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Methylotrophic activities of *Acenatobactor spp*. from Lonar lake

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ABSTRACT

Lonar Lake is a saline ecosystem formed entirely on basalt rock. It is a completely closed system a favors the growth of alkalophiles and halophiles. The *Acenatobactor* play a major role for conserving environment by utilizing toxic C1 carbon compound and reduces the pollution produced by methanol, green houses gases etc. In present study, methylotrophic bacterial isolates were isolated from water and sediment of Lonar Lake using minimal salt medium containing 2% (v/v) methanol as sole carbon and energy source. One bacterial strains were isolated and characterized morphologically, biochemically and identified as *Acenatobactor* by 16S rRNA sequencing. These *Acenatobactor* strains were further screened for its ability to utilize methanol by Spectrophotometric method. Results showed that the isolates ALP3 of *Acenatobactor* strains are found to be efficient methanol utilizer and could be effectively use for bioremediation by reducing methanol at polluted sites and also helpful in reducing C1 compound, and global warming gases from environment.

Keywords: Lonar Lake, Methylotrophs, Bioremediation, Methanol and *Acenatobactor*

INTRODUCTION

The Lonar Lake is a saline and hyperalkaline ecosystem formed entirely on basalt rock by meteor impact. The crater is located in India ~ 550 km east of Mumbai and Arabian Sea is 150 m deep and 1830 m across (Fredrikson, 1973) with raised rim up to the 100m in width and 20m to 30 m high and alkalinity of the Lake water is attributed to the high content of sodium carbonate (Thakker and Ranade, 2002). The Lonar Lake is unique in the world for its alkalinity (pH 10) and salinity (NaCl 0.9%) of the water but it was seen that chlorides and salinity of the Lake water is decreasing day by day (Tambekar *et al*., 2010). Microbiological studies using culture-dependent and culture-independent strategies have identified and characterized both bacterial (Kanekar *et al*.,1999, 2002; Nilegaonkar et al., 2002; Wani et al., 2006; Surakasi *et al*., 2007) and archaeal (Thakker and Ranade, 2002;

Surakasi, 2007; Surakasi *et al*., 2007) communities in the Lonar Lake water and sediment.

Methanotrophs are a unique group of methylotrophic bacteria, which utilize one-carbon (C1) compounds such as methane, methanol and methylamine which constitute an important component of microbe-driven food web chains in many ecosystems. Methylotrophic bacteria, are phylogenetically distributed across diverse phyla such as Gammaproteobacteria (type I methanotrophs), Alphaproteobacteria (type II methanotrophs) (Trotsenko and Murrell,2008), filamentous methane oxidizers (Stoecker *et al*., 2006; Vigliotta *et al*., 2007) and Verrucomicrobia (Dunfield *et al*., 2007; Pol *et al*., 2007; Islam *et al*., 2008) and contribute significantly in biogeochemical cycling of carbon by facilitating the incorporation of C1 compound-derived carbon into biomass (Anthony, 1992; Chistoserdova *et al*., 2009). The global cycling of methane and related C1 compounds further affects the important environmental phenomena related to climate change.

The toxicity of methanol has been widely documented and their disastrous effect towards human and environment is greatly concerned. Currently utilization of C1 compound received great attention from many people from industries and researcher due to their toxicity. The present study aimed to isolate and characterize methylotrophic bacterial spp from Lonar Lake which can remediate methanol by which there may reduction in C1 compound pollution in the environment. The phylogenic analysis of these methylotrophic bacterial isolates could be very much helpful in future research, related to microbial diversity and reduction in pollution level of global gases from environment.

