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BIODEGRADATION OF CRUDE OIL BY BACTERIA ISOLATED FROM FISHING HARBOURS OF KOZHIKODE DISTRICT, KERALA, INDIA

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ABSTRACT

Hydrocarbon contamination is a major threat of marine ecosystem. The release of petroleum products by shipping activities and accidental oil spills severely affects the flora and fauna in aquatic environment and result in the imbalance of ecosystem. 5 strains of crude oil degrading bacteria were isolated from the sea water samples of Beypore and Chaliyam fish landing harbours of Kozhikode district. They have shown excellent lipase activity in tributyrin agar plates. The potential of the isolates for crude oil degradation was examined in Bushnell-Haas agar, where crude oil served as the sole carbon source. Selected isolates showed significant growth on the medium. Morphological and biochemical characterization of bacterial isolates was done. The isolates were identified as *Bacillus thuringiensis, Cronobacter sakazakii, Proteus vulgaris, Lysinibacillus boronitolerans* and *Oceanimonas doudoroffii* by 16s gene sequencing. Since fish landing harbours are generally more exposed to petroleum products, the isolates obtained from Beypore and Chaliyam are extremely adapted to survive in oily environment with potential lipase activities. The five bacterial strains isolated in this present study have promising potential to be used as bioremediators. Microbial remediation technology is regarded as an eco-

friendly and efficient technology. Thus, the products of this present study have application in bioremediation and environmental protection.

Keywords: Crude oil, bioremediation, oil spill, lipase activity, marine environment

INTRODUCTION

Environmental pollution by petroleum hydrocarbons and its adverse effects are among the most ominous problems that the world is grappling today. Past century witnessed a global surge in oil pollution due to industrial development, urbanization and so forth. Microbial degradation is one of the foremost routes in the natural removal of these oil products from contaminated environments. Microorganisms in the aquatic environment quickly respond to the changing environmental conditions. They can regulate nutrient cycling, decomposition and mineralization **[1]**. Marine bacteria have potential to survive in high halophilic conditions by using unique extracellular enzymatic activity. Enzymes such as protease, lipase, amylase, pectinase etc. acts as potential catalyst in biological and biochemical system **[2]**. The unique features of marine microbes makes them excellent source in the production of antibodies, biosurfactants, enzymes and their utilization in biodegradation and bioremediation **[3]**.

The marine environment is highly susceptible to pollution by oil spills and hence it is an important source of potential lipolytic microorganisms capable of degrading hydrocarbons. Microbial lipases gained special attention in various industries. Bacterial lipases are mainly produced by submerged fermentation. Microbial lipases gained special attention due to their stability, selectivity and substrate specificity **[4]**.Oil spills in marine waters may occur regularly during the exploration, production, refining, transport and storage of petroleum and petroleum products. This contributes to the organic pollution in marine environment and these products are classified as hazardous wastes due to their mutagenic, cytotoxic and carcinogenic effects **[5]**.

Petroleum hydrocarbons are the most alarming pollutants. It mainly includes hazardous compounds like n-alkanes, cycloalkanes and cyclic aromatic hydrocarbons. Lipolytic bacteria degrade petroleum components into small inorganic compounds **[6]**. Petroleum degrading bacteria are often exploited for bioremediation of petroleum oil contaminated environments **[7]**. Bioremediation with petroleum hydrocarbon degrading bacteria is widely

regarded as an eco-friendly and efficient technology. The cultivation of desired bacteria is done by biotechnological process **[8]**. Recently, microbial remediation technology has developed rapidly and achieved much attention. Advances in biotechnology have confirmed that several compounds present in petroleum hydrocarbons are consumed by microorganisms as sole source of carbon. These hydrocarbons are both a target and a product of microbial metabolism. There are reports about oil degrading bacteria from both oil contaminated soils and water from Kerala **[9, 10]**. But studies on oil degrading bacteria at specific sites like harbours which are continuously exposed to petroleum hydrocarbons are meagre. Hence the area for the present study gains importance as it is continuously exposed to the petroleum pollutants by various activities, being a harbour.

