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Isolation, Characterization and Description of Hydrocarbon Degrading Thermophilic *Aeribacillus pallidus* Strain MCM B-882 from Oil Reservoirs, India

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A B S T R A C T

Thermophilic alkalitolerant bacterial strain MCM B-882 was isolated from oil well formation water in India. MCM B-882 was Gram variable, aerobic, motile, endospore forming and rod shaped. The MCM B-882 cells were occurring either solitarily or arranged in pairs and are 0.38 (0.33-0.47) x 2.75 (1.52-2.88) µm in size. MCM B-882 grows in the pH range of 6.0-10.0 and optimally at 7.0; temperature range of 45 – 65°C and optimally at 60°C; as well as in salt (NaCl) range of 1- 4% and optimally at 2%. The fatty acid spectrum of MCM B-882 showed isopentadecanoic, palmitic, and isoheptadecanoic acids were the predominant species. The 16S rDNA analysis revealed that strain MCM B-882 is closely affiliated with genus *Aeribacillus* as *Aeribacillus pallidus*. The G+C content of it's DNA was 42.24 mol%. There is significant difference in physiological properties, fatty acid profiles, G+C content between *Aeribacillus pallidus* MCM B 882 and reported phylogenetically closest *Aeribacillus pallidus* DSM 3670^T and also *A. pallidus* strain TD1. MCM B-882 can oxidizes hydrocarbons which was confirmed using INT assay. It produces biosurfactant when grown in presence of hydrocarbons. *A. pallidus* strain MCM B882 may be a good candidate to be used effectively in MEOR process as well as in bioremediation processes.

Introduction

An oil reservoir commonly called as petroleum reservoir is a subsurface pool of hydrocarbons that has been enclosed in the porous rock formations. The survival of living organisms in petroleum reservoir depends on the composition of ecology as well as physico-chemical characteristics of the environment (Magot *et al*., 2000). Temperature plays important role in the development of microbial ecology in petroleum reservoirs. The presence of metabolites from the biodegradation of oil in petroleum reservoirs provide an indication for active microorganisms in the petroleum reservoir (Roadifer, 1987). Diverse group of microorganisms having different metabolic potential have been reported from oil reservoirs. Thermophily of microorganisms and their optimum temperature depend on *in situ* temperature and other physiological and

ecological considerations. Aerobic and facultatively anaerobic microorganisms have been reported in petroleum reservoir samples (Adkins *et al.*, 1992; Zvyagintseva *et al*., 1995; Voordouw *et al*., 1996). Microaerophilic microorganisms belonging to the different genera like *Campylobacter*, *Oceanospirillum* and *Thiomicrospira* have been detected (Voordouw *et al*., 1996; Telang *et al*., 1997). Bacteria living in oil reservoirs have many applications in the field of petroleum biotechnology. These bacteria can be useful for microbial enhanced oil recovery (MEOR) (Banat *et al*., 1995; Helmy *et al*., 2010; Nazina *et al.*, 2007), in microbial de-emulsification, microbial desulfurization, microbial de-nitrogenation, enzymatic upgrading of petroleum fractions and pure hydrocarbons (Van Hamme *et al*., 2003). Petroleum reservoirs due to its unique environment can be the good source for the isolation of novel microorganisms. Members of the genus *Aeribacillus* are aerobic, thermophilic, alkalitolerant, motile, Gram positive rods that occur singly, in pairs or in chains (Minana-Galbis *et al.*, 2010). *Aeribacillus pallidus* is the only species from genus *Aeribacillus*. (formerly *Geobacillus pallidus).* Isolation of different starins of *Aeribacillus pallidus* from sewage, production water from oil resrvoirs, hot springs, oil-contaminated soil, and from a deep geothermal reservoir have been reported (Poltaraus *et al*., 2016). *A. pallidus* strain GS3372 isolated from deep geothermal reservoir from of Gross Schoenebeck, in the North German Basin and it's full genome has been analyzed. The GS3372 contain genes for heavy metal resistance and assimilation of different types of carbon sources (Filippidou, *et al*., 2015).