MATERIAL AND METHODS

Isolation of Bacterial Strain by Enrichment Method The sediment and water samples were collected from different sites of Lonar Lake (Buldhana District in Maharashtra, India). The medium containing ingredient (g/l): $NaNO_3$ 2.5g, KCl 0.1g, KH_2PO_4 3g, K_2HPO_4 7g, $CaCl_2$ 0.01g, $MgSO_4$ 0.5g, $FeSO_4$ 0.116g, H_3BO_3 0.232g, $CuSO_4$ 0.41g, $MnSO_4$0.008g, $(NH_4)_6$ Mo_7O_{24}, 0.008g, and $ZnSO_4$ 0.174g, 20 ml methanol, is used for isolation of methytrophic bacteria (Haddad *et al*., 2009) The medium was then incubated at 37^0C on a rotary shaking incubator at 100 rpm for 3 days. The subculturing was made for 5 times for enrichment and isolation of pure culture was done on solid nutrient agar plate. Well isolated colonies were selected and stored at 4^0C as stock culture (Tambekar *et al*., 2010).

Identification of Isolates

The isolates were characterized by morphophysiological and cultural characteristics. The biochemical tests were performed by rapid detection kit (Hi-media, KB001 and KB009). The kit contents the tests namely, indol, methyl red, voges prauskar, citrate, lactose, xylose, maltose, fructose, dextrose, galactose, raffinose, trehalose, melibiose, sucrose, L-arabinose, mannose, inulin, sodium gluconate, glycerol, salicin, glucosamine, dulcitol, inositol, sorbitol, Rhamnose, cellobiose, melizitiose, á-methyl mannose, xylitol, ONPG, esculin, arabinose, citrate, malonate, sorbase, nitrate reduction, urea hydrolysis and starch hydrolysis. The molecular identifications were performed by 16S rRNA sequencing at NCCS, Pune.

Methanol Utilization Studies For estimation of methanol, the bacterial isolates were grown in the nutrient broth and incubated overnight at 37 ^{0}C. This 24 h old culture was inoculated in mineral salt medium containing 2% (v/v) methanol as a sole source of carbon and energy (Tambekar *et al*., 2013). Preliminary studies were carried out bacterial inoculums in medium containing 2% (v/v) methanol. The methanol utilization was determined by analyzing residual methanol at 480 nm in the medium after the each interval 24 h up from 0 h to 96 h by using UV- visible spectrophotometer (Zhan *et al*., 2010).

RESULTS AND DISCUSSION

Lonar Lake represents an extreme environment with high pH and moderate salinity. It is the only known depression in the region and hence may serve as a drain for excess runoff from anthropogenically influenced surrounding areas. However, the contribution of such natural or anthropogenic factors towards elevated phosphate and nitrate levels in the lake sediments warrants further investigation. Lonar Lake water is green throughout the year because of dense cyanobacterial bloom dominated by *Arthrospira* (Surakasi *et al*., 2007). Sediment and water samples were chosen as the source of bacterial isolation and these were enriched in minimal medium using 2% (v/v) methanol as sole source of carbon and energy for one month by repeated subculture after every 96 h.

Those bacterial isolates able to grow on medium containing 2% (v/v) methanol as carbon source were identified as methylotrophs and subsequently isolated in pure culture. The experimental outcome of morphological and biochemical characterization proved that all isolates are gram negative and identified as *Acenatobactor (*Table 1) and represented as ALP3.

These isolated were analysed for 16s rRNA and identified as *Acenatobactor*. The *Acenatobactor* have best potential to utilize methanol. These isolates were able to grow at temperature up to 42^0C.

In this investigation, a new method for the direct determination of methanol using sodium nitroprusside (SNP) is used (Zhan *et al*., 2010). It has been reported that SNP can react with nucleophilic agent such as primary and secondary aliphatic amine however; no studies in the literature to date have been reported on the reaction of SNP and alcohol. This experiment results showed that SNP can react with methanol to form colored product. Absorbance of product is linear with certain extent of the concentration of methanol.

These morpho-biochemically characterized bacteria were identified by 16S rRNA sequencing and phylogenetic tree was constructed. The result of the phylogeny showed that methylotrophic strains isolated from Lonar Lake were related to phylum Proteobacteria. According to 16S rRNA gene sequences, these isolates showed a high level of similarity with the genus *Acenatobactor*.

