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Research Article

HALOMONAS DESIDERATA A HYDROCARBON DEGRADING BACTERIA ISOLATED FROM AN UNCONTAMINATED SAMBHAR LAKE, RAJASTHAN (INDIA)

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ABSTRACT

Water and soil sample were collected from an uncontaminated Sambhar Lake, Rajasthan (India) were used for isolation and characterization of halophilic bacteria able to degrade 2T engine oil a hydrocarbon mixture. Enrichment technique was used to isolate bacterial strain ZHE 05 in presence of 50 gl^{-1} NaCl and 1% 2T engine oil at 37°C. ZHE05 strain was rod shaped gram negative, motile, positive for oxidase and catalase. Phenotypic characters and phylogenetic analysis based on 16s rRNA gene of isolate ZHE05 showed that it was related to member of halomonas genus. The initially hydrocarbon degrading activity of strain ZHE05 was observed by CO_2 evolution experiment. The degradation of several compounds present in 2T engine oil was confirmed by GC-MS analysis. The significantly amount of heterogenic compound was degraded which is present in refined petroleum product such as 2T racer oil and diesel oil under the same condition of salinity and temperature. Degradation activity of strain ZHE05 was observed on pure aliphatic compound hexadecane which show significant degradation of aliphatic compound.

INTRODUCTION

Saline and hyper saline environment such as marine ecosystem, natural saline lakes or industrial effluents are frequently contaminated with petroleum hydrocarbons (LeBorgne *et al*, 2008). The contaminates made up of the mixture of alkanes, aromatic, resins and asphanates which are generally environment persistent and exhibit toxic and adverse effect on aquatic and terrestrial life. The bioremediation is most effective and important mechanism for hydrocarbons elimination. Conventional microorganism(s) cannot perform bioremediation process to eliminate hydrocarbon at high salt concentration. The solution of outcome problem is the use of halophilic or halotolerant organism which can use efficiently their enzymatic machinery at high salt concentration for bioremediation (Fathepure BZ, 2014). Halophilic bacteria have advantage of their growth over wide saline concentration, as well as in simple media. A bacterial strain DQD3-30T related to genus halomonas isolates from daquing oil field, china (Wu *et al*, 2008). It was able to degrade crude components over wide range of NaCl concentration. Similarly Mnif *et al* (2009) have isolated halotolerent bacterial strain C2SS100 related to genus halomonas from production water Tunisia. Strain C2SS100 has capability to degrade various petroleum products such as crude oil, diesel fuel and lubricant oil in presence of 10% salinity condition. (Nicholson and Fathepure, 2005; Tapilatu *et al*, 2010) have isolated microbial community that provides strong support about biodegradation by inhabiting microorganisms in uncontaminated habitat. Biodegradation of hydrocarbons has been extensively studies by normal bacteria, little by halophilic bacteria, but a few is known about the degradative ability of organism to natural hypersaline environments that have no history of contamination (Nicholson and Fathepure, 2005). The aim of the present study was to isolate and identify the archeal strains from Sambhar Lake, Rajasthan that could degrade 2T engine oil.

MATERIALS AND METHODS

Culture media

The minimum salt medium (MSM) contained 0.73 g KH_2PO_4, 2.2 g K_2HPO_4, 1.0 g NH_4Cl, 0.2 g $MgCl_2$ $6H_2O$, per litre of distilled water and supplemented with 50 g NaCl per litre. The pH was adjusted to 7 with 10 N KOH solution. The medium was sterilized by autoclaving at 121°C for 15 min. 2T engine oil was added at 1% (v / v), as carbon and energy source.

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Chemicals were used in experiments including, hexadecane purchased from Sigma-Aldrich, (USA) while 2T engine oil and diesel fuel obtained from Hindustan petroleum (HP) pump, Jaipur (india).

Enrichment and isolation procedure

Soil and water sample was collected from Sambhar Lake, Rajasthan with no contamination history. Hydrocarbon degrading microorganisms were isolated by selective enrichment technique.MSM broth was used in enrichment technique supplemented with 5% NaCl (w/v) and 1% (v/v) 2T engine oil. The 2T engine oil substrate used in enrichment method was a mixture of hydrocarbons. Soil and water sample directly added to enrich media after incubated for 14 days at 37º C and 120 rpm. 1ml of cultured broth from primary culture were transfer to fresh enriched MSM broth; in order to after second enrichment 1ml of cultured broth was plated after appropriate dilution on Nutrient agar plates and incubated at 37 ºC. After 72 h of incubation pure colony were plated by repeated single colony method.