In the present study we documented the isolation and characterization of thermophilic hydrocarbon degrading *Aeribacillus pallidus* MCM B-882. The strain was identified on the basis of morphological, chemotaxonomic, phylogenic and molecular systematic analysis. This strain was further screened to assess it's ability to degrade hydrocarbons and produce biosurfactants.

Materials and Methods

Sample collection

Formation water samples were collected from ten oil wells in Mehsana (23.5880° N, 72.3693° E) Bechraji (23.4989° N, 72.0439° E) and Kalol (23.2464° N, 72.5087° E) regions, Gujarat (India). Samples were maintained at 4°C until further use. Temperature and pH of each sample was recorded at the point of collection.

Enrichment and isolation of hydrocarbon degrading bacteria

Bacterial strains used in all experiments were isolated by selective enrichment technique from formation water. Mixture of formation water samples was used as an inoculum in a minimal medium; contained per liter of distilled water: KH_2PO_4, 1.0 g; KH_2PO_4 1.0g; NH_4Cl, 1.0g; $MgSO_4.7H_2O$, 0.05g; NaCl, 5.0g; yeast extract 0.0025g; trace element solution,10 ml was supplemented with 1% v/v crude oil as the sole carbon and energy source. The pH of the medium was kept 8.0. For isolation of bacteria from enrichments aliquots of the enrichment culture was streaked on the minimal medium lacking carbon source containing 2% gelrite and 0.2% $MgCl_2.6H_2O$ were made in 65 ml capacity serum bottles. Mixture of n-alkanes (C_{12}-C_{18}) was absorbed on a Whatman filter paper and then placed in the headspace of the slant bottle. The plates were incubated at 60°C. After 48 hour incubation colonies

were isolated by using a single colony isolation procedure.

Scanning electron microscopy

Actively growing bacterial cells were harvested and fixed overnight in glutaraldehyde (2%, at 4°C). The cells were subsequently dehydrated in increasing concentrations of ethanol (from 5 to 100%). Cells were then loaded on the SEM stubs and dried and coated with gold using a plasma SEM coating unit. The samples were examined with a scanning electron microscope (JEOL JSM 6360 A) (Fischer, *et al.*, 2012).

INT (p-iodonitrotetrazolium) assay

An INT (3.5 mg/ml) stock was prepared. Biomass from enrichment culture was harvested, washed thrice with 1X PBS and re-suspended in 300 µl minimal medium containing 50µl INT stock. As a carbon source 10 µl of crude oil was added and kept for incubation at 60°C. Results were noted after 2 hrs of incubation (Malatova, 2005).

Biochemical characterization

Hi-Carbohydrate Kit (HiMedia, India) was used for the evaluation of fermentation characteristics of the isolates. The kit contained 35 fermentable carbohydrate substrates. The fermentation tests were performed as follows: Actively growing culture was used as an inoculum. The inoculum density was adjusted to A600~ 0.5 and 20 µl were inoculated in each of the test well. The results were recorded after 48h of incubation at 45°C. Ability of cultures to ferment carbohydrate was recorded in terms of change in the colour and interpreted according to the manufacturer's instructions.

FAME analysis

To determine the fatty-acid composition of bacteria, biomass grown on LB medium at 60°C for 18 h was used (Popova *et al.*, 2002). Fatty acids were extracted by saponification (sodium hydroxide and methanol) and methylated with acidic methanol followed by solvent extraction (hexane and methyl tertiary butyl ether).The fatty acid methyl esters (FAME) were analyzed on the GC system with hydrogen as a carrier gas and flame ionization detector (Disha Life sciences Pvt. Ltd., Ahemadabad, India).

