Isolation and Molecular Detection of Methylotroph from Lonar Lake

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ABSTRACT

Lonar Soda Lake is situated in the Buldhana district of the Maharashtra state. It is one of the largest craters and is the only crater which is formed due to high velocity meteoritic impact on basaltic rock, about 50,000 years old. Methylotrophic bacteria are unique in their ability to utilize methanol as a sole source of carbon and energy. The methylotrophic strain was isolated by using minimal salt medium containing 2% methanol as single source of carbon and energy. This isolate was further characterized morphologically, biochemically and identified by 16S rRNA sequencing. The result of sequencing showed that the strain belongs to phylum proteobacteria and species is *Pseudomonas aeruginosa*. This *Pseudomonas* species was further screened for its ability to utilize methanol by spectrophotometric method. Results showed that the *Pseudomonas aeruginosa* found to be highly efficient methanol utilizer and could be use for bioremediation of methanol polluted sites. This study is useful to control global warming and to reduce pollution of methanol and other C1 compounds.

Key words: Lonar Lake, Methylotrophs, *Pseudomonas*.

INTRODUCTION

Lonar crater (Latitude 19° 58', Longitude 76° 36') is a simple, bowl-shaped, near-circular crater formed by meteor impact (Fredriksson *et al.,* 1973) around 52 000 years ago (Sengupta *et al.,* 1997) in the Deccan volcanic flood basalts situated in village at Lonar, Buldhana district, Maharashtra, India. The crater has an average rim diameter of 1830 m and a rim-to-floor depth of about 150 m (apparent depth) (Fredriksson *et al.,* 1973). A salinity (NaCl 0.9%) and alkalinity (pH 10) of this creates an extreme environment for the growth of halophilic and alkaliphilic bacteria. Water enters the Lake through rain, ground water seepage and the springs situated at the edge of the Lake. Alkalinity of the Lake is attributed to the high content of sodium carbonate and hence was used, previously as source of washing soda (Thakker and Ranade, 2002; Tambekar *et al.,* 2010). Lonar Lake water is green throughout the year because of dense cyanobacterial blooms (Surakasi *et al.,* 2007). Decomposition of cyanobacterial biomass in soda lakes is likely to produce high quantities of methane, methanol, methylamine and dimethylsulfide favouring the surveillance of methylotrophs in this lake (Jones *et al.,* 1998).

Methanotrophs are a unique group of methylotrophic bacteria, which utilize methanol as sole source of carbon and energy (Trotsenko and Murrell, 2008; Olivier *et al.,* 2005). Methane oxidizing bacteria (MOB) include species in the Alpha proteobacteria (type II MOB) and in the Gamma proteobacteria (type I MOB) (Bowman, 2000). The aerobic methane oxidizing bacteria or methanotrophs are a group of bacteria that grow on methane. They are a subset of the methylotrophic bacteria which can grow on a number of different one carbon compounds including methane, methanol, methylated amines and methylated compounds containing sulphur (Anthony, 1992; Lidstrom, 2006). Methylotrophic bacteria are phylogenetically distributed across diverse phyla, contributes significantly towards the biogeochemical cycling of carbon by facilitating the incorporation of C1 compound-derived carbon in to biomass (Anthony, 1992; Chistoserdova *et al*., 2009). The global cycling of C1 compounds further affects the important environmental phenomena related to climate change. The aim of present study to isolate methylotrophic bacteria present in Lonar Lake which can be degrade the industrial pollutant methanol and C1 compound.

MATERIALS AND METHODS

Sampling sites and sample collection: Sediment, water and matt samples were collected respectively from selected sites of Lonar Lake in sterile containers during August, 2012. They were labeled and transported to laboratory for further analysis.

