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Application of hydrocarbon degrading halophilic bacterial strains as an indicator to locate crude oil reservoirs in Atlantis II deep in Red Sea

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Abstract

Sediment and water samples were collected between 2098 -2151m in Atlantis II deep of red sea during cruses Pelagia- 64PE350-1, Poseidon- P408. The samples obtained from located in volcanic and tectonic zone were screened for hydrocarbon degrading halophilic bacterial strains and also to highlight the importance of using halophiles as an indicator for oil reservoirs in deep brines of Red Sea. Out of 40 strains isolated 12 strains were capable of degrading hydrocarbons (phenanthrene) under saline condition (4%). The hydrocarbon degrading strains present in the sediment samples were identified as *Micrococcus* sp, *Pseudomonas stutzeri*, *Pseudomonas* sp, *Vibrio* sp, *Bacillus* sp, *Bacillus subtilis* and *Bacillus megaterium*. The mixture of the bacterial strains as a halophilic consortium potentially degraded phenanthrene and fluorine completely in 4 days. The consortium also degraded 93% of pyrene in 10 days and 77% of benzo(k)fluoranthene in 12 days under saline condition. was used in the present to analyse the hydrocarbon degradation potential under saline condition. This result gives insights about the screening for presence of hydrocarbons (oil seeps) in such deeps by isolation of oil degrading halophilic bacteria as indicator organism.

Keywords: Hydrocarbon degradation, Crude oil, halophiles, Indicator, Bacillus

Introduction

The common natural sources for PAHs in environment is oil seeps, volcanic eruptions, petroleum spills and others (Bisht et al. 2014). Crude oil consist of alkanes, asphaltene resins and both low and high molecular weight PAHs (polycyclic aromatic hydrocarbons). Crude oil undergo natural biodegradation process by the indeginous microorganisms in the marine or terrestrial environment (Kumari et al. 2012, Hassanshahinan et al. 2012). Previous studies clearly revealed crude oil contamination through different sources contribute to serious environmental problems especially in soil and water (Ikhajiagbe and Anoliefo, 2011, Piubeli 2012). The hydrocarbons in the crude oil exist in the environment with high toxicity for longer period due to their hydrophobic nature (Arulazhagan and Vasudevan 2009). Contamination of water with hydrocarbons wastes stimulates indigenous microbial population, which are capable of utilizing the hydrocarbon substrates as their sole carbon and energy sources, thereby degrading the contaminants (Darsa et al. 2014). Okerentugba and Ezeronye (2003) indicated the presence of high number of oil degrading microorganisms in oil polluted environment. Different strains of *Marinobacter*, *Ochrobactrum*, *Pseudomonas*

and *Bacillus* were reported for their ability to degrade hydrocarbons in the field of oil pollution treatment (Varjani et al. 2013, Bisht et al. 2014, Pugazhendi et al. 2017). Borah and Yadav (2014) reported biodegradation of crude oil, kerosene, diesel oil and used engine oil in 28 days without any nutritional supplements (nitrogen and phosphorus) by a novel *Bacillus cereus*. The previous studies detailed different bacterial strains as a potential hydrocarbon degrader and not as a biomarker for locating oil reservoir. All the studies confirmed that hydrocarbon degrading bacteria can be formula to control the petroleum hydrocarbon pollutions. The present study was aimed to use the hydrocarbon degrading strain as an indicator to trace the oil reservoirs in the Red Sea deep areas. Also to detail the potential of the bacterial strain to degrade hydrocarbons under saline condition.

Materials and Methods

Sea Water and sediment sampling

Water and sediment samples were collected by rosettes attached with Niskin-bottles and 10cm of sediment from the top of 3m gravity corer collected for microbiological study. Latitude and Longitude of the sampling location was (21° 20.565'N and 38° 04.755'E) respectively.