Molecular systematics

The total genomic DNA was isolated using GenElute Genomic DNA isolation kit (Sigma, USA) as per the manufacturer's instructions and used as template for amplification of 16S rDNA genes in PCR reactions. The genomic G+C ratio and Tm was examined using Light Cycler 2 thermocycler (Roche, Germany) as per the method described by Xu *et al*., (2000). Primers FDD2 (5'-CCGGATCCGTC GACAGAGTTTGATCITGGCTCAG-3') and RPP2 (5'-CCAAGCTTCTAG ACGGIT ACCTTGTTACGACTT-3') (Muyzer *et al.*, 1993) were used for bacterial 16S rDNA amplification. PCR amplification was performed in a 50 µl PCR mixture containing 1.5U Taq DNA polymerase, 10x buffer, 50 pmol of each primer, and 200 µM dNTP. PCR amplifications were performed in Mastercycler Gradient Thermal Cycler (Eppendorff, Germany) under the condition of initial denaturation for 95°C for 5 min, 35 cycles of 94°C for 1 min, annealing at 60°C for 1 min with bacterial primers and extension at 72°C for 1 min with a final elongation step of 72°C for 5 min. PCR amplification of 16S rRNA gene fragments was confirmed on 1.5% agarose gel containing ethidium bromide (0.1 mg/mL). The resulting 16S rRNA gene amplicons were purified using polyethylene glycol

precipitation (Sambrook and Russel, 2001). Purified product was used as template for cycle sequencing reaction using ABI Prism BigDye Terminator Cycle Sequencing Ready Reaction kit (Applied Biosystem, Foster City, CA, USA). Post cycle sequencing clean-up was done by Big Dye Clean Up method, according to the manufacturer's instructions (Invitrogen, India) and subjected to sequencing in an automated sequencer (3100 Avant Gene Analyser, Applied Biosciences, USA).

The 16S rDNA nucleotide sequence of an isolate was deposited in the GenBank nucleotide databases under accession JN701184. This sequence was compared with reference sequences available in the GenBank database using the BLAST algorithm to establish phylogenetic affiliation. Sequences alignments were performed using the software CLUSTAL W (http://www.ebi.ac.uk/) (Thompson *et al*., 1997). MEGA software v 5.2 (Tamura *et al*., 2011) was employed to construct the phylogenetic tree based on Kimura two-parameter model (Kimura, 1980) and neighbor-joining algorithm (Saitou and Nei, 1987). Bootstrap analysis with 1000 replicate runs was applied to assign confidence levels to the nodes in the trees.

Results and Discussion

Enrichment and isolation of hydrocarbon degrading bacteria

The temperature of the formation water samples when collected was in between 60-70°C and pH was 8.0. The enrichment of hydrocarbon degrading bacteria was performed at 60°C. Enrichment cultures were agitated at 130 rpm for 20 days. Positive growth was determined by an increase in the turbidity of the flasks containing crude oil as a sole carbon and energy source compared to the negative control flasks. After 20 days of incubation the cell density reached up to 5 x 10^7cells/ml (initial cell density ≈ 10^3 cells/ml). Crude oil used as a sole source of carbon and energy, dispersed uniformly during the enrichments probably due to the production of the biosurfactants by growing consortium of microorganisms (Fig.1). It was reported that hydrocarbon-degrading microbes produce a variety of biosurfactant molecules.

Biosurfactants can effectively reduce the interfacial tensions of oil and water *in situ* as well as the viscosity of the oil (Liu *et al*., 2004). The thermophilic consortium of putative oil degrading bacteria obtained from the enrichment flask was serially diluted and spread on slope bottle (Fig.2). Isolated colonies were obtained after two days of incubation at 60°C. One colony was selected for further study and it was designated as MCM B-882.