Measurement of CO_2 from 2T racer oil

Degradation of hydrocarbon activity was confirmed by measuring the evaluated CO_2 from 2T engine oil during microbial utilization. Two set of flasks in which each set contain four different flasks were setup for experiment and both sets have 100 ml MSM medium, 1 ml culture but they differ in carbon source where SET-II have carbon source while other SET-I have not. It is anticipated that KOH solution has ability to absorb carbon dioxide from environment. Test tube filled with 15 ml of 0.1 M KOH solution was fitted in flask and were sealed with rubber cork and aluminum cap. Test tubes filled with KOH in both sets were pulled after scheduled time of 7, 14, 21 and 28 days for measuring evaluated carbon dioxide. The amount of CO_2 trapped in KOH was accurately titrated according to (Pathak *et al*, 2012). The difference in mililiters was converted into micromoles of evolved carbon dioxide during microbial degradation of hydrocarbons.

GC-MS analysis

Sample (50 ml) of culture of halomonas ZHE05 containing hydrocarbons and an abiotic control were extracted according to Geerdink *et al* (1996). Samples were first centrifuged at 10,000 rpm for 10 min and cell pellet were discarded and entire supernatant were used for hydrocarbon extraction. The pH of supernatant was adjusted to pH 13 with 10 N NaOH and the suspension was extracted with 10 ml of diethyl Ether in a separatory funnel. The diethyl phase was collected from the flask and the extract was dehydrated with 1 to 2 g of Na_2SO_4. The diethyl phase was evaporated, dissolved in equal volume of hexane and then analyzed by gas-chromatography mass-spectrometry. GC-MS was performed with Thermo GC 1300 and "TSQ 8000 "Triple Quadrupole GC-MS MS SYSTEM with auto sampler Al 1310. Gas Chromatography 1300 fused with a GC column TG-5MS AMINE. The column length was 30 m with internal diameter 250 µm; coated film 0.25µm. The conditions were as follows: PTV Temp. Program: 70 °C, hold 2.00 min, 10 °C/min to 270 °C, hold 10 min. Carrier gas helium flow rate 1ml/min, split ratio 1:50. GC is equipped with auto-sampler AI 1300 and sample volume was 1µ litre. The Elutes were automatically passed into a mass spectrometer. GC mass Spectrum analysis was conducted using TSQ8000 with transfer line temperature 280°C and ion source temperature 230°C in EI mode. Mass scan time was 4 min with full Scan MS. The mass spectrum was also equipped with a computer fed NIST mass Spectra data library. Chemical constituent components of the extracts were identified by matching the peaks with Computer NIST MS libraries and confirmed by comparing mass spectra of the peaks of literature.

Characterization of strain ZHE05

The growth of bacterial strain ZHE05 was evaluated at various salt concentrations at 0 to 15 % (w/v) in enriched MSM medium. Optical density was measured at 600 nm, according to Barathi and Vasudeven (2001) and mid log growth data used to specified growth rates. The growth curve for each compound measured after adaption time and strain four times cultivated for each compound in MSM medium. The pH and temperature requirement for growth were determined by adjusting the final pH of medium 6,7,8,9 or 10 with HCl and NaOH (37° C) and by incubation at 32 to 42° C (32, 37 and 42° C) (5% NaCl and pH 8.0). The gram reaction was determined by HiMedia gram reaction kit according to the manufacturer's instruction. The oxidase activity of strain was determined by oxidation of 1% p-aminodimethylaniline. H_2S production, indole test and motility were performed on SIM agar media (sigma-aldrich). Hydrolysis of gelatin and tween 80 were performed according to the procedure of Williams *et al* (1983) on ASW medium at 30°C. Methyl red and Voges-proskaur were performed by using methyl red and Barritt's regent (Barritt, 1936; Mata *et al*, 2002). Phenylalanine deaminase test was examined according to Mata *et al* (2002). All biochemical tests were performed in duplicates.