Halophilic Mineral Salt Medium (HMSM)

The halophilic mineral salt medium contained the following ingredients such as Ammonium chloride (2.5 g), Potassium dihydrogen phosphate (5.46 g), Disodium hydrogen phosphate (4.76 g), Magnesium sulphate (0.2g) and sodium chloride (40g). Prior to sterilization the medium pH (7.4 ± 0.2) was adjusted using 1M NaOH solution. Phenanthrene (50 ppm) was used as the sole carbon source during the screening and enrichment of hydrocarbon degrading halophilic bacterial strain. Phenanthrene (PHE) and Fluorene (FL) from low molecular weight substance was employed to investigate the degradation ability of the halophilic bacterial consortium. Pyrene (PY) and benzo(k)fluoranthene (BKF) was used from high molecular weight PAHs.

Halophilic consortium (HC)

The bacterial strains screened individually for potential degradation of hydrocarbons (PHE and PY)under saline condition (4%). Mixture of the potential bacterial strains resulted in the formation of halophilic consortium. This halophilic consortium was investigated for biodegradation of low and high molecular weight PAHs under saline condition.

Biodegradation of Hydrocarbons

The experimental set-up for biodegradation of hydrocarbons consist of dual control namely abiotic (HMSM+PHE) and biotic control (HMSM+HC) flasks. 100 mL conical flask was used in the study, where PHE present in ethyl acetate was allowed to evaporate allowing the hydrocarbon crystals for biodegradation. Medium (22 mL) and HC (3 mL) was added in the flasks. Test flask contained HMSM+PHE+HC kept in shaker at 150 rpm and every 24 h the samples were extracted. Ethyl acetate (v/v) was used to extract the hydrocarbons present in the abiotic and test flasks. Twice the sample was extracted to improve the extraction efficiency. The extracted solvent phase was passed through anhydrous sodium sulphate and filtered in syringe filter. The filtrate was stored in HPLC (high performance liquid chromatography) vials. The samples were analysed in HPLC and GCMS (Gas Chromatograph Mass Spectrometry). All the experiments were performed with duplicates.

HPLC analysis

Biodegradation of hydrocarbons was analysed in HPLC (Agilent, USA) with respective hydrocarbon standards obtained from sigma Aldrich with 98% high purity. C_{18} general purpose column and acetonitrile with 1 mL/min flow rate was used as stationary and mobile phase.

GCMS analysis

GCMS (Shimadzu, Japan) was used to analyse the metabolites formed during the mineralization of hydrocarbons under saline condition. Silica fused capillary column and helium (carrier gas) was used as the stationary and mobile phase. Complete temperature was set and operated as detailed by Pugazhendi et al. 2017). The metabolites formed during biodegradation was identified by using GCMS internal library search.

Phylogenetic analysis

The bacterial DNA of the isolated strains and different experiments were isolated using Qiagen DNA isolation Kit following the manufacturer protocol. The

offshore drilling rig (core) samples also subjected to DNA extraction. Extracted DNA samples were amplified in thermal cycler (Applied Biosystem, USA) with 27f and 1492R primers (Arulazhagan and Vasudevan 2009). The high throughput sequencing was performed targeting V4–V5 region of the 16S rRNA gene by using primer set 515-532U 5′-GTGYCAGCMGCCGCGGTA-3′ and 909-928U 5′-CCCCGYCAATTCMTTTRAGT-3′ (26). The amplification was executed at annealing temperature of 65 °C for 30 amplification cycles. Further the second PCR with 12 cycles was performed by adding an index sequence (Arulazhagan et al. 2017). Illumina MiSeq cartridge loaded with purified PCR product for sequencing was prepared as per the manufacturer guidelines. GeT PlaGe Sequencing Center of the Genotoul Life Science Network in Toulouse, France (get.genotoul.fr) performed the sequencing for bacterial DNA. The DNA nucleotide sequence for each individual bacterial sample was matched by BLAST (Basic Logarithmic Assign Search Tool) against all available bacterial 16S ribosomal gene DNA sequences in the genes database. Upon completion of the BLAST search, the results of the search were obtained, analyzed and recorded. All the nucleotide sequences were submitted to NCBI (National Center for Biotechnology Information) and the accession number for each strain was obtained.