Morphological characterization

The colonies of MCM B-882 on solid media were rounded with wavy edges (1-3 mm in diameter) smooth, convex, transparent, colorless, structurally homogeneous and viscous. Cells of MCM B-882 were Gram-variable, rod-shaped occurring either solitarily or arranged in pairs and were 0.38 (0.33-0.47) x 2.75 (2.0-3.80) µm in size. The vegetative cells of strain were straight and motile. MCM B-882 cells displayed pleomorphism, a characteristic feature of thermophiles. Sporulating cells appeared at the end of the growth retardation phase (Fig. 3).

Nutritional requirements and physiological properties

MCM B-882 was an obligate thermophile, which exhibited oxidase and catalase

positive characters. It could grow under micro-aerobic conditions (in a rubber stoppered bottles half-filled with liquid medium and N_2 gas in the head space). The strain showed good growth on LB medium as well as in minimal medium with glucose. It was capable of utilizing a wide range of carbohydrates, alcohols, polycarboxylic acids, and hydrocarbons. Hydrocarbon utilization was confirmed by INT assay. INT (a p-iodonitrotetrazolium dye precursor) gets reduced to form brown colored formazan dye subsequent to bacterial utilization of oil as carbon and energy source. MCM B-882 was able to utilize glucose, maltose, lactose, mannose, sucrose, glycerol, salicin, glucosamine, sorbitol, mannitol, esculin and D-arabinose as source of carbon and energy. These morphological and physiological properties are summarized and compared with other phylogenetically closest affiliates (Table 1). MCM B-882 could grow in the pH range of 6.0-10.0 and optimally at 7.0; temperature range of 45 – 65°C and optimally at 60°C; as well as in salt (NaCl) range of 1- 4% and optimally at 2%.

FAME analysis

The fatty acid spectrum of MCM B-882 lacked hydroxyl acids (Table 2), which is typical of Gram-positive bacteria. Branched fatty acids prevailed in this strain. Iso-pentadecanoic, isopalmitic, and isoheptadecanoic acids were the predominant species.

The prevalence of isopentadecanoic and isoheptadecanoic acids was earlier established in other representatives of the genus *Geobacillus.* The major fatty acid composition of MCM B-882 revealed isopentadecanoic acid (i15:0) as major component followed by isopalmitic acid. (i16:0).

DNA Characterization

The G+C content in the DNA of strain MCM B-882 was 42.24 mol %, a value close to those reported for the species of the genus *Geobacillus.* The Tm value was found to be 84.

Phylogenetic Analysis

The nucleotide sequence of a large portion of the 16S rDNA gene (1369 nucleotides) was determined. A comparative analysis of the nucleotide sequence of the 16S rRNA gene revealed that MCM B-882 strain affiliated to the genus *Aeribacillus* as *Aeribacillus pallidus* strain MCM B-882. Based on the phylogenic tree, the strain was most closely affiliated to *A. pallidus* strain TD1 (Fig.4).

Comparison of MCM B-882 with the closest phylogenetic affiliates

The phylogenetic analysis revealed that strain MCM B-882 was affiliated with genus *Aeribacillus* as *Aeribacillus pallidus*. *A. pallidus* DSM 3670^T is the only type strain and species of genus *Aeribacillus*. *Bacillus pallidus* first described by Scholz and colleagues in 1987 was renamed in 2004 by Banat and colleagues as "*Geobacillus pallidus*" (Yasawong *et al*., 2011). The same strain was subsequently transferred to the new genus *Aeribacillus* as *A. pallidus* by Minana-Galbis *et al* (2010) on the basis of 16S rDNA sequence divergence and as well as unique phenotypic characteristics (Minana-Galbis *et al.*, 2010). Another strain *A. pallidus* TD1 was reported by Yasawong *et al* (2011). *A. pallidus* TD1 as well as MCM B-882, in the present study, contained linear and branched fatty acids, and very few unsaturated fatty acids; branched saturated fatty acids were dominant.