Enrichment of samples and isolation of bacterial strains: The collected sample such as sediment(1g), matt (1g) and water (10 mL) samples were inoculated 250mL conical flask containing 100mL minimal salt medium having composition (g/l) $NaNO_3$ 2.5; KCl 0.1; KH_2PO_4 3.0; K_2HPO_4 7.0; $CaCl_2$ 0.01; $MgSO_4.7H_2O$ 0.5; $FeSO_4.7H_2O$ 0.116; H_3BO_3 0.232; $CoCl_2.6H_2O$ 0.41; $CuSO_4.5H_2O$ 0.008; $MnSO_4$.H2O 0.008; $(NH_4)6$ Mo_7O_{24} 0.022; $ZnSO_4$ 0.174 with 2% methanol as a sole source of carbon (Haddad *et al.*, 2009). The medium was then incubated at 37^0C at 100 rpm on rotary shaker for 3 days and repeated 5 time subculturing was made in the respective medium. After repeated subculturing, the bacterial growth was sub cultured on nutrient agar plate for isolation of methylotrophs. After overnight incubation well isolated and differentiated colonies were transfered on Nutrient agar slant and maintained at 4^0C for further study.

Morphological, biochemical identification of isolate: The isolate was characterized by morphological and cultural characteristics. While rapid detection kit (Hi-media) was used to perform biochemical tests. The kit involves indole, 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, α-methyl mannose, xylitol, arabinose, citrate, malonate, sorbase, nitrate reduction, urease and starch hydrolysis and identified by 16S rRNA sequencing from NCCS, Pune.

Study on methanol utilization: For determination of methanol utilization of bacterial isolate was grown in nutrient broth and incubated overnight at 37^0C. This culture was then inoculated in minimal salt medium containing 2% methanol as sole source of carbon and energy. The methanol concentration was determined by analyzing samples at each 24 h to 96 h by using UV- Visible spectrophotometer at 481 nm (Zhan *et al.,* 2010*).* The effect of environmental parameters on methanol utilization efficiency was also studied.

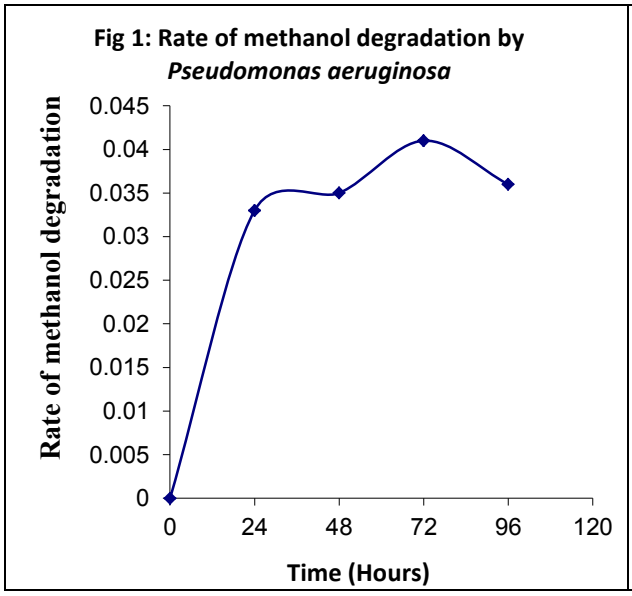
RESULTS AND DISCUSSION

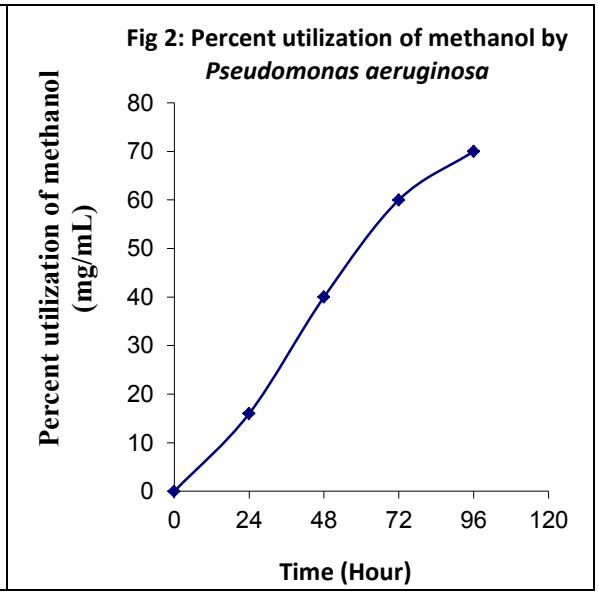
Methylotrophic bacteria are unique organisms with the ability to use compounds with single carbon atom as single sources of carbon and energy, thus playing a role in global carbon cycling. While studying the methylotrophic bacteria from Lonar Lake, a total of four samples comprising of sediment, matt and water samples were collected and processed on minimal salt media containing 2 % methanol as carbon source. After five times subculturing in minimal salt medium containing 2% methanol then inoculated on Nutrient agar plate. Then well isolated colonies are transfer on Nutrient agar slant for further characterization. The bacterial strain were analyzed for standard biochemical test and further confirmed by 16S rRNA sequencing. The isolate was gram negative, short rod, aerobic and motile. Xylose, dextrose, galactose and mannose sugar was fermented and citrate utilized (Table 1). Potentially novel haloalkaliphilic methanogens related to the genera *Methanosarcina*, *Methanocalculus* and *Methanoculleus* have also been isolated in culture from the Lonar lake sediments.