Results and Discussion

The present study was aimed to obtain a link between the hydrocarbon degrading strains and the location of crude oil reservoir. Samples collected from Atlantis II deep of Red Sea was mixed and screened for hydrocarbon degrading strain. Among 40 strains, 12 strains showed potential mineralization of PHE (50 ppm) under saline condition. The phylogenetic study confirmed the genus of the hydrocarbon degrading halophilic strains such as *Micrococcus* sp strain JAM1 (MF716470), *Pseudomonas stutzeri* strain JAM2 (MF716471), *Pseudomonas* sp strain JAM3 (MF716472), *Pseudomonas stutzeri* strain JAM4 (MF716473), *Vibrio* sp strain JAM5 (MF716474), *Vibrio* sp strain JAM6 (MF716475), *Bacillus subtilis* strain JAM7 (MF716476), *Bacillus* sp strain JAM8 (MF716477) *Bacillus subtilis* strain JAM9 (MF716478), *Bacillus subtilis* strain JAM10 (MF716479), *Bacillus megaterium* strain JAM11 (MF716480), *Bacillus* sp strain JAM12 (MF716481). All the 12 strains were analysed for degradation capability using PHE and FL (50 ppm) under saline condition (Table 1).

The results revealed Bacillus and Pseudomonas were the dominant strains in the samples and potentially utilized both PHE and FL in 5 days when compare to Vibrio strains under saline condition. *Pseudomonas stuzeri* reported as gram negative bacterium with wide distribution in the environment (Lalucat et al., 2006; Grigoryeva et al., 2013) showed that these bacteria are known to be nutritionally versatile and especially interesting because of specific metabolic ability (denitrification, degradation of aromatic compound, nitrogen fixation). Also *Pseudomonas stuzeri* was isolated from industrial hydrocarbon sludge with capability to fixing nitrogen (Grigoryeva et al., 2013).

Table 1. Biodegradation of PHE and Fl by halophilic bacterial strains under saline condition.

Halophilic Strain	PHE	Time	FL	Time
	Degradation (%)	day	Degradation (%)	day
JAM1	96 ± 1.1	5	100	5
JAM2	100	5	98 ± 1.2	5
JAM3	100	5	100	5
JAM4	100	5	97 ± 1.5	5
JAM5	94 ± 2.1	7	96 ± 1.4	7
JAM6	92 ± 1.3	7	95 ± 1.7	7
JAM7	100	5	100	5
JAM8	97 ± 1.2	5	98 ± 1.7	5
JAM9	100	5	100	5
JAM10	100	5	100	5
JAM11	94 ± 2.2	5	98 ± 1.1	5
JAM12	98 ± 1.2	5	97 ± 1.5	5

The present study results were also in agreement with previous research by Smith et al. 2012 reported *Vibrio parahaemolyticus* able to grow on PAHs present in crude oil. Previous studies reported *Bacillus* and *Pseudomonas* as potential hydrocarbon degrading strains (Varjani et al.2013, Das et al. 2017), still limited studies were performed under saline condition. Biodegradation of hydrocarbons in marine environment was limited by salinity, temperature, oxygen and nutrients (Atangana et al. 2003, Jamal and Pugazhendi 2018).

The crude oil drilling rig core samples collected from the Atlantis II deep sea subjected to phylogenetic study and the results confirmed the presence of *Bacillus* (76%), *Pseudomonas* (19%) and *Vibrio* (5%). Thus the halophilic strains present in the sediment and water sample act as an indicator to locate the crude oil reservoir in the Red Sea marine Environment.

Biodegradation of PAHs under saline condition

Further the mixture of the halophilic strains was used to analyse the degradation potential on low and high molecular weight PAHs. The study revealed above 90% of the low molecular weight PAHs degradation in 3 days. The results showed complete degradation of low molecular weight PHE and FL (100 ppm) in 4 days under saline condition (Fig. 1 and 2).