MATERIALS AND METHODS

Study area and sample collection

Sea water samples were collected from Beypore (11.1718°N 75.8095°E) and Chaliyam (11.1560°N 75.8113°E) harbours of Kozhikode district, Kerala. Samples of sea water from 30cm below the surface and 50cm away from the shoreline were collected during February 2020. Triplicate samples were collected in sterile amber colored polyethylene bottles with 20m distance between each other according to the protocols. All the samples were transported aseptically and processed within 2 hours of collection.

Isolation of bacteria

The seawater samples were serially diluted (10^{-1} to 10^{-3}) and spread plated on to nutrient agar plates and incubated at 28 ± 2ºC for 24 hours. The individual colonies were picked from the plate, purified by quadrant streaking and transferred to nutrient agar slants for further studies.

Screening for the extracellular lipase enzyme production

Lipase activity of bacterial isolates was tested on tributyrin agar plates (1% tributyrin as substrate) **[11]**. Plates were spot inoculated and incubated at room temperature (28°C) for 24 hours. Presence of clearance zone was noted as positive for lipase activity.

Screening for hydrocarbon degradation capacity

Bacterial isolates showing lipase production was plated on sterile Bushnell-Haas agar plates (0.02g $MgSO_4.7H_2O$, 0.02g of $CaCl_2$, 1g of KH_2PO_4, 1g of K_2HPO_4, 1g of NH_4NO_2 and two drops of $FeCl_3$). 1% triphenyl tetrazolium chloride (TTC) was

added to the media as indicator dye. The isolates were spread plated and 1ml sterile crude oil was over layered. All the plates were incubated at 28 ± 2ºC for 3 days. Formation of pink colonies was considered as positive.

Characterization of selected bacterial isolates

Morphological characterization of selected bacterial isolates was done by Gram's staining method. Biochemical characterization was carried out using Indole test, Methyl Red test, Voges-Proskauer test and Citrate Utilization test **[12]**. DNA extraction from bacterial isolates was done by genomic DNA isolation kit following manufacturer's instructions. For bacterial identification, isolated genomic DNA was used as template in the PCR amplification for 16S rRNA gene using the universal primers 27 forward (5'- AGA GTT TGG ATC MTG GCT CAG -3') and 1492 reverse (5'-CGG TTA CCT TGT TAC GAC TT -3') **[13]**. The gel purified PCR products were then sequenced following Sanger's dideoxy chain termination method with AB1 3730XL Automated sequencer. The obtained forward and reverse sequences were trimmed and aligned by using Clustal W. The consensus thus obtained was taken for searching similarity with other sequences in NCBI database using the BLAST.

RESULTS

A total of 16 distinct bacterial colonies were isolated from sample taken from Site-I, Beypore. They are represented as MSI. Whereas 15 colonies were picked from Site-II, Chaliyam water sample and denoted as MSII **(Figure 1)**. Among 16 colonies from Site-I, 11 isolates showed lipase activity on tributyrin agar media whereas 7 isolates from Site-II were found to have lipase activity **(Figure 2)**. The diameter of clearance zone around the bacterial colonies were measured and recorded in mm **(Table 3)**. The oil degrading capacity of selected bacterial strains was assessed by growth in the Bushnell-Haas agar media **(Figure 3)**. A total of 4 lipolytic bacterial isolates (MSI-3, MSI-4, MSI-8 and MSI-9) obtained from Beypore harbor (Site 1) shown crude oil degradation capacity and exhibited maximum growth in the specific media. But only one bacterial strain (MSII-7) from Chaliyam harbor (Site II) was able to utilize the hydrocarbon that resulted in the formation of pink colonies. These isolates were designated as MT 01, MT 02, MT 03, MT 04 and MT 05 respectively for the further studies. The morphological features of the selected bacterial isolates were determined by using

Gram's staining and biochemical tests for their identification and evaluation of biological properties. The bacterial isolates were identified by molecular methods using 16S rRNA gene sequencing. The PCR products amplified with 27F and 1492 R primers were subjected for electrophoresis separation in 1% agarose gel to view DNA bands of 1500 bp. The purified PCR products were analysed for sequencing by Sanger's dideoxy termination method with ABI 3730XL automated sequencer. The electropherogram were analysed in BioEdit software and sequences were aligned by using ClustalW for final sequence. The final consensus sequence obtained was subjected for BLAST in NCBI database for species level identification in terms of sequence similarity and the obtained sequences were submitted in NCBI GenBank **(Table 3)**.