Table 1: Morphological, Cultural and Biochemical Characteristic of Bacteria Isolated From Lonar Lake

	Character		**Character**
ALP3	Culture Code	ALP3	Culture Code
Acenatactoobr	Bacterial isolates on the basis of 16S rRNA sequencing	*Acenatactoobr*	Bacterial isolates on the basis of 16S rRNA sequencing
-	Dulcitol	S	Source
-	Glycerol	W	Colony Colour
-	Salicin	WSCE	Colony Morphology
-	Glucosamine	-	Gram Reaction
-	Sodium gluconate	CB	Shape
-	Inositol	G	Arrangement
-	Sorbitol	-	Endospore
-	Mannitol	-	Motility
-	Adonitol	-	Catalase
-	Methyl d-glucoside	-	Oxidase
-	Ribose	-	Indol
-	Rhamnose	-	Methyl red
-	Cellibiose	-	Voges Praskaur
-	Melizitose	+	Citrate
-	Methyl d-mannose	-	Lactose
-	Xylitol	-	Xylose
+	ONPG	-	Maltose
-	Esculin	-	Fructose -
-	D-arabinose	+	Dextrose
-	Malonate	-	Galactose
-	Sorbose	-	Raffinose
-	Nitrate Reduction	-	Trehalose
-	Urease	-	Melibiose
14MM	Starch Hydrolysis	-	Sucrose
+	Growth at 6.5% NaCl	-	Larabinose
-	Growth at 4^0C	-	Mannose
+	Growth at 42^0C	-	Inulin
+	Growth at pH10		
Note: Sed-Sediment, W-Water, Gr-Green, Wh-white, I-Irregular, SR-Short rod, S-Single.			

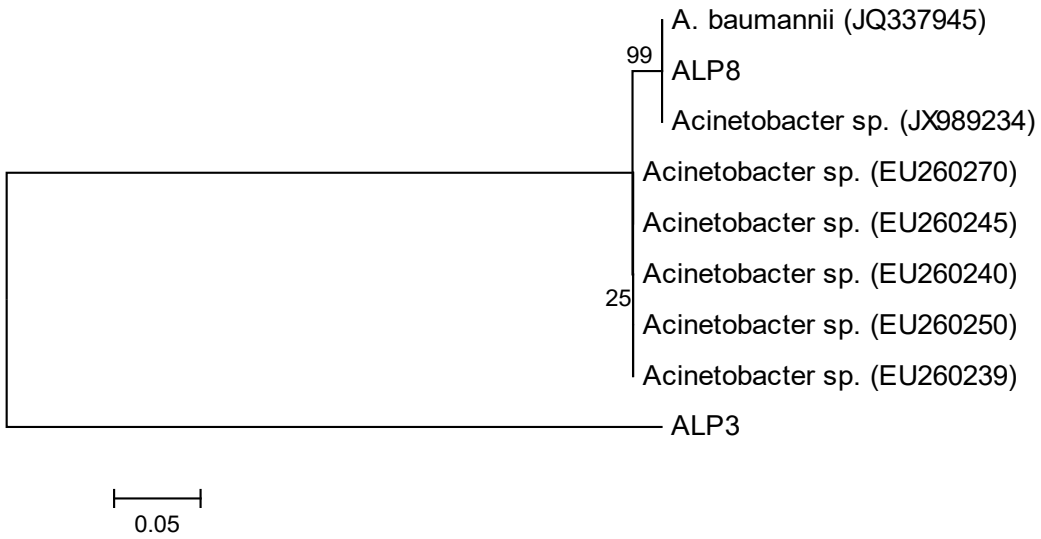

Figure 1: Phylogenic Tree of *Acenatobactor* Species Isolated from Lonar Lake : Phylogenetic tree for two methanotrophic bacteria isolated from Lonar Lake based on 16S rRNA gene comparisons and some of their closest phylogenetic relatives. The tree was constructed for the isolates ALP3 and ALP8. The phylogenic tree was constructed by neighbor-joining method. The number on the tree indicates the percentage of bootstrap sampling derived from 1,000 replications.