The result of 16S rRNA showed that the organism was found to be *Pseudomonas aeruginosa*. The isolate when studied for percent utilization and rate of degradation of methanol it was found to be 70% and 0.036 mg/l. The effect of various environmental parameters on methanol utilization efficiency was also studied and it was found that the optimum temperature for organism is 37^0C, while on 20 and 40^0C, the percent utilization and rate of degradation slows down (Fig. 1 and 2). In case of pH the methanol degraded up to pH 8, while below 7 and above 9 the rate of degradation was declines. Tambekar *et al*., (2011) also isolate the methylotrophic bacteria from Lonar Lake and reported *Acinetobacter, Achromobactrum Xylosonidans*, *Ochromobactrum* among these organisms *Pseudomonas aeruginosa* was found to be efficient methylotroph.

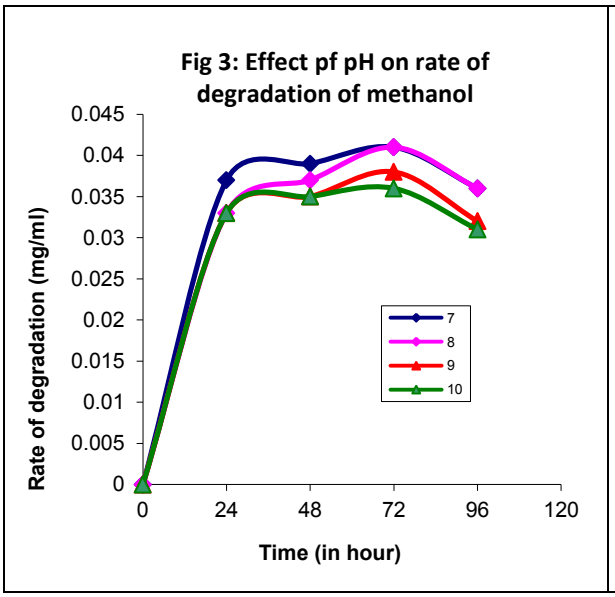
In this investigation a new method for direct determination of methanol using sodium nitroprusside (SNP) is developed.

It has been reported that SNP can react with nucleophilic agent such as primary and secondary amines however no studies in the literature to date have been reported on the reaction of SNP can react with methanol to form colored product absorbance of product is linear with certain extent of the concentration of methanol compared with others method, this method is very simple rapid and reliable. The methanol utilization efficiency of several strain were examined spectro photometrically. The experiment designed to find out methanol utilization percent utilization and rate of utilization after 24h, 48h, 72h and 96h respectively.





Studies on optimization of environmental parameters showed that the optimum pH for percent methanol utilization and rate of degradation is pH 7 at which organism utilizes 70% methanol and rate of degradation was found to be 0.036 mg/mL. However at pH 9 and pH 10 rates of degradation and percent utilization was found to be decreases gradually. The percent utilization was found to decrease from 70 % to up to 62 %. In case of rate of degradation the rate decreases from 0.036 mg/mL to up to 0.031 mg/mL (Fig 3 and 4).



