



Biodegradation of hydrocarbons by halophilic bacteria isolated from the saltpans of Thoothukudi district, India

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Abstract

Many halophilic microorganisms have evolved unique properties of a considerable biotechnological importance. In this study, two halophilic bacteria were isolated from the solar saltpans of the Thoothukudi district, located in Tamil Nadu, India, and were investigated for their hydrocarbon degrading abilities. These isolates were assigned names as GD30 and DM27; and then were subjected to morphological and molecular characterization, finally identified as *Oceanobacillus oncorhynchi* and *Pseudomonas stutzeri*, respectively. Among both isolates, DM27 specifically showed maximum growth and degradation activity of hydrocarbons (i.e. diesel and naphthalene). This hydrocarbon degrading ability was assayed by using 2, 6-dichlorophenol-indophenol (DCPIP) as an indicator. Our overall results demonstrated the potentiality of both halophilic bacterial isolates (*Oceanobacillus oncorhynchi* and *Pseudomonas stutzeri*), for biodegradation of hydrocarbons in contaminated saline soil.

Keywords: Hydrocarbons, halophiles, biodegradation, Thoothukudi saltpans

1. Introduction

Hydrocarbons are introduced into our environment through their extensive use as fuels and chemicals; as well as through leaks or accidental spills during exploration, production, refining, or transportation. The release of hydrocarbons into aquatic environments that contain low concentrations of inorganic nutrients, often produces unfavorable carbon/nitrogen or carbon/phosphorous

ratios for microbial growth (Van Hamme *et al.*, 2003). Poisoning due to hydrocarbons such as benzene and petroleum usually occurs accidentally by inhalation or ingestion of these cytotoxic chemical compounds. These compounds are degraded by enzyme system of microorganisms that utilize hydrocarbons as sole sources of carbon and energy. This biodegradation activity could take place over

wide range of temperatures; the rate of biodegradation however, generally decreases with decreasing temperature. This effect of temperature was also complicated by other factors such as the composition of the microbial population (Zhu *et al.*, 2001).

The most important bacterial genera in biodegradation of hydrocarbons in soil and sea water were; *Achromobacter*, *Acinetobacter*, *Alcaligenes*, *Arthrobacter*, *Bacillus*, *Flavobacterium*, *Nocardia*, *Pseudomonads*, and *Corynebacteria*. Meanwhile, among significant sea water yeasts and fungi that decompose hydrocarbons were; *Aureobasidium*, *Candida*, *Rhodotorula* and *Sporobolomyces* spp. (Antic *et al.*, 2006; Cervantes-Gonzalez *et al.*, 2007). These active microorganisms influence degradation by altering the chemistry at the interface between the environment and the hydrocarbon.

Current study is a pioneer effort based on the use of halophilic bacteria through investigating their morphological and physiological characteristics, in order to establish their affiliation with hydrocarbons biodegradation (i.e. diesel and naphthalene). We reported that the effective degrading strains belonged to *Oceanobacillus* and *Pseudomonas* spp.; however, the second strain showed more degrading potential.

2. Materials and methods

2.1. Isolation of halophilic bacteria from the saline soil samples

In this study, saline soil samples were collected from solar salt pans of Thoothukudi district, Tamil Nadu, India. The collected soil samples were serially diluted in the range of 10^{-1} to 10^{-7} in a series of test tubes according to Mayavu *et al.*, (2014). The samples were spread plated on Mineral salt (MM63) medium with 6g/100 ml of NaCl, pH of medium was adjusted to 8-9. Plates were incubated at 30°C for 3-7 days, and colonial appearances were followed.

2.2. Morphological test

Thin smear of the bacterial colonies were prepared on clean slides. The slide was heat fixed, covered with crystal violet for 3 min., and then rinsed with dist. water and air dried. The smear was covered with iodine for 1 min., rinsed with water, decolorized with 95% ethanol, washed with water, finally counterstained for about 30 sec with safranin, and then washed with water. Slides were examined under oil immersion (1,000x).

2.3. Biochemical assays

The halophilic isolates were screened with many biochemical assays such as; Indole, Methyl-red, Voges-proskauer, Citrate utilization, Catalase, Oxidase, utilization of D-Galactose, D-Fructose, D-glucose, D-xylose, Sucrose and Lactose, using standard procedures of Bergey's manual of determinative bacteriology (Holt *et al.*, 1994).