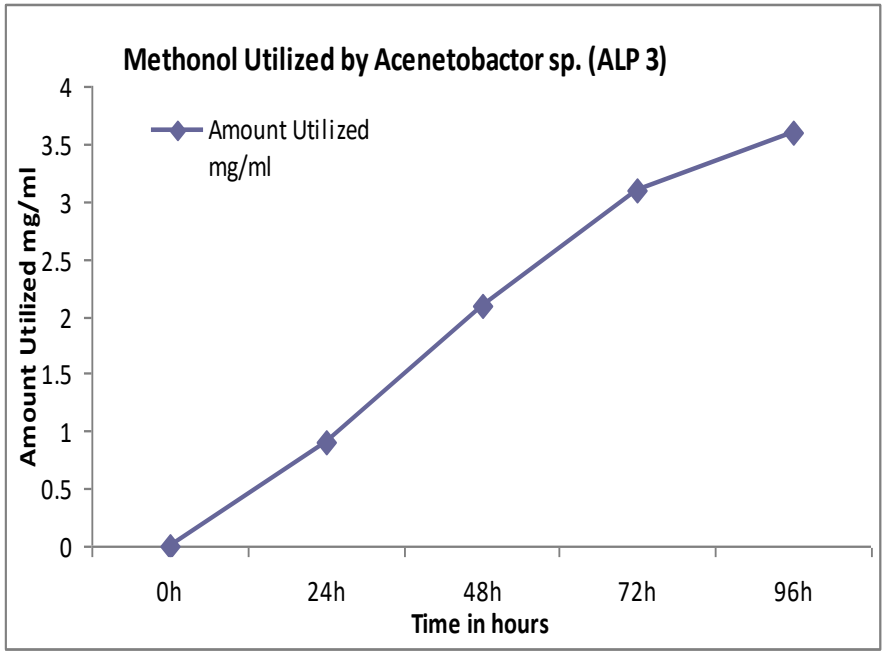

Figure 2: Percent Utilization of Methanol by *Acenatobactor* Species

Sediment is the potential source to isolate *Acenatobactor*. Antony *et al*, (2010) identified *methylmicrobium*, *methylophaga* and *Bacillus spp* as predominant methanotrophs from sediments of Lonar Lake. The utilization performances of selected strain were examined by spectrophotometric method. The experiment designed to find out the percent utilization and rate of utilization after 24h, 48h, 72h and 96h. In these studies, methanol estimated for 96h by spectroscopic method at each 24h time interval. Percent utilization and rate of degradation of methanol for these isolates was found, *Acinetobacter* Sps. indicated 72% methanol degradation at pH 7 and at 37°C temperature and showed 64% methanol degradation at 3% salt concentration. Experiment also showed that *Acenatobactor sp* ALP3 utilize methanol 0.9, 2.1, 3.1

and3.6 mg/mL after 24h time interval and rate of utilization of methanol was about 0.037 for isolates of *Acenatobactor sps* (Figure 2). The rate of utilization of methanol was also almost same for bacterial isolates Tambekar *et al*., (2011) reported that Methylotrophic bacteria not previously recorded bacterial species from Lonar Lake to utilize methanol as carbon source.

The findings of this study provide a window into the diversity of bacterial community members which are methane degrading from the Lonar Lake. These isolated bacterial species may be used to combat industrial pollution of methanol or to control global warming which may found better choice for studies like methane, methanol or toxic chemical degradation to combat Global warming. Till date several works are in progress to isolates efficient microbial strain that have ability to utilize methanol. We report here a new *Acenatobactor* (ALP3) was found to be a potential strain to utilize methanol as sole source of carbon and energy. This work has provided a useful guideline in evaluating potential methanol utilizer isolated from Lonar Lake.

CONCLUSION

The unexplored site of alkaline Lonar Lake contains many methanogenic and methylotrophic genera which might be helpful for the remediation of pollution environment. In present study isolation strategy for methylotrophic bacteria was used and potential methanol utilizing bacterial isolates of *Acenatobactor* were isolated which can be helpful for remediation of site with pollution of C1 compounds and provides a new unexplored site for researcher.

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