Table.1 Physiological characteristics of MCM B-882, *A. pallidus* DSM 3670T and *A. pallidus* TD1 (Yasawong *et al*., 2011)

Characteristic	MCM B-882	*A. pallidus* DSM 3670^T	*A. pallidus* TD1
Cell width (um)	0.75	0.8-0.9	0.4
Cell length (um)	2.0-3.80	2.0-5.0	2-40
Motility	+	+	+
Temp range for growth (°C)	45-65	30-70	45-67
G+C (mol%)	42.24	39-41	38.9
Acid produced from:			
Cellobiose	-	-	+
Maltose	+	d	+
Mannose	+	-	+
Sucrose	+	ND	+
Trehalose	+	d	+
Xylose	-	-	+
D-Arabinose	+	-	-
Ribose	-	-	+
Citrate used	-	-	+
Hydrolysis of:			
Casein	-	-	-
gelatin	-	-	-
Starch	-	+^{w}	-
Alkane utilization	+	ND	-

Note: +, Positive; -, negative; +w, weakly positive; d, variable between strains; ND, not determined

Table.2 Cellular fatty acid composition (% w/w) of MCM B-882, *A. pallidus* DSM 3670T and *A. pallidus* TD1 (Yasawong *et al*., 2011)

Fatty Acid	MCM B-882 (%)	*A. pallidus* DSM 3670^T (%)	*A. pallidus* TD1 (%)
i 14:0 isomyristic acid	2.57	1.6	0.36
14:0 myristic acid	1.59	8.5	1.87
a 14:0 anteisomyrisyic acid	-	1.6	-
i 15:0 isopentadecanoic	27.86	6.2	16.30
a15:0 anteisopentadecanoic	7.28	4.9	4.50
15:0 pentadecanoic	-	1.2	0.33
i 16:0 isopalmitic acid	17.16	9.3	11.5
16:1 w7c alcohol	9.26	-	0.14
16:1 w11 c alcohol	5.40	-	0.65
16:0 palmitic acid	3.12	50	25.04
17:1 iso w 10 c	4.86	-	
i 17:0 isoheptadecanoic acid	7.32	4.0	17.95
a 17:0 anteisoheptadecanoic	10.01	6.5	19.74
17:0 heptadecanoic acid	6.73	-	0.19
18:0 stearic	-	2.1	1.03
i18:0 iso	-	-	0.29