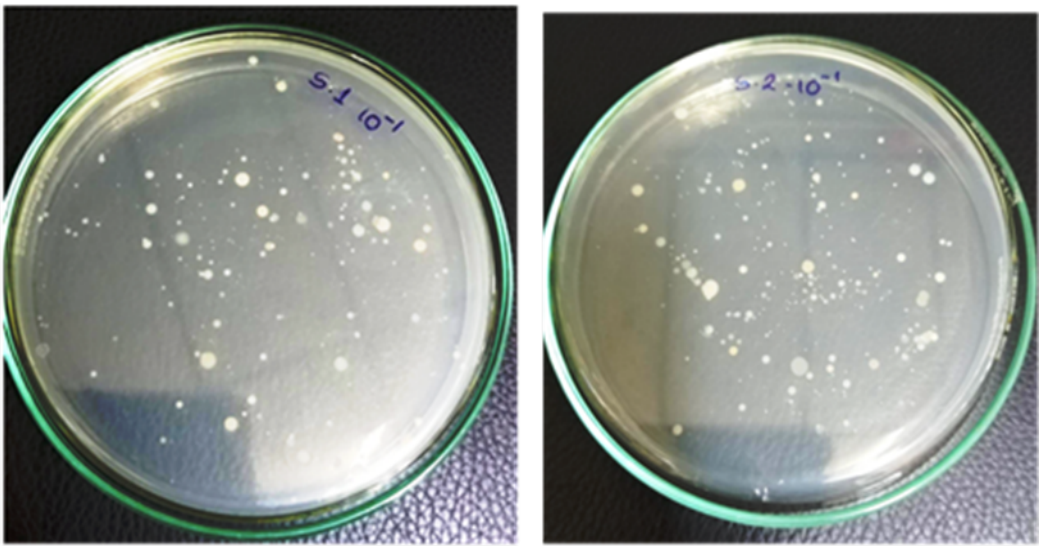

Figure 1: Spread-plate culture of bacterial colonies during the present study

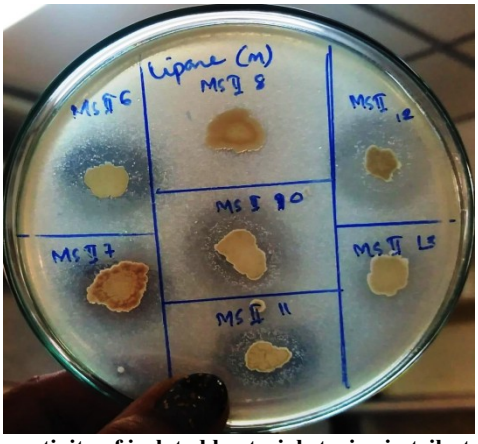

Figure 2: Lipase activity of isolated bacterial strains in tributyrin agar plates

