biosurfactant production (Table 2).

RIBOSOMAL DATABASE PROJECT

GTCACTTCACGCGTTAGCTGCGCTACCAAGGCCCGAAGGCCCCAACAGCTAGTTGACATCGTTTAGGGCGTGGACTACCA
GGGTATCTAATCCTGTTTGCTCCCCACGCTTTCGTGCATGAGCGTCAGTGTTATCCCAGGAGGCTGCCTTCGCCATCGGTG
TTCCTCCGCATATCTACTCATTTCACTGCTACACGCGGAATTCCACCTCCCTCTGACACAC
Achromobacter xylosoxidans strain CBMAI 709 16S ribosomal RNA gene,

Lineage:
Results for Query Sequence: seqmatch_seq, 212 unique oligos
norank Root (10) (match sequences)
domain Bacteria (10)
phylum "Proteobacteria" (10)
class Betaproteobacteria (10)
order Burkholderiales (10)
family Alcaligenaceae (10)
genus Achromobacter (10)

S000000970 - not calculated 0.953 1381 Alcaligenes sp.; 159; AJ002804
S000012321 - not calculated 0.953 1393 Alcaligenes sp.; 151; AJ002802
S000106588 - not_calculated 0.953 1405 Achromobacter xylosoxidans subsp. xylosoxidans; F; AJ491845
S000107277 - not_calculated 0.953 1405 Achromobacter xylosoxidans subsp. xylosoxidans; A1; AJ491839
S000108346 - not_calculated 0.953 1397 Achromobacter xylosoxidans subsp. xylosoxidans; E; AJ491844
S000118379 - not_calculated 0.953 1402 Alcaligenes faecalis subsp. faecalis; 6818m-E; AJ508999
S000354949 -not_calculated 0.958 1407 uncultured bacterium; KRA30+05; AY081976
S000824400 -not_calculated 0.967 1151 Achromobacter xylosoxidans subsp. xylosoxidans; CBMAI 709; DQ413030
S001600795 - not_calculated 0.967 1362 uncultured Achromobacter sp.; F1 jan. 7; GQ416668
S001744292 - not calculated 0.958 1326 [Streptomyces] sp. SDAP 101; GQ408915

After the confirmation of biosurfactant production isolates BW1 was further studied for the hydrocarbon degradation potential by petroleum ether estimation method [15, 16].

Table 2: properties of selected biosurfactant producing bacteria

Isolate	Drop Collapse test	Oil spreading Test	Emulsification Index test	ST of supernatant (mN/m)	ST of media with oil (mN/m)
BW1	+	+	58%	51.60	64.65

Hydrocarbon degradation potential of the isolate shows the percent degradation of about 14.46% for first 24h incubation but as incubation period increases the percent degradation increases upto 25% but rate of degradation of the hydrocarbon decreases as the incubation period increases i.e. percent degradation rate is inversely proportional to the rate of degradation (Fig. 1 and 2)). Although, the largest application of biosurfactant is the oil industry, for petroleum production and incorporation into oil formulations, oil spill bioremediation, removal of oil sludge from storage tanks and enhanced oil recovery[18] (Desai and Banat, 1997) selected isolates will be used for the bioremediation of the petroleum contaminated site as the organism has oil degradation capacity.

In the present study, the potential of the ***Achromobacter xylosoxidans*** to produce the biosurfactant from the alkaline Lonar Lake is indicative of the treasure of the diverse industrially important microorganism which may be exploited further for commercial point of view. This study suggests that the Lonar Lake is the extreme environment or system for the growth of organism. Biosurfactant obtained from organisms isolated from such environment might be useful in extreme environments such as temperate marine compartments and industrial systems where extremes of temperature are integral elements.

Fig. 1: Rate of degradation (mg/ml) of oil

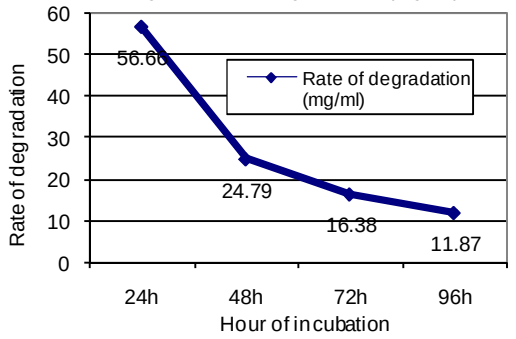
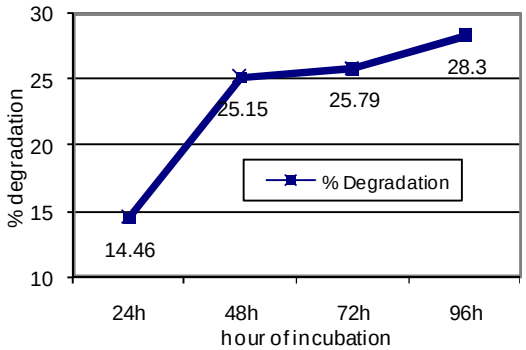


Fig. 2: Percent degradation of oil



Conclusively, this study has shown that *Achromobacter xylosoxidans*, an organism isolated from Lonar Lake has ability to survive on such harsh environment and also has degradative potential for oil. Because of the substrate diversity of this organism it is adjudged as a good candidate for bioremediation of polluted sites and a potential resource for surface- active molecules of industrial importance.

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