Antimicrobial susceptibility test for strain ZHE05 were performed on the Mueller-Hinton agar by disc diffusion (Kirby-Bauer) method (Hudzicki J, 2009) using HIMEDIA Dodeca Universal-V. Dodeca Universal -V is an inert flat circular ring having 12 discs of 6 mm diameter on its projections. Azithromycin (AZM) 30µg, Rifampicin (RIF) 5µg, Penicillin (P) 10Unit, Piperacillin (PI) 100µg, Augmentin (AMC) 30µg, Ampicillin 10µg, Roxithromycin (RO) 30µg, Erythromycin (E) 15µg, Ampicillin (AMP) 10µg, Cloxacillin (COX) 1µg, Amoxycillin (AMX) 10µg and Vancomycin (VA) 30µg.

16S rRNA sequencing and phylogenetic analysis

Bacterial Genomic DNA was isolated by using the InstaGeneTM Matrix Genomic DNA isolation kit. Using below 16S rRNA Universal primers gene fragment was amplified using MJ Research Peltier Thermal Cycler. The Universal primers 27F and 1492R (27F, 5C-AGAGTTTGATCMTGGCTCAG-3C; 1492R, 5C-TACGGYTACCTTGTTACGACTT-3C) were used to obtain a PCR product of approximately 1,400bp in the case of bacteria. Include a positive control (*E.coli* genomic DNA) corresponding to base position 8-1542, based on Escherichia coli numbering of the 16S rDNA gene (Winker and Woese, 1991). The PCR product was sequenced using the 518F and 800R (518F, 5'-CCAGCAGCCGCGGTAATACG-3'; 800R,**5'C**TACCAGG GTATCTAATCC-3') primers. Sequencing reactions were performed using a ABI PRISM® BigDyeTM Terminator Cycle Sequencing Kits with AmpliTaq® DNA polymerase (FS

enzyme) (Applied Biosystems). The full multiple sequences were aligned using the program MUSCLE 3.7 (Edgar RC, 2004). The resulting aligned sequences were cured using the progam Gblocks 0.91b. (Talavera and castresana, 2007). Finally, the program PhyML 3.0 aLRT was used for phylogeny analysis and HKY85 as Substitution model. The program Tree Dyn 198.3 was used for tree rendering (Dereeper *et al*, 2008).

RESULTS

Soil and water samples were collected from uncontaminated Sambhar Lake, to isolate 2T engine oil degrading bacterial strains which could degrade engine oil under saline condition. Enriched medium have 2T engine oil and 5% w/v NaCl became white turbid and the yellow oily layer become clear, indicating the bacterial growth and degradation of this substrate. CO_2 evolution experiment confirmed hydrocarbon degrading activity in Sambhar Lake. CO_2 evolution data shown in Table 1 reveals that CO_2 evolution in SET-II was significantly high than SET I due to degradation of 2T engine oil as carbon and energy sources. During isolation six different strains were picked in consortium out of which a pure culture designated ZHE05 selected for hydrocarbon degradation studies under saline condition. The selection of strain was based on foaming production and efficiently degrading ability to engine oil in MSM medium Contained of 50 g l^{-1} NaCl.

Table 1 Evolution of CO_2 during 2T engine oil degradation by ZHE05 microbial strain

		SET-I	SET-II
ZHE05	07 days	120	625
	14 days	145	680
	21 days	140	670
	28 days	95	510

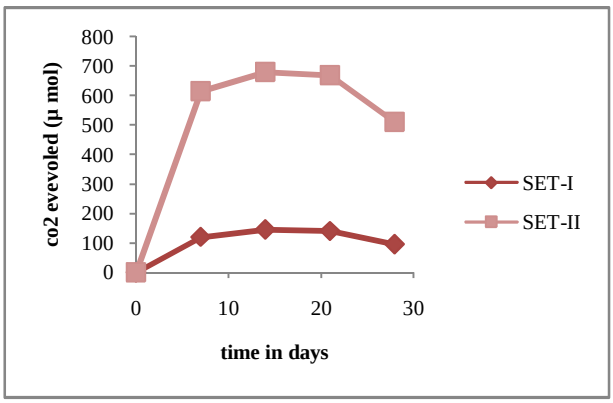

Figure 1 graphical presentation of CO_2 evolution during 2T engine oil degradation