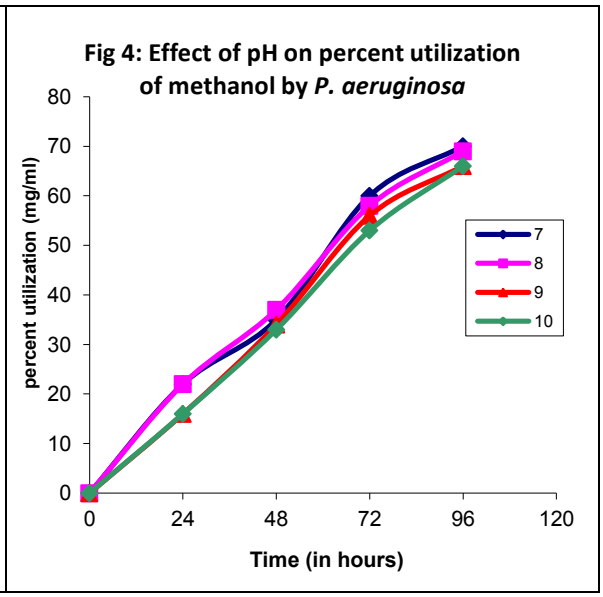


Fig 5: Effect of temperature on rate of degradation of methanol

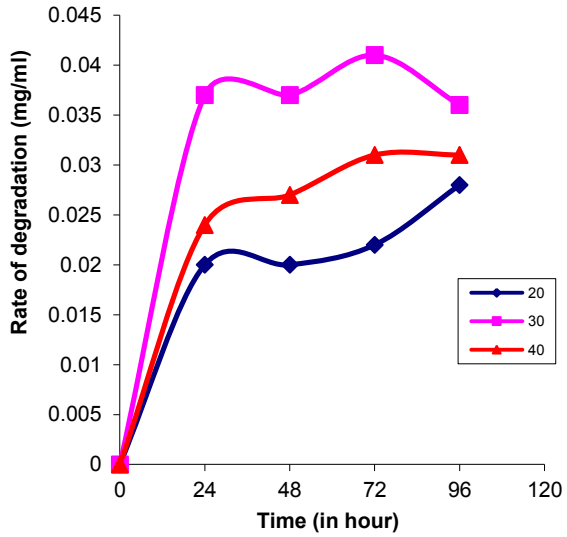
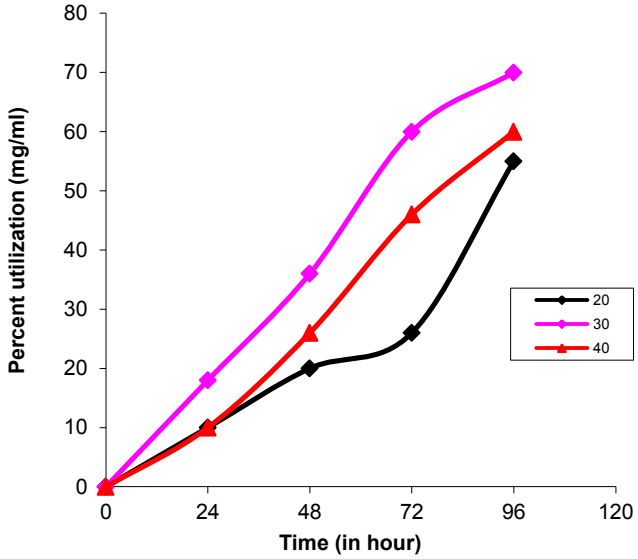


Fig 6: Effect of temperature on percent utilization of methanol



RIBOSOMAL DATABASE PROJECT

>Met1

GTCGACTTATCGCGTTAGCTGCGCCACTAAGATCTCAAGGATCCCAACGGCTAGTCGACATCGTTTACGGCGTGGACTACCA
GGGTATCTAATCCTGTTTGCTCCCCACGCTTTCGCACCTCAGTGTCAGTATCAGTCCAGGTGGTCGCCTTCGCCACTGGTGTTC
CTTCCTATATCTACGCATTTCACCGCTACACAGGAAATTCCACCACCCTCTACCGTACTCTAGCTCAGTAGTTTTGGATGCAGTT
CCCAGGTTGAGCCCGGGGATTTCACATCCAACTTGCTGAACCACCTACGCGCGCTTTACGCCCAGTAATTCCGATTAACGCTT
GCACCCTTCGTATTACCGCGGCTGCTGGCACGAAGTTAGCCGGTGCTTATTCTGTTGGTAACGTCAAAACAGCAAGGTATTAA
CTTACTGCCCTTCCTCCCAACTTAAAGTGCTTTACAATCCGAAGACCTTCTTCACACACGCGGCATGGCTGGATCAGGCTTTCG
CCCATTGTCCAATATTCCCCACTGCTGCCTCCCGTAGGAGTCTGGACCGTGTCTCAGTTCCAGTGTGACTGATCATCCTCTCAG
ACCAGTTACGGATCGTCGCCTTGGTAGGCCTTTACCCCACCAACTAGCTAATCCGACCTAGGCTCATCTGATAGCGTGAGGTC
CGAAGATCCCCCACTTTCTCCCTCAGGACGTATGCGGTATTAGCGCCCGTTTCCGGACGTTATCCCCCACTACCAGGCAGATTC
CTAGGCATTACTCACCCGTCCGCCGCTGAATCCAGGAGCAAGCTCCCTTCATCCGCTCGACTTGCATGTGTTAGGCCTGCCGC
CAGCGTTCAATCTGAGCCATGATCAAG