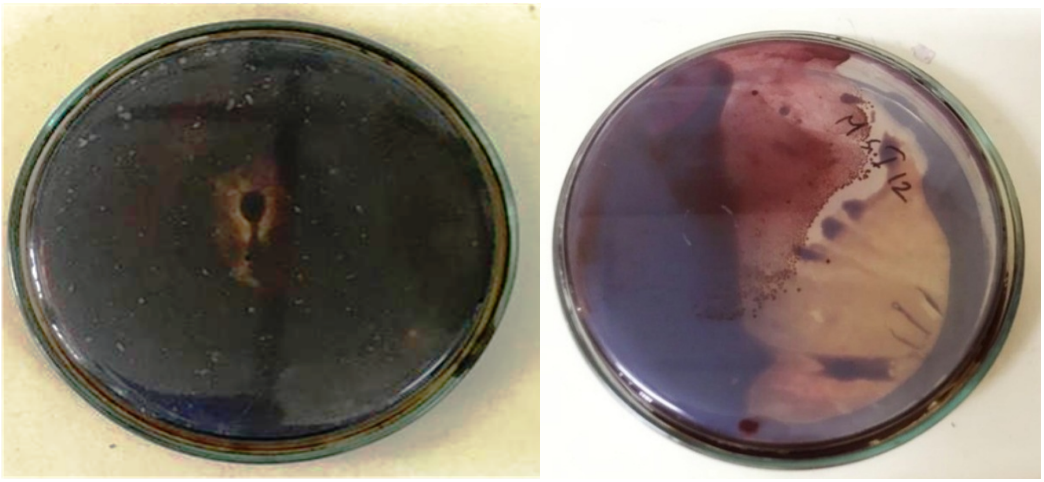

Figure 3: Crude oil degradation activity of bacterial strains in Bushnell-Haas media

Table 1: Bacterial isolates showing lipolytic activity obtained during the present study

Beypore Harbour (Site I)		*Chaliyam Harbour (Site II)*	
Bacterial isolate	**Clearance zone (mm)**	**Bacterial isolate**	**Clearance zone (mm)**
MSI-1	**12**	**MSII-1**	**12**
MSI -2	**11**	**MSII -2**	**09**
MSI -3	**17**	**MSII -3**	**10**
MSI -4	**13**	**MSII -4**	**11**
MSI -5	**09**	**MSII -5**	**08**
MSI -6	**12**	**MSII -6**	**12**
MSI -7	**11**	**MSII -7**	**14**
MSI -8	**18**		
MSI -9	**17**		
MSI -10	**10**		
MSI -11	**11**		

Table 2: Morphological and biochemical characterization of the selected bacterial isolates from the harbour marine water

Properties	*MT 01*	*MT 02*	*MT 03*	*MT 04*	*MT 05*
Gram staining	+	-	-	+	-
Shape	**Rod**	**Rod**	**Rod**	**Rod**	**Rod**
Indole Test	+	-	+	-	-
Methyl Red	-	-	+	-	-
Voges Proskauer	+	+	-	-	-
Citrate utilization	+	+	-	-	-

Table 3: Molecular identification and GenBank accession of bacterial strains in the present study

S. No.	*Bacterial strain*	*Organism*	*NCBI GenBank Accession*
1	**MT01**	***Bacillus thuringiensis***	**MW 199078**
2	**MT02**	***Cronobacter sakazakii***	**MW 201482**
3	**MT03**	***Proteus vulgaris***	**MW 199102**
4	**MT04**	***Lysinibacillus boronitolerans***	**MW 199099**
5	**MT05**	***Oceanimonas doudoroffii***	**MW 199091**

DISCUSSION

The present study involves assessment of microbial lipase enzyme as an indicator for biodegradation of crude oil. The study was carried out using sea water samples taken from fishing harbours of Beypore and Chaliyam. The sites were continuously exposed to oils and other

hydrocarbon compounds discharging from the boat at the sites. Crude oil is a complex mixture of polar and non-polar compounds. It contains a greater number of non-polar compounds than polar. Carbon atom of these polar and non-polar substrates serves as the growth substrates for microbial enzymes using degradation of hydrocarbon **[14]**. Increasing number of bacteria with the progress of time is used to determine the extent of their ability to adapt and degrade crude oil components **[15]**. The evaluation of oil biodegradation was carried out by examining the colonies developed on Bushnell-Haas agar plate after incubation. Mainly 5 potential bacterial strains were isolated for crude oil degradation in this study. For identification, bacteria were subjected to molecular characterization using 16S rRNA gene sequencing method. The organisms identified from Beypore are *Bacillus thuringiensis, Coronobacter sakazakii, Proteus vulgaris* and *Lysinibacillus boronitolerans,* whereas from Chaliyam is *Oceanimonas doudoroffii*.