Characterization of strain ZHE05

Bacterial strain ZHE05 was a aerobic, Gram- negative, rod shaped, motile, H_2S producing, oxidase and catalase positive. Bacterium strain Colony of strain was large, white cream and smooth in morphology. Strain ZHE05 shows positive activity for Methyl Red (MR), phenyl alanine deaminase, hydrolysis of gelatin and tween80, positive for citrate utilization whereas negative for Voges-proskauer test. Biodegradation of 2T engine oil was evaluated on various salt concentrations ranging From 0 to 150 g l^{-1}, with an optimum at 30-70 g l^{-1}NaCl. Strain ZHE05 was able to grow over temperature range of 32-45°C with optimal growth at 37°C. The salinity range for growth of strain studies in the presence of some carbohydrate at a concentration of 5 g l^{-1} showed fermentation of the glucose, fructose, sucrose and glycerol but not lactose and galactose.

In antimicrobial susptability test strain ZHE05 was only resistance against Cloxacillin (COX) while sensitive to other antibiotics Azithromycin (AZM), Rifampicin (RIF), Penicillin (P), Piperacillin (PI), Augmentin (AMC), Ampicillin, Roxithromycin (RO), Erythromycin (E), Ampicillin (AMP), Amoxycillin (AMX) and Vancomycin (VA).

The nearly complete 16S rRNA gene sequence (1475 bp) of strain ZHE05 was determined. A phylogenetic tree was constructed (Fig. 2) that was based on 1475 unambiguous bp. The 16s rRNA gene sequence of strain ZHE05 was deposited in the NCBI gene bank under Accession no KT893874. The phylogenetic result showed that strain ZHE05 was a member of genus halomoas and closely related to *halomoans daqingensis* (EF121854) (Gang Wu *et al* 2008). Differential Physiological and biochemical characteristic of strain ZHE05 and other hydrocarbon degrading strains of the genus halomonas are given in table 2.

Table 2 Physiological and biochemical characteristic of strain ZHE05 and other closely related type of strain of the genus halomonas

Characteristic	halomonas sp. ZHE05	halomonas sp. C2SS100 EU601735 (S. Mnif *et al*. 2009	Halomoans daqingensis (EF121854) (Gang Wu et al 2008)
Morphology	short rod	short rod	short rod
Pigmentation	white-cream	yellow- cream	ND
pH range	7-9	5.5-9	8-10
pH optimum	7-8	--	9
Temperature range ()	32-42	30-45	10-50
Temperature optimum	37	37	30
NaCl range (%, w/v)	0-15	0-15	1-15
NaCl optimum (%, w/v)	3-7	3-8	5-10
Oxidase	+	+	+
Catalase	+	+	+
H_2S production	+	ND	--
Indole production	--	ND	+
Phenyl alanine deaminase	+	ND	--
MR-VP test	+/--	ND	--/--
Hydrolysis of:			
Gelatin	+	ND	--
Tween 80	+	+	+
Citrate utilization	+	ND	+
Production of acid from			
Glucose	+	+	ND
Fructose	+	+	ND
Lactose	--	--	--
Galactose	--	ND	+
Mannitol	--	+	--
Sucrose	+	+	+
Origin	Sambhar Lake, India	oil field production water, Tunisia	Daqing oilfield, China

+, positive, –, negative, ND, not determined

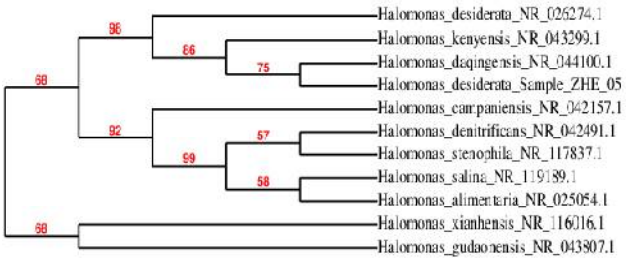

Figure 2 phylogenetic tree based on 1475 unambiguous nucleotides of the 16S rRNA sequence, showing the position of strain ZHE05 **(KT893874)** among related to genus halomonas. Bootstrap values, expressed as percentage of 100 replications, are shown in (red colour) middle of each branch.