Fig.1 Dispersion of oil by biosurfactant produced via bacterial consortium

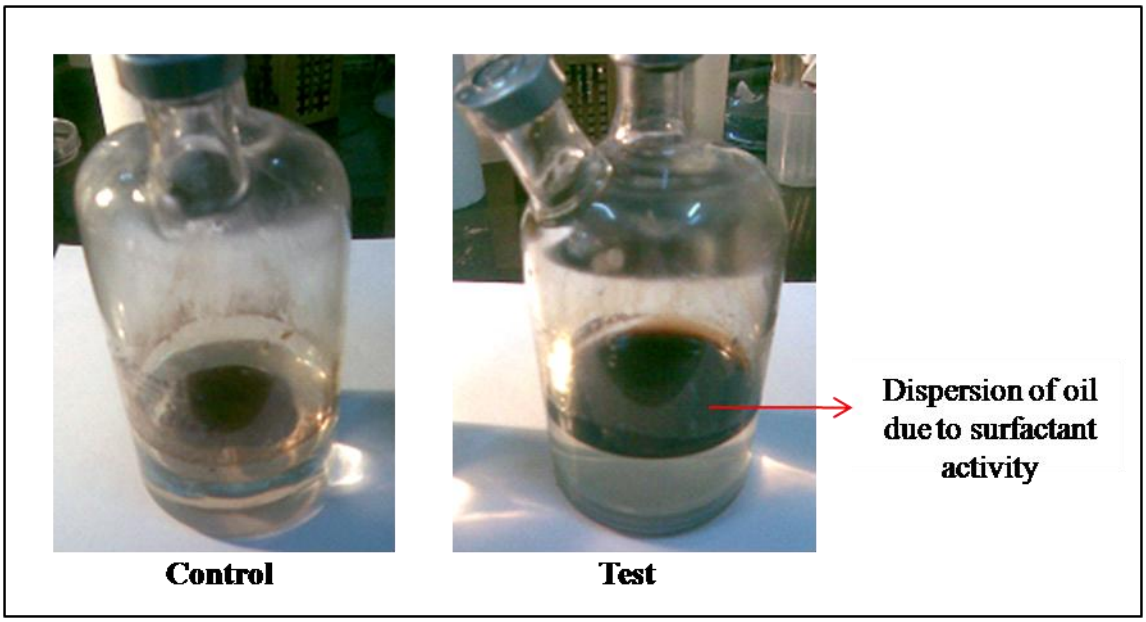


Fig.2 Experimental set up for the utilization