Lineage:

Results for Query Sequence: seqmatch_seq, 828 unique oligos

rootrank Root (10) (match sequences)

domain Bacteria (10)

phylum "Proteobacteria" (10)

class Gammaproteobacteria (10)

order Pseudomonadales (10)

family Pseudomonadaceae (10)

genus Pseudomonas (10)

S000008931	-	not_calculated	0.999	1452	Pseudomonas sp.; OLB-1; AJ387904
S000333003	-	not_calculated	0.999	1421	Pseudomonas aeruginosa; X13; AY631241
S000412778	-	not_calculated	0.999	1457	Pseudomonas aeruginosa; Z5; AY548952
S000428754	-	not_calculated	0.999	1416	Pseudomonas aeruginosa; ATCC 10145; AF094713
S000497702	-	not_calculated	0.999	1461	Pseudomonas aeruginosa PAO1; AE004501
S000497705	-	not_calculated	0.999	1461	Pseudomonas aeruginosa PAO1; AE004844
S000651692	-	not_calculated	0.999	1292	Pseudomonas aeruginosa; PGSL 03; DQ420635
S000711168	-	not_calculated	0.999	1461	Pseudomonas aeruginosa PAO1; AE004091
S000711172	-	not_calculated	0.999	1462	Pseudomonas aeruginosa PAO1; AE004091
S000735437	-	not_calculated	0.999	1452	Pseudomonas aeruginosa UCBPP-PA14; CP000438

Temperature was also found to affect both percent utilization and rate of degradation. The optimum temperature for percent utilization and rate of degradation was found to be 30ºC to 37^{0}C. However the organism showed results at 30ºC the rate of degradation and percent utilization was found to be 0.036 mg/mL and 70% mg/mL respectively. At 20ºC the rate of degradation and percent utilization was found to be 0.028 mg/l and 55% respectively. At 40ºC there was also gradual decrease in percent utilization and rate of degradation (Fig 5 and 6) .

Table 1: Morphological and biochemical characteristic of *P. aeruginosa* from Lonar Lake

Colony characters	Pigment	Green	**Hydrolysis of**	Urea	-	**Utilization of**	Rhamnose	-
	Shape	Irregular		Starch	-		Cellobiose	-
Morphology of bacteria	Gram reaction	-	**Utilization of**	Lactose	-		Raffinose	-
	Shape	Short rod		Xylose	A		Melibiose	-
	Arrangements	Single		Maltose	-		Sucrose	-
	Motility	+		Fructose	-		L-arabinose	-
Biochemical characters	Catalase	-		Dextrose	A		Mannose	A
	Oxidase	+		Galactos	A		Mannitol	-
	Indole	-		Trehalos	-		Adonitol	-
	Methyl red	-		Inulin	-		Ribose	-
	Voges Prauskar	-		Dulcitol	-		Sorbitol	-
	Citrate	+		Salicin	-		Glucosamine	-
	Nitrate	+		Inositol	-		Sodium gluconate	-

A = acid , Negative= -

The alkaline Lonar Lake contains many methanogenic and methylotrophic genera which might be helpful for the remediation of polluted environment. The isolated strain from alkaline Lonar Lake was identified as *Pseudomonas aeruginosa* showed the ability to utilize methanol as a source of carbon and found to be utilize upto 70% methanol.

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