2.4. Hydrocarbon degradation assay

The 2 recovered halophilic isolates (GD30 and DM27) were subjected to hydrocarbon degradation assays using two classes of hydrocarbons namely; naphthalene and diesel fuel, using 2,6-dichlorophenol-indophenol (DCPIP) as an indicator (Oliveira *et al.*, 2012). Both isolates were grown in minimal salt (MM63) medium supplemented with glucose (10 g/l), yeast extract (1.0 g/l), and then incubated at 25°C for 72 h with shaking at 120 rpm. After incubation, the culture was centrifuged at 10,000 g for 10 min. The pellet was re-suspended in phosphate buffer and again centrifuged to remove all residues of culture medium. The pellet was finally re-suspended in phosphate buffer, the optical density (OD) was then adjusted to McFarland 0.5. The biodegradation assay was carried out in sterile Eppendorf tubes. Each tube contained 20 µl of the suspended isolate, 168 µl of minimal salt MM63 medium, 12 µl of DCPIP and 1 µl of each hydrocarbon (naphthalene and diesel), separately.

Tubes were incubated at 30°C, and then the OD readings were taken at 600 nm using a spectrophotometer after 72 h of incubation. The percentage of reduction of DCPIP was obtained using the following equation:

$$\text{DCPIP reduction (\%)} = \frac{(\text{Initial O.D} - \text{Final O.D})}{\text{Initial O.D}} \times 100\%$$

2.5. Molecular identification

The halophilic isolates were identified based on 16S rRNA gene sequencing. Briefly, DNA was extracted that served as a template for the 16S rRNA gene amplification using universal forward primer (27F) (5'- AGAGTTTGATCMTGGCTCAGTAC-3'), and reverse primer (1492R) (5'-GGYTACCTTGTTACGACTT-3') (Biozone, India, Pvt. Ltd.) referring to standard polymerase chain reaction (PCR) protocol. The amplified gene product (1 Kb) was checked on a 1.5% agarose gel against a 1 Kb DNA ladder. The BLAST search program was employed to find nucleotide sequence homology.

3. Results

Saline soil samples were collected from the coastal areas of Thoothukudi district. Thirty four colonies with different sizes and shapes on mineral salt medium (MM63) were recovered after incubation period. The selected white colored colony was named as GD30, whereas, the reddish brown colony was named as DM27. Results of Gram staining showed that isolate GD30 was a Gram positive rod shaped bacteria; on the other hand, isolate DM27 was a Gram negative rod shaped one. The selected pure cultures were subjected to various biochemical characterization assays. Isolate GD30 showed positive reaction to Methyl red, Citrate, Catalase, Oxidase, Urease, D-fructose, D- glucose, sucrose, and negative reaction to Indole, VP test, D-galactose, Lactose, and D-xylose. Whereas, isolate DM27 was positive to Indole, Methyl red, Citrate,

Catalase, Oxidase, Urease, D-galactose, D- fructose, D-glucose, sucrose, lactose, D-xylose, and negative to VP test.

The OD was measured at 600 nm before and after inoculating the hydrocarbons (diesel and naphthalene), along with DCPIP as an indicator dye. Both isolates (GD30 and DM27) showed gradual decrease in OD and a change in color was observed (Table 1). Moreover, GD30 and DM27 isolates caused reduction in % of DCPIP by (17, 14%), (28, 41%) for diesel and naphthalene, respectively. 16S rRNA gene sequence analysis revealed that isolate GD30 was a member of the genus *Oceanobacillus*, whereas isolate DM20 was a member of the genus *Pseudomonas*. The closest phylogenetic relatives of the isolates GD30 and DM20 were *Oceanobacillus oncorhynchi* and *P. stutzeri*, with a sequence similarity of 92% and 90%, respectively. The accession numbers for both halophilic isolates were assigned as; LT221188, ABI26690, respectively (Table 2).

4. Discussion

Most previous studies on biodegradation of hydrocarbons have been performed using bacterial isolates obtained from soil, but very few isolates obtained from marine soil sediment were studied.