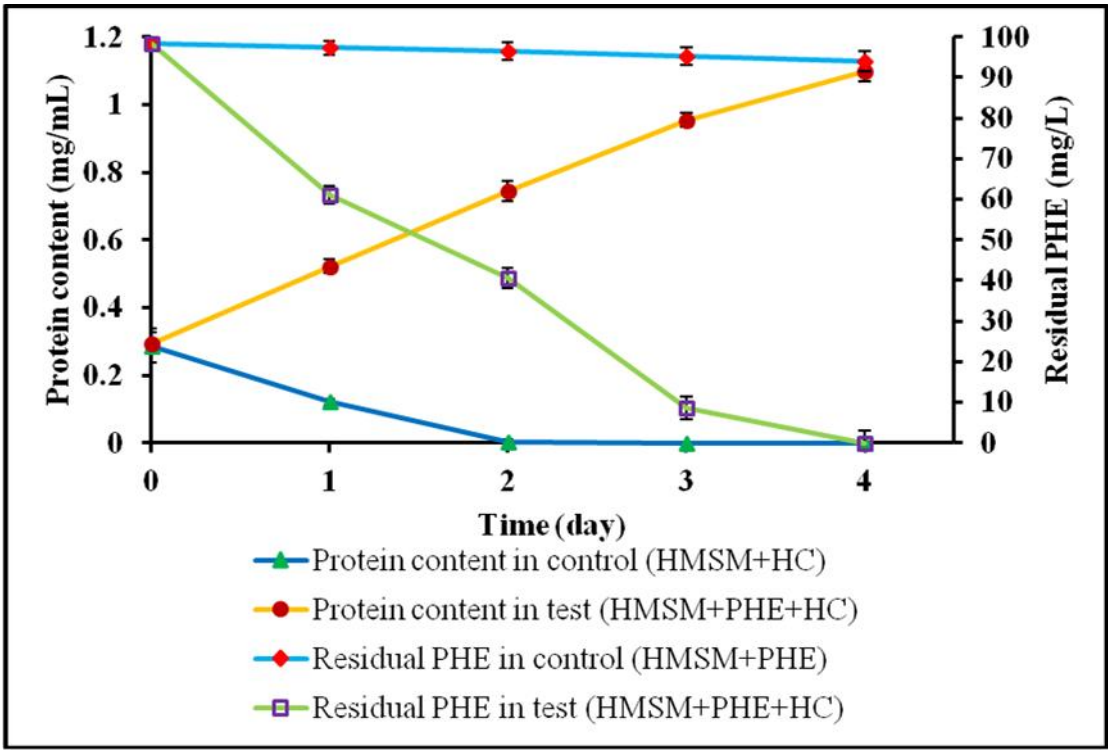

Fig. 1 Degradation of PHE by the halophilic consortium under saline condition.