Several works have been done by many researchers for the isolation of potent lipase digesting bacteria. Highly thermostable and potent bacterial strain specifically Bacillus can be used for industrial application **[16]**. *Bacillus thuringiensis* is soil borne gram positive bacteria, which are efficient producers of lipase enzyme and act as good indicator of crude oil. The viable count of *B. thuringiensis* produces efficient bioemulsifiers that can enhance the solubility and biodegradation of petroleum hydrocarbons. These exhibit the ability to dismantle crude oil through clear emulsion layer of crude oil **[17]**. Biosurfactant secreted by bacteria are more effective than chemical surfactant in enhancing the solubility and biodegradation of petroleum hydrocarbons. They significantly reduce the hydrophobicity and increase the rate of hydrocarbon biodegradation. *Bacillus thuringiensis* show 89.97% of hydrocarbon degradation on Bushnell - Haas broth **[18]**. The *C. sakazakii* is a rod shaped gram-negative pathogenic bacterium, which was formerly known as *E. sakazakii*. *C. sakazakii* helps in degrading highly dangerous organic pollutants such as poly aromatic hydrocarbons (PHA). *C. sakazakii* MM045 was proven for effective degradation of phenanthrene and pyrene, low and high molecular weight hydrocarbons from engine oil contaminated soil **[19]**. *Proteus vulgaris* is a gram-negative bacterium present in water or soil habitats. They acquire various metabolic abilities allowing adaptation to different

environmental conditions. Thus, these microorganisms have immense applications in bioremediation and environmental protection **[20]**. The bacterium *P. vulgaris* SR-1 utilize hydrocarbon and biodegrade Bonny light crude oil by as much as 78% in the presence of 1.0% NaCl after 96 hours. The gene responsible for hydrocarbon degradation could be located on (9.1kb) the plasmid it harbors **[21]**. *P. vulgaris* was reported as the first pyrene degrading bacteria. *P. vulgaris* 4Bi was excessively sufficient for positive improvement of bacterial growth and pyrene removal. Pyrene represent high molecular weight aromatic hydrocarbon that are difficult to degrade **[22]**. *Lysinibacillus* are highly efficient group of micro-degraders of crude oil. They are gram positive rod-shaped motile bacteria. The strain obtained in the present study is *L. boronitolerans*. Only few studies are reported regarding the biodegradation potential of *L. boronitolerans.* The bacterial strains were reported to be isolated from hydrocarbon contaminated soil of automobile workshops in Lapai. Here, the bacterial strain utilized 1% engine oil as the sole carbon and energy **[23]**. *Lysinibacillus* was able to form a biofilm on the control polyethylene. Polyethylene gets biodegraded via conversion of carbonyl groups into unsaturated hydrocarbon **[24]**.

Oceanimonas doudoroffii is the only bacterium isolated from Chaliyam site in the present study. It is a gram-negative bacterium usually present in sea water. Xenobiotics compounds released through natural and anthropogenic activities results in the pollution of environment. *Oceanimonas* sp. GK1 was reported for biodegradation of xenobiotic compounds such as phenol. The isolate utilizes phenol via the ortho cleavage pathway as carbon source **[25]**. All the isolates obtained during the present study are potential degraders of petroleum hydrocarbons and can be utilized commercially for bioremediation purposes.

CONCLUSION

Five oil degrading bacteria were isolated from fishing harbours of Beypore and Chaliyam at Kozhikode district. They were found to be highly efficient lipase producers with potential to degrade crude oil as sole source of carbon. The isolates were identified as *Bacillus thuringiensis, Coronobacter sakazakii, Proteus vulgaris, Lysinibacillus boronitolerans* and *Oceanimonas doudoroffii*. Biodegradation is of crucial ecological significance as this contributes to bioremediation processes. Reducing the hydrocarbon in a contaminated

environment is a significant challenge. The growth and oil degrading potential of the isolates at different conditions need to be optimized further. Further characterization of the oil degrading property of these isolates might give a lead to its potential application in bioremediation.

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