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BIOREMEDIATION OF PHENOL BY HALOALKALIPHILIC MICROORGANISMS ISOLATED FROM LONAR LAKE

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Abstract: The alkaline Lonar Lake situated in Buldhana district of Maharashtra state, India, ranking third in the world based on diameter and its high alkalinity (pH 10.5). The aim of the present study was to isolate phenol degrading bacteria from Lonar Lake. From twelve sediment and water samples collected from four different sites of Lonar Lake two bacterial strains were isolated by using peptone water phenol medium. The bacterial strains were biochemically characterized and identified by 16S rRNA sequencing. The result of the sequencing showed that the isolates belong to phylum Firmicutes and the species were *Staphylococcus arlettae* (SDT1) and *Staphylococcus* species (SDT2). The isolated strains were further characterized for there potential to degrade phenol and the phenol degradation was determined by the spectrophotometric method 4-amino antipyrine. Result of the study showed that the isolates (SDT1) and (SDT2) degrade 64% and 75% phenol in the peptone water phenol medium at laboratory level. Thus the isolated gram positive bacteria can be exploited as a candidate of choice for the bioremediation of phenolic effluent.
Keywords: Lonar Lake, Phenol bioremediation and *Staphylococcus arlettae*.

INTRODUCTION

Lonar crater (lat. 19"58'45", long. 76'34') is unique eco-system in the world situated in Buldhana district of Maharashtra, India. The lake is known to be a unique inland saline lake in Asia. It has a circular periphery and diameter of 2 km around the top of the banks and 1.2 km at the bottom. The lake has a high saline level (2649mg/l sodium chloride) and a high level of alkalinity (2605 mg/l calcium carbonate) (Kanekar *et al.,* 1999). It is a natural reservoir of saline water. The Lake harbors diverse microbial flora of alkaliphilic microorganisms growing at pH 8 to 10 or at high salt concentration. The alkaliphilic bacteria isolated from Lonar lake include the genera of *Bacillus* sp*., Flavobacterium* sp*., Aeromonas* sp*., Micrococcoccus* sp*., Archaeobacteria* sp*., Natronobacterium and Natronococcus*, are

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responsible glycosyl transferase pectinases, phosphatases etc., which have industrial applications (Joshi *et al.*, 2007; Tambekar and Dhundale, 2012).

Although contamination of soils and waters with chemically synthesized phenolic compounds is a serious environmental problem, their remediation may be possible using physical, chemical and biological methods, such as bioremediation (Leung *et al.*, 1997; Errampali *et al.*, 1997). Phenol or phenolic compounds are widely distributed in the environment partly as a result of natural processes and more importantly, due to human and industrial activities. These compounds originate mainly from industrial processes such as resin manufacturing, oil refineries, petrochemicals, pharmaceuticals, dyes, textiles and plastic industries (Kumaran and Paruchuri, 1997). As they are persistent in nature and due to its toxic, mutagenic and carcinogenic characteristics they are classified as highly hazardous chemicals (Buckley *et al*., 2000).

Phenol is known to be toxic to both aquatic and terrestrial life including human being and necessary to remove from industrial effluents before discharging them into the environment. Some microorganisms are endowed with the property of degrading phenol but meager data are available on the use of these organisms for treatment of phenol containing industrial waste effluent (Kanekar *et al.*, 1999; Catia *et al.*, 2010). Since some of the phenol-bearing industrial waste waters are alkaline in nature, it was thought worthwhile to explore the use of alkaliphilic bacteria for the removal of phenol. Naturally the alkaliphilic bacteria reside at soda lakes and alkaline springs, but these are uncommon. The most notable lakes and springs for alkaliphilic bacteria are Ashanti Lake, Bosumatwi, Ghana, New Quebec, North America, Lonar, Maharashtra, India, and Canyan Diable, Arizona. Some alkaline environments have been described by Grant and Tindall, (1986). The phenol degrading bacteria present in the Lonar Lake have not been studied in detail. Therefore attempt was made to apply culture dependent strategy to explore the diversity of phenol degraders from Lonar Lake and identification of these degraders based on cultural characters and 16S rRNA analysis.

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.

Enrichment of samples: Sediment, matt (1g) and water (10ml) samples from Lonar Lake were separately inoculated in 250ml Erlenmeyer's flask containing 100ml peptone water phenol medium having composition peptone 0.5% phenol 5% NaCl 5% and were incubated at 37^0C at 200 rpm on rotary shaker for 7days and same procedure was successively repeated 5 time for enrichment of bacterial culture (Kanekar *et al*., 1999).

Isolation and biochemical characterization: After enrichment the broth were inoculated on solid nutrient agar plate and well isolated and morphologically different colonies were selected after incubation and stock culture were prepared. All these isolates were further characterized by standard biochemical test according to Bergey's manual of systematic bacteriology.