of n-alkanes on solid media

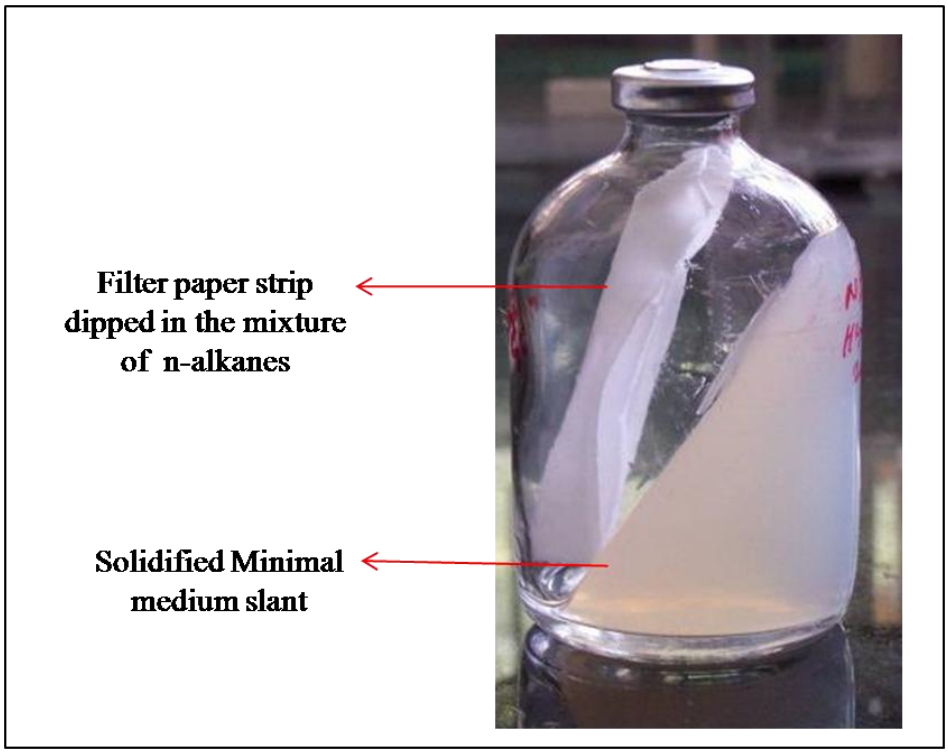
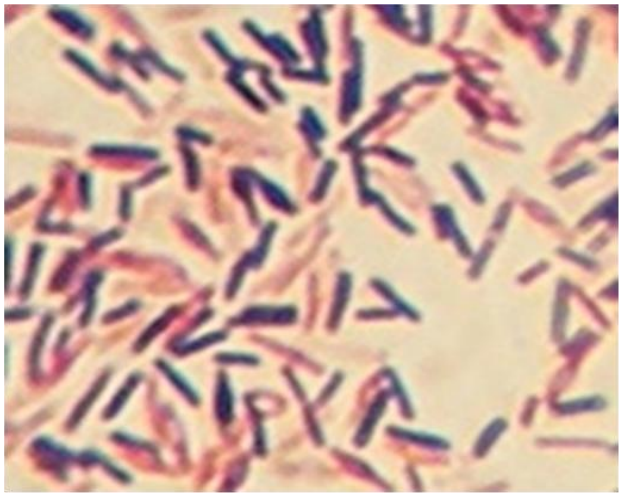
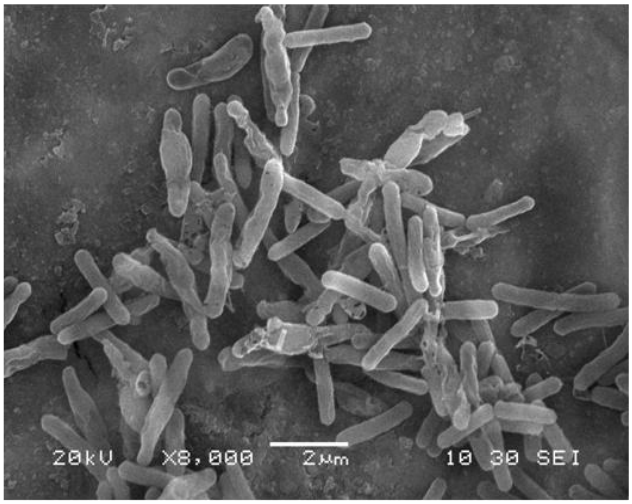