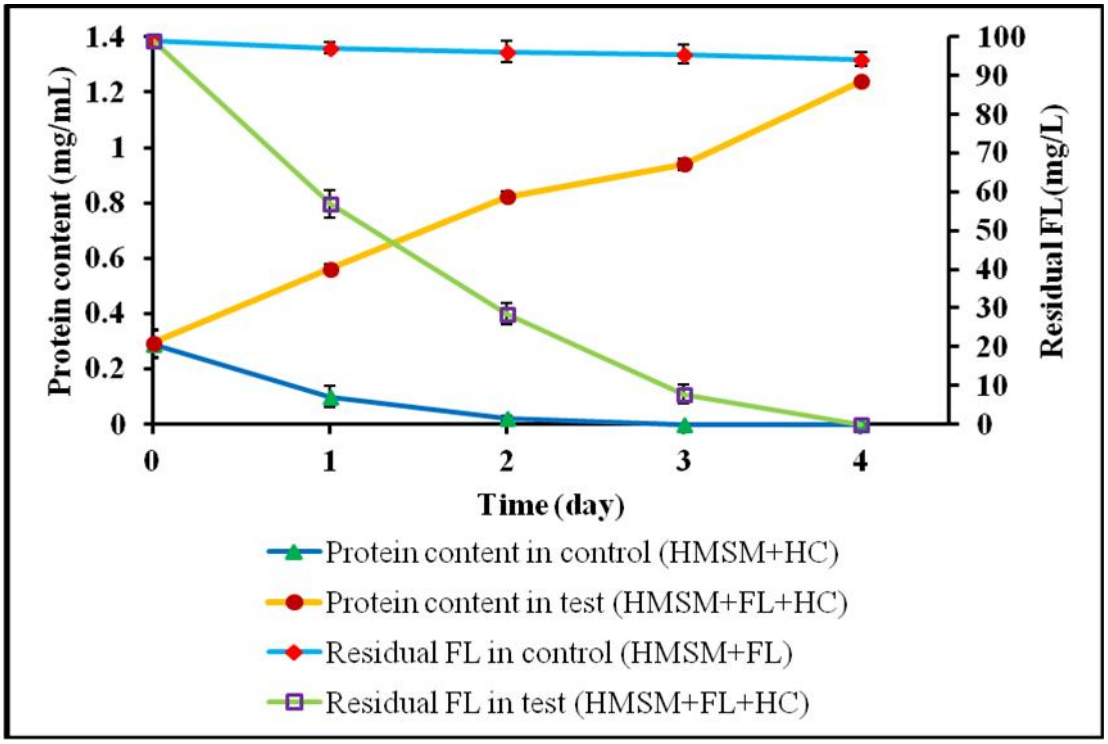

Fig. 2 Degradation of FL by the halophilic consortium under saline condition.

The halophilic consortium recorded fastidious organism in utilizing the low molecular weight PAHs. Compare to previous research studies (Jamal and Pugazhendi 2018) the halophilic consortium utilized FL at faster rate. The metabolites formed during degradation of PHE and FL subjected to GCMS analysis recorded the presence of benzoic acid and benzene-1, 2-dicarboxylic acid, where in agreement with the previous studies by Tsai et al. (2009) and Pugazhendi et al. (2017).

High molecular weight compounds such as PY (100 ppm) and BKF (10 ppm) degradation was a major challenge for the halophilic consortium. The consortium showed above 80% and 93% degradation of PY in 8 days and 10 days respectively under saline condition.

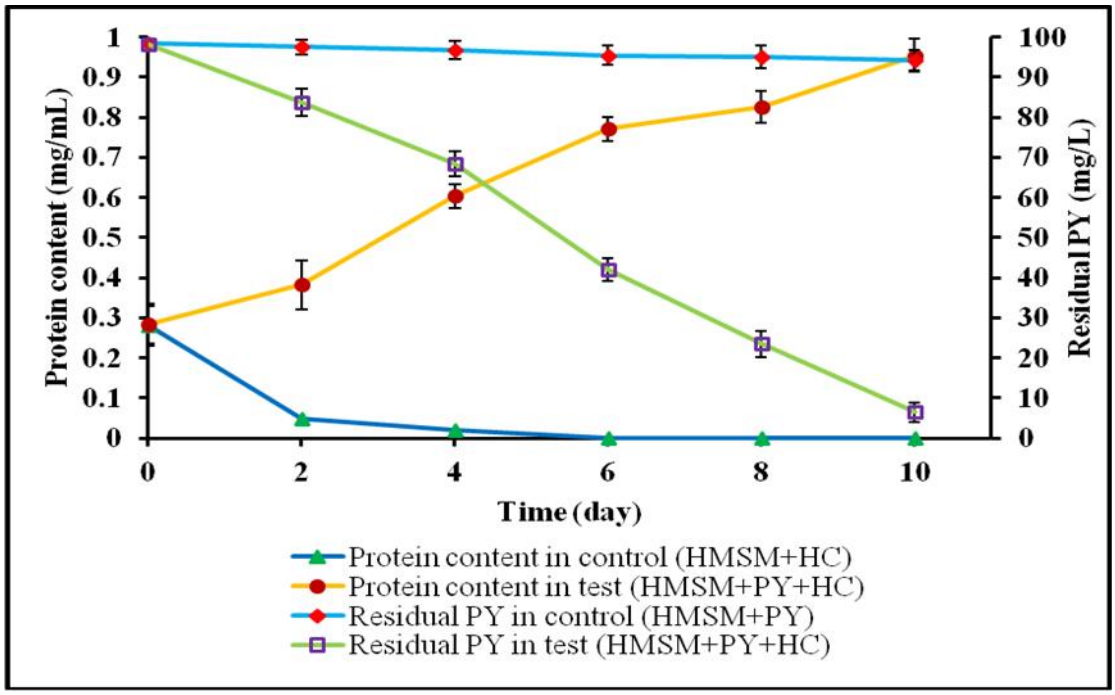

Fig. 3 Degradation of PY by the halophilic consortium under saline condition.