Biodegradation of 2T engine oil

Strain ZHE05 was able to degrade 2T engine oil 1% (v/v) in MSM broth and agar plates, in presence of 50 g l^{-1} NaCl. The growth of strain on 2T engine oil and diesel fuel was followed by measuring the OD at 600nm at different culture's time (Fig. 3). GC-MS analysis of abiotic control contained 2T engine oil showed it was a mixture of different hydrocarbons. Strain ZHE05 was actively able to degrade total mixture of hydrocarbons present in 2T engine oil after 14 days of incubation. The result was confirmed by diminution or total disappearance of the corresponding peak of each compound, as (shown Fig 4). The strain ZHE05 degrades higher than of Diesel fuel present in culture after two weeks incubation.

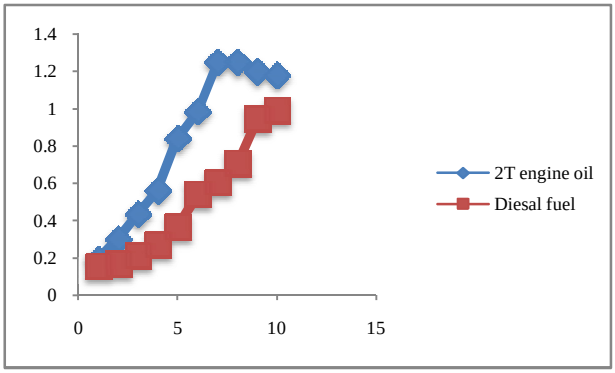

Figure 3 Growth of strain ZHE05 in MSM medium containing ( ) 2T engine (1% v/v), ( ) diesel fuel (1% v/v) determined by measuring the optical density at 600 nm.

In addition to capacity of strain ZHE05 to grow with 2T engine oil could grow on other petroleum product such as diesel fuel in presence of 50 g l^{-1} NaCl. GC-MS analysis and growth curve showed that 2T engine oil was the best substrate to support bacterial growth and strain ZHE05 obtained maximum growth within 7 days where as growth in presence of diesel oil showed extended leg time and approximately 10 days were required to reach maximum growth. Bacterial strain ZHE05 was evaluated its ability to grow on variety of simple hydrocarbons as sole carbon and energy source, in presence of 50 g l^{-1} NaCl. The strain able to degrade 16 carbon containing aliphatic hydrocarbon, Hexadecane (1% v/v). It was also degrade aromatic compound naphthalene and phenethrene however activity was weak. ZHE05 was able to grow rapidly in MSM medium supplemented with (1% v/v) hexadecane as the sole carbon and energy source in presence of 50 g l^{-1} NaCl. Hexadecane was 80% degraded after 14 days incubation. After 3 days incubation GC-MS study shows the formation of new peaks (data not shown).