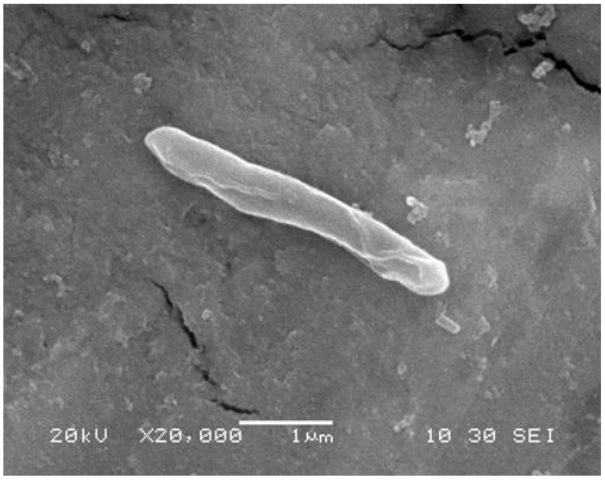
Fig.3 Microscopic observation of MCM B-882 :(A) Gram staining, (B) and (C) are scanning electron microscopic images



A

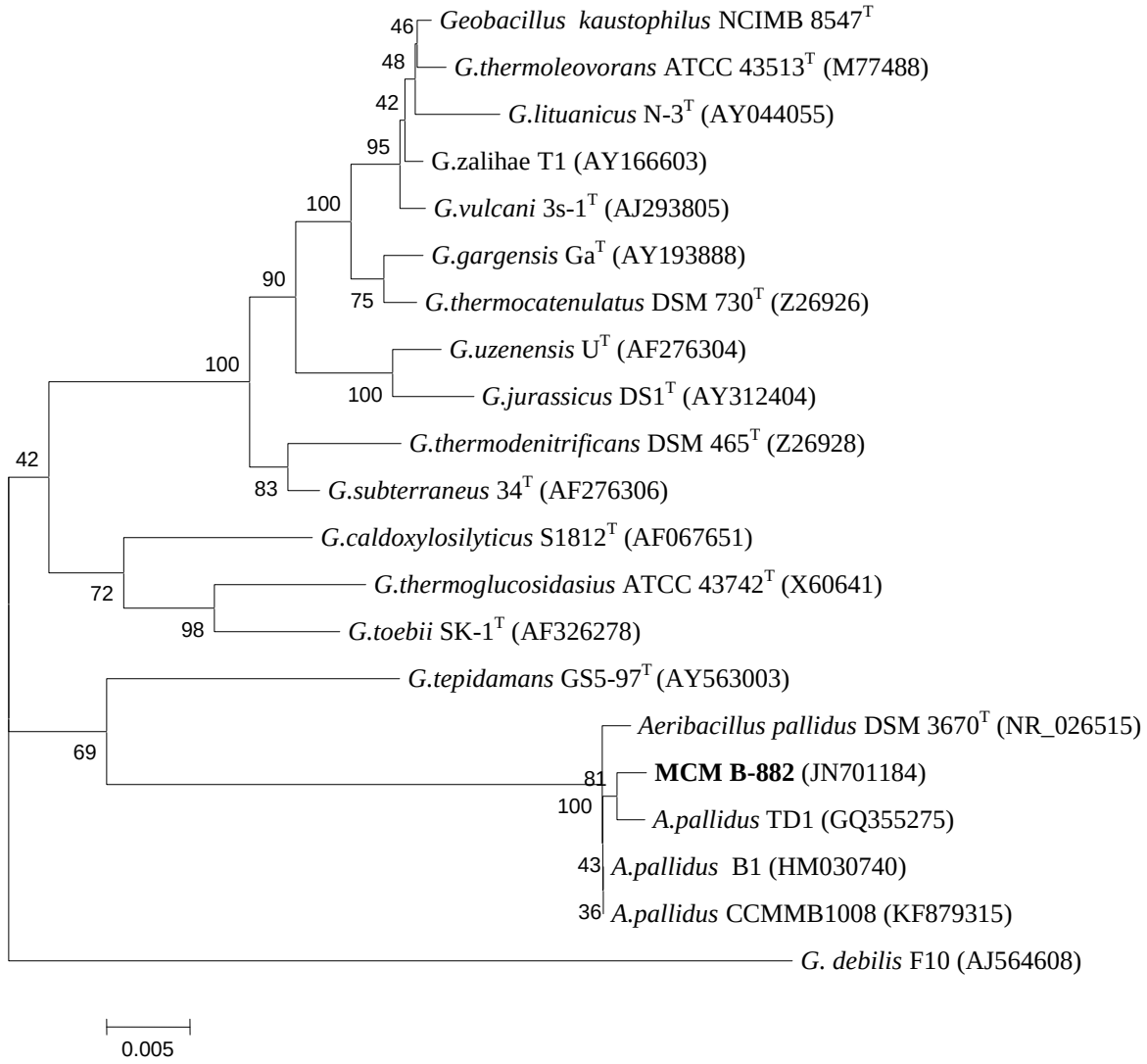


B



C

Fig.4 Phylogenetic tree showing the position of strain MCM-B 882 within the radiation of the genera Geobacillus and Aeribacillus. Bootstrap values (expressed as percentages of 1000 replications) are shown at branching points.



By contrast, no unsaturated fatty acids were detected in strain *A. pallidus* DSM 3670^T and linear fatty acids were dominant. Palmitic acid was the main constituent (25% and 50% respectively) in TD1 and DSM 3670^T. However, palmitic acid constituted only 3.12% of the cellular fatty acids in MCM B-882. Further 16:1 w7c alcohol constituted 9.26% of the cellular fatty acids in MCM B-882 but the same fatty acid was absent in DSM 3670^T. Uniqueness of MCM B-882 was further confirmed in DNA base composition studies which revealed 42.24 mol% G+C of MCM B-882 as significantly different from 38.9% and 39-41% reported for TD1 and DSM 3670^T respectively. Carbohydrate utilization pattern observed for MCM B-882 was significantly different from that for TD1 as well as DSM 3670^T. Only strain TD1 was able to produce acid

from cellobiose, ribose, and xylose where as both TD1 and MCM B-882 produced acid from arabinose and mannose. DSM 3670^T did not produce acid from any of these five carbohydrates.

A. pallidus strain MCM B-882 is a thermophilic, alkalitolerant and hydrocarbon oxidizing bacterium. Therefore it may be used in MEOR processes for the recovery of crude oil from oil reservoirs.

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