Halophilic consortium potentially degraded 76% of BKF in 12 days under saline condition (Fig. 4). After 12 days decline in growth of the consortium and absence of degradation was recorded. Thus the high molecular weight and salinity stressed the halophilic consortium which resulted in reduction in the progress of biodegradation process. Very few studies were performed on biodegradation of high molecular weight PAHs under saline condition (Arulazhagan et al. 2010, Jamal and Pugazhendi 2018)

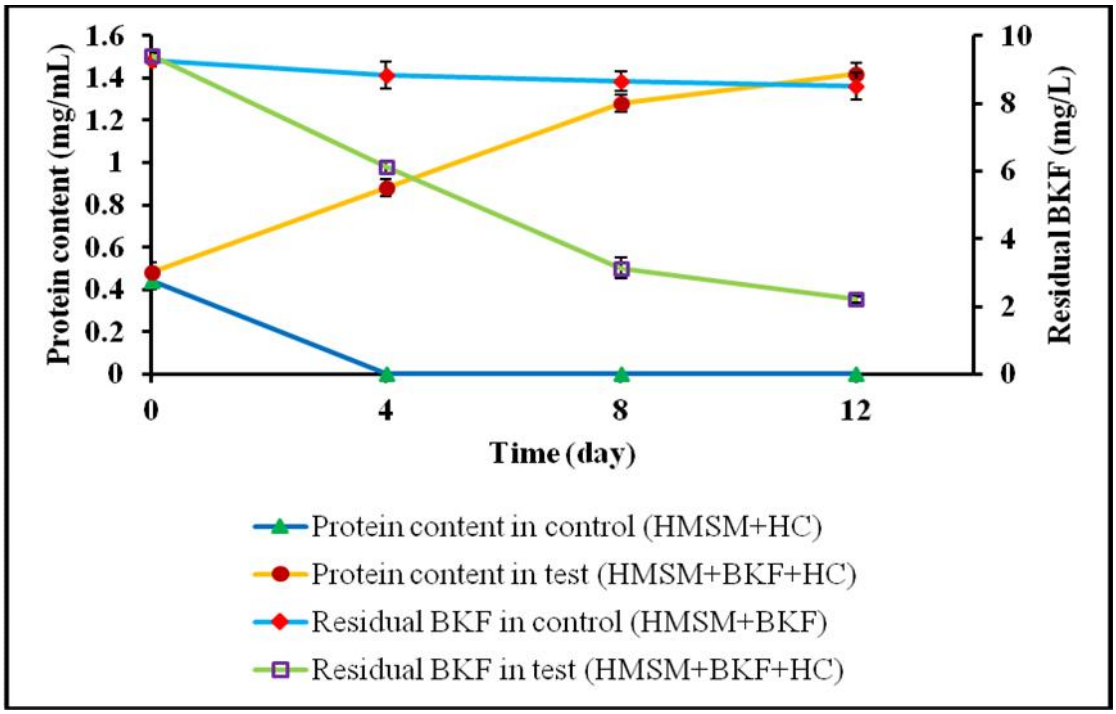

Fig. 4 Degradation of BKF by the halophilic consortium under saline condition.

Conclusion

Thus the present study clearly confirmed all the strains were potential hydrocarbon degraders under saline condition. The halophilic consortium acted as a fastidious hydrocarbon degrader which can be employed in the treatment of petroleum contaminated wastewater and coastal environment. The presence of *Bacillus*, *Pseudomonas* and *Vibrio* both in the oil drilling core rig samples and corresponding to the sediment samples clearly revealed these organisms can be used as an indicator to locate the oil reservoir.

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