Identification of bacteria on the basis of 16S rRNA sequencing: Phenol degrading bacterial cultures were identified by using the sequence of the gene encoding 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 15,000bp) were compared with sequences in the Gene bank database of NCBI using the BLAST network service (Altschul *et al*., 1997).

Determination of phenol degradation potential: For study the phenol degradation potential of the bacterial isolates was estimated by preparing standard graph for different concentration of phenol (1 µg /100ml-5µg/100ml) and the degradation rate was determined by inoculating the culture broth in the flask containing peptone water phenol medium having 5µg/100ml concentration of phenol. The flasks were incubated for 96h and the degradation rate of phenol was estimated using 4-amino antipyrine method at each 24h interval by taking absorbance at 460nm wavelength on UV-VIS spectrophotometer (Mohammed *et al*., 2003). From the absorbance recorded the percent utilization, rate of degradation and amount utilized/hour was determined.

RESULTS AND DISCUSSIONS

Total twelve samples comprising of sediment, matt and water were collected from Lonar Lake, from which two bacterial strains were isolated in peptone water phenol medium having 5µg/100ml concentration of phenol, the isolates were characterized biochemically by

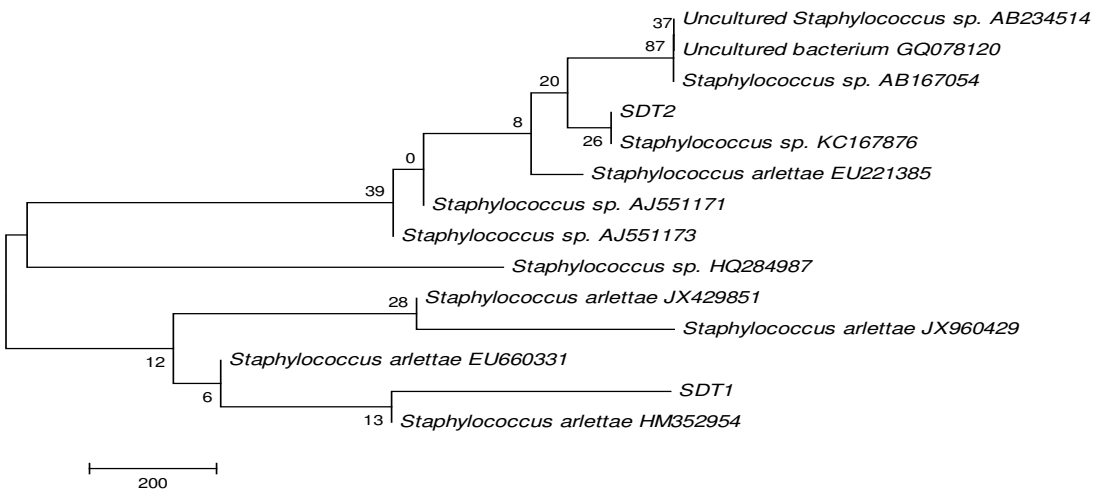
Hi-media *Staphylococcus* Rapid detection Kit (KB 004). Both isolates SDT1 and SDT2 were gram positive non motile cocci. The isolates ferment maltose, trehalose, mannitol and sucrose with acid production while the difference in sugar fermentation was observed in arabinose, raffinose and lactose (Table 1). The biochemically characterized isolates were identified by 16S rRNA sequencing from NCCS, Pune. The results of biochemical tests and sequencing showed that the isolates belong to phylum Firmicutes and were identified as *Staphylococcus arlettae* (SDT1) and *Staphylococcus* sp. (SDT2). Tambekar *et al.,* (2012) isolated the phenol degrading *Pseudomonas stutzeri* from the samples collected from Lonar Lake, while Kanekar *et al.*, 1999 isolated alkaliphilic bacteria from the sediment of Lonar Lake, which have the capacity to degrade phenol. They were identified as *Arthrobacter sp., Bacillus cereus, Citrobacter freundii, Micrococcus agilis* and *Pseudomonas putida*. These microorganisms use both aerobic and anaerobic pathway for phenol degradation while the aerobic biodegradation of phenol had been studied by Abdullah *et al.*, (2010). The phenol degrading *Staphylococcus aureus* strain was also isolated by Butani *et al*., (2012) from the effluent sample of Amla Khadi, located in Unkaleshwar, India.