Currently, we isolated and identified diesel and naphthalene degrading bacteria recovered from saline sediments in Thoothukudi district, Tamil Nadu. Results of their biochemical characteristics showed that none of them fit exactly with the published descriptions of members of the *Oceanobacillus* or *Pseudomonas* groups (Yumoto *et al.*, 2005; Lalucat *et al.*, 2006). Likewise, Saju *et al.*, (2011) isolated and characterized several halophilic bacteria such as *Vibrio fischeri*, *Haloacillus salinus*, *Halobacterium salinarium*, *Bacillus subtilis* and

5.

Table 1: Detection of hydrocarbons degradation by the 2 halophilic isolates (GD30 and DM27) using DCPIP as an indicator after 72 h of incubation

Isolate ID	Diesel		DCPIP reduction (%)	Naphthalene		DCPIP reduction (%)
	Initial OD	Final OD		Initial OD	Final OD	
GD30	1.375	1.140	17 %	1.558	1.127	28%
DM27	1.260	1.081	14%	1.399	0.819	41%

*Only the initial and final OD values were considered for calculating the % of DCPIP reduction

Table 2: Identification of both isolated halophilic isolates (GD30 and DM27) based on 16S rRNA gene sequence

Isolate ID	Isolate genus name	Number of nucleotides of 16S rRNA gene	Accession number of 16S rRNA gene	Closely related taxa	Sequence similarity (%) of 16S rRNA gene
GD30	<i>Oceanobacillus</i>	1497	LT221188	<i>Oceanobacillus oncorhynchi</i>	92%
DM27	<i>Pseudomonas</i>	1150	ABI26690	<i>Pseudomonas stutzeri</i>	90%

Staphylococcus citreus, from salt pans of Kovalam, Kanyakumari district. Certain biochemical characters were related to their potential use in biotechnology. The search for ideal microorganisms that could be used in bioremediation of saline ecosystems contaminated with aromatic and aliphatic hydrocarbons, is in progress all over the world. Fathepure, (2014) during studying the microbial degradation of petroleum hydrocarbons in hypersaline environments reported that, many oily and saline environments were posing a high environmental risk due to their toxic, mutagenic and carcinogenic properties. Since there was only a little information on the ability of halophiles to treat hypersaline environment contaminated with aromatic hydrocarbons, the present study was carried out in order to document the efficacy of halophilic bacteria to degrade hydrocarbons (i.e. diesel and naphthalene), using DCPIP as an

indicator. DCPIP is an electron acceptor that becomes reduced (decolorized) when redox reactions occur during microbial degradation of hydrocarbons. This significant reduction reaction was attributed to the conversion of NADH to NAD⁺ during microbial metabolism of polyaromatic hydrocarbons under hypersaline conditions.

Isolate GD30 (*Oceanobacillus oncorhynchi*) showed a hydrocarbon degrading activity toward naphthalene (28% DCPIP reduction), compared with diesel fuel (17% DCPIP reduction). Meanwhile, isolate DM27 (*P. stutzeri*) demonstrated a higher hydrocarbon degrading potential towards naphthalene (41% DCPIP reduction), compared with diesel fuel (14% DCPIP reduction). Similar study has been carried out by Selvarajan *et al.*, (2017), where halophilic isolates were screened for their ability to degrade hydrocarbons (diesel, naphthalene

and benzantracene). Six bacterial isolates were tested; however, *Salinivibrio* sp. showed the highest potency to metabolize all the three hydrocarbon classes, with significant activity against benzantracene (70% DCPIP reduction).

In this study, 16S rRNA sequence of both halophilic isolates (GD30 and DM27) was analyzed to ensure their accurate taxonomic position. On the basis of phenotypic characteristics and comparison of partial 16S rRNA gene sequence, both isolates were identified as *Oceanobacillus oncorhynchi* and *P. stutzeri*, respectively. In a similar study, Kumar *et al.*, (2012) reported the morphological, biochemical and 16S rRNA analysis of halophilic bacteria such as; *Oceanobacillus*, *Bacillus*, *Halomonas* and *Staphylococcus* genera, isolated from salt pans.

Conclusion

The present study reported the potentiality of some halophilic bacteria such as; *Oceanobacillus oncorhynchi* and *P. stutzeri* to cause hydrocarbon biodegradation; making them potentially important tools for bioremediation of hydrocarbon contaminated sites. Thus it is concluded that microbial biodegradation can be considered as a key component strategy for hydrocarbon bioremediation, due to their efficient degradability and low toxicity.

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Conflict of interests

Authors declare no conflict of interests

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