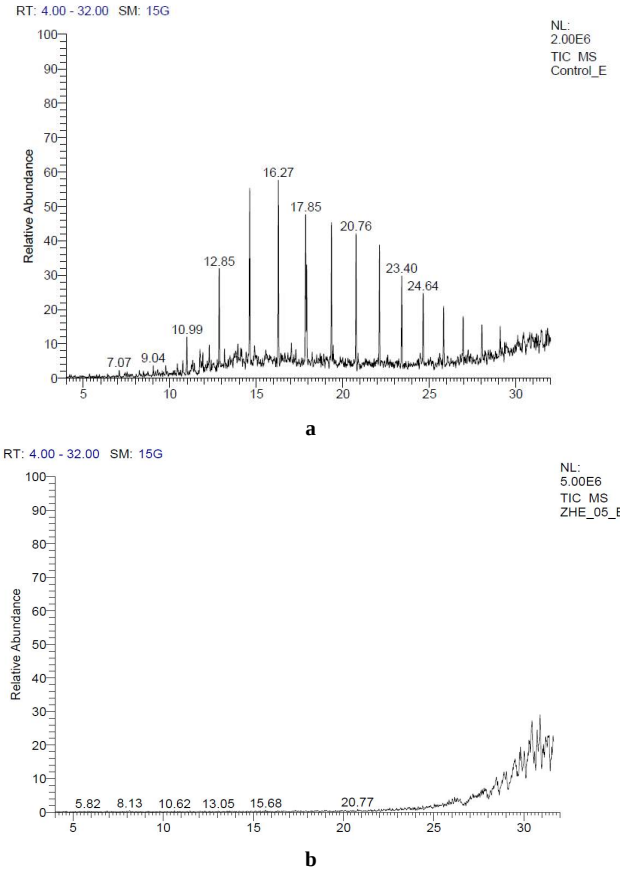

Figure 4 GC-MS profiles of 2T engine oil in MSM medium after incubation (a) represent without strain while (b) with strain ZHE05 at 37°C for 14 days.

DISCUSSION

The present study clearly revealed that organism inhabiting saline habitat could rapidly degrade various or mixture of hydrocarbons. In last decade's researcher have been focused to find out bioremediation capacity of halophilic bacteria inhabiting uncontaminated saline habitat. A few bacterial strains have isolated which have a capability of hydrocarbon degradation. Subsequently Nicholson and Fathepure (2005) have reported that microbial community lives in uncontaminated saline habitat Okhalama which could rapidly degrade benzene and toluene in presence of 3-14.5% NaCl concentration. These studies demonstrated complete mineralization of $^{14}CO_2$ by enrichment culture in the presence of 14.5% NaCl. Similarly Tapilatu *et al* (2010) have isolated several archeal strains from shallow crystallizer pond (Camargue, France) that degrade n-alkane in presence of 22.5% NaCl. Of these strain MSNC 2 pointed to genus Haloarcula and three other strains MSNC 4, MSNC 14, and MSNC 16 were related to genus Haloferax.

However, strain ZHE05 was able to degrade 2T engine oil in presence of 50 g l^{-1}. This strain isolated from an uncontaminated Sambhar Lake after enrichment of 2T engine oil, is an aerobic, moderately halotolerent, gram negative, motile, rod shaped, catalase +, oxidase + and phylogenitically closely related to genus halomonas. An approach to extend the optimal utilization for biodegradation, halophilic bacterial

consortium enriched from an oil contaminated saline soil which have two novel catechol 2,3-dioxygenases (C23O1 and C23O2) were cloned and over expressed in *E.coli* BL21 (DE3) (Guo *et al*, 2015). *E.coli* with the halotolerant feature was show growth over a range of 0-30% salinity and they show more degradation at high salinity than absence of salinity.

Strain ZHE05 has also the capability to degrade other petroleum product such as diesel fuel in the presence of 50 g l^{-1}. The capacity to degrade 2T engine oil is higher than diesel fuel. Most of component of present in 2T engine oil rapidly reduced within a week and completely degraded in 14 days. The result of GC-MS chromatogram shows that peaks represent various hydrocarbons in 2T engine oil completely disappeared after 14 day's incubation. GC-MS analysis of diesel fuel showed that it contains major n-alkane of C10-C24, and minor cyclic compound.

Biodegradation is significantly affected by adaption of bacterial strain on organic substrate. Growth study and GC-MS analysis reveals that four times cultivated bacterial strain on 2T engine oil rapidly degrade hydrocarbon within a week and totally in two weeks. Subsequently initially exposure to diesel fuel bacterial strain shows less growth and degradation was also lower but after adaption biodegradation was increased and significantly higher. Biodegradation of hexadecane was quantified by GC-MS analysis. The result revealed that 80% hexadecane was degraded within 14 days cultures at previously described condition of salinity.

During incubation period foaming and emulsification of 2T engine oil was visualized in the culture broth inoculated with ZHE05 which suggested that strain produces biosurfactant to emulsify hydrocarbons and renders their accessibility for bacterial degradation (Cavalo *et al*, 2004).

These observations suggest that isolated bacterial strain from an uncontaminated Sambhar Lake, ZHE05 was related to genus *Halomonas* and capable to degrade 2T engine oil as sole carbon and energy source. The capability of degradation aliphatic and aromatic hydrocarbon make it potential organism to consider as a bioremediation in petroleum contaminated sites. The halophilic character of bacterium considers as additional feature to use in saline and marine petroleum contaminated environments for bioremediation.

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