Table 1: Morphological and biochemical characteristic of bacteria isolated from Lonar Lake

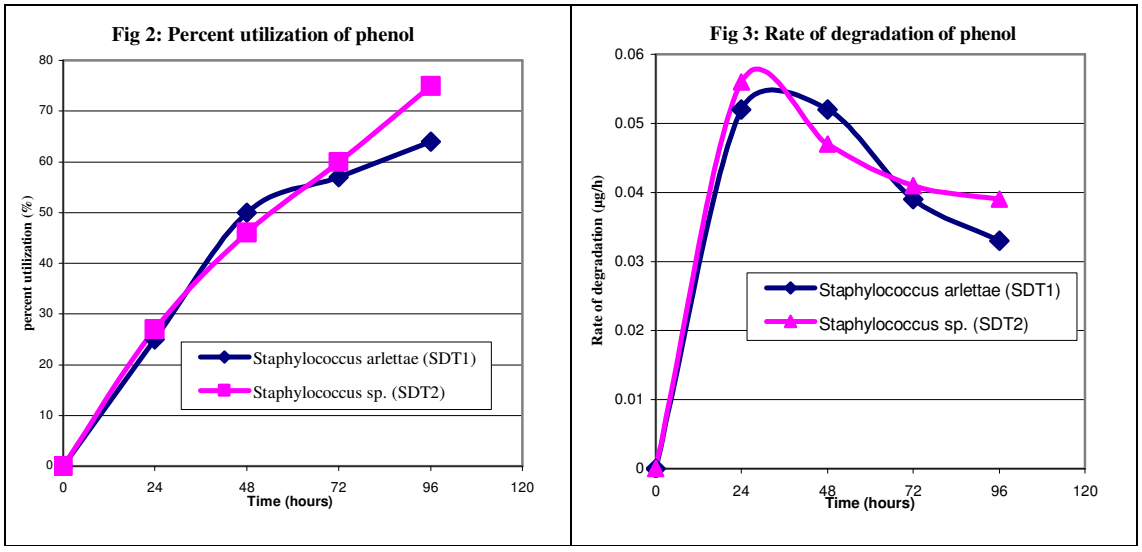
Test	Isolates		Test	Isolates	
	Staphylococcus arlettae (SDT1)	*Staphylococcus* sp. (SDT2)		*Staphylococcus arlettae* (SDT1)	*Staphylococcus* sp. (SDT2)
Shape of colony	Circular	Circular	ONGP	+	-
Colour of colony	White	Yellow	Alkaline phosphatase	-	-
Gram staining	+	+	Urease	-	-
Shape	Cocci	Cocci	Arginine utilization	-	-
Arrangement	Clustered	Clustered	Mannitol utilization	A +	A +
Catalase	+	+	Sucrose utilization	A +	A +
Oxidase	+	+	Lactose utilization	A +	A -
Indol	-	-	Arabinose utilization	A +	A -
Methyl Red	-	-	Raffinose utilization	A -	A -
VP **test**	-	+	Trehalose utilization	A +	A +
Citrate utilization	+	-	Maltose utilization	A +	A +

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 SDT1 and SDT2 were affiliated to phylum Firmicutes with genera *Staphylococcus* (Fig 1). The highest similarity values with the sequences of SDT1 relate with *Staphylococcus arlettae* HM352954 while the SDT2 shows maximum similarity with *Staphylococcus* sp. KC167876. Bastos *et al*., (2000), isolated phenol degrading strains from Amazonian soil samples and were identified by 16S rRNA sequencing as *Candida tropicalis* and *Alcaligenes faecalis*.

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 when characterized for there phenol degradation potential the isolates SDT1 utilize 64% of phenol from the culture broth having 5µg/100ml concentration of phenol before incubation, while the isolate SDT2 utilize 75% phenol from the medium having same amount of the phenol. The rate of degradation of phenol was increasing for first 24h incubation as the bacterial cultures were in continuous phase of division. The rate of degradation decreased slowly after the 24h incubation (Fig 2 and Fig 3). The isolate *Staphylococcus aureus* removed phenol upto 800ppm from initial concentration 1000ppm Butani *et al*., (2012).



Mrozik *et al.*, (2003), demonstrated that phenols and their compounds are the most recalcitrant and persistent organic chemicals in the environment. Vidyavathi *et al*, (2000) reported phenol degradation by *Nocardia* that resulted in complete degradation of phenol (100 ppm) within 96 hours. The isolates form the present study also reduces the phenol to a permissible limit and can be used in the bioremediation purpose. The salinity and the alkalinity of the Lonar Lake is higher as compare to the industrial effluent, so the isolates from such environment sustain to the environment having high pH and salt concentration. Chakraborty *et al.,* (2010) investigated the biodegradation of phenol by native bacterial strains isolated from coke oven processing waste water. In present study the isolates SDT1 and SDT2 showed the similar rate of phenol degradation as they belong to a common genus, but the results were taken at laboratory scale and optimum conditions were provided for the study. For field application of remediation of polluted sites the isolates may face the adverse condition with rapid change in the availability of nutrient and pollutants, so further processing and proper enrichment can help the isolates to improve the ability to remediate phenol. Present study showed that alkaline Lonar Lake harbors diverse microbial flora, which is endowed with the potential to degrade variety of chemical pollutants which helps to develop a new line of research in the field of bioremediation.

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