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Plasmid Incidence in Four Species of Hydrocarbonoclastic Bacteria Isolated from Oil Polluted Marine Environment

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Abstract: The present investigation is on evaluation of biodegradation potential of four species of hydrocarbonoclastic bacteria, *B. megaterium*, *C. kutscheri*, *L. delbrueckii* and *P. aeruginosa* and screening for plasmids. For biodegradation study, the strains were cultured in mineral salt medium with 0.1% of crude oil for seven days. Biodegradation results inferred that, *P. aeruginosa* showed 85.15% of crude oil degradation, followed by *B. megaterium* (78.5%), *C. kutscheri* (76.4%) and *L. delbrueckii* (71.6%). Strains were grown for 24 h in Luria-Bertani broth for plasmid study. Plasmid study revealed that, *P. aeruginosa* harbored two plasmids with molecular weight of 4.2 and 3.8 kb. The other strains namely *L. delbrueckii*, *C. kutscheri* and *B. megaterium* were found to have single plasmid with respective molecular weight of 3.8, 4.2 and 4.1 kb. Complete loss of biodegradation potential was observed when the plasmids of these strains cured with acridine orange.

Key words: Biodegradation, plasmids, hydrocarbonoclastic bacteria

INTRODUCTION

Environmental pollution is a cause of major concern affecting ecosystems globally. Oil spill, an offshoot of this environmental pollution caused by spillage from tankers, release of effluents and offshore drilling activities is adversely affecting the aquatic ecosystems (Atlas, 1981). Chemical or physiological means of controlling oil spill are rapidly being developed, but they have the disadvantage that, these chemicals leave toxic wastes which may not be biodegradable. Physical means are only useful in clearing small areas. Use of eco-friendly methods such as bioremediation using microbes is the recent concern for environmental pollution. The adaptation of microbes to survive in the polluted environment is mainly based on their genetic make up. Plasmids that have been found to harbor genes encoding the transformation of environmental pollutants are known as catabolic plasmids. From single step reaction to multi step pathways, plasmids appear to be versatile mean for microorganisms to gain metabolic capacities for the exploitation of otherwise unavailable resources (Anthony *et al*., 2000). Heterotrophic bacteria have been observed to exhibit a higher incidence of plasmid DNA in hydrocarbon contaminated environments, such as offshore fields (Hada and Sizemore, 1981), riverine sediment polluted by coking plant discharges (Burton *et al*., 1982; Day *et al*., 1988) ground water contaminated by aromatic hydrocarbons (Ogunseitan *et al*., 1987) and a dystrophic lake containing naturally high concentrations of aromatic humic compounds (Schutt, 1989). Some of the plasmid mediated bacterial utilization of various carbon compounds which could be found in the complex mixture of crude oil (Chakrabarty, 1976). Presence of catabolic genes responsible for the degradation of naphthalene in plasmid of *P. putida* was reported by Park *et al*. (2003). With the information in above literature, the present study was carried out to investigate the presence of plasmids in four species hydrocarbonoclastic bacteria and their biodegradation ability of crude oil.

MATERIALS AND METHODS

Isolation and identification of hydrocarbonoclastic bacteria (HCB): *Bacillus megaterium*, *Corynebacterium kutscheri*, *Lactobacillus delbrueckii* and *Pseudomonas aeruginosa* were isolated from water samples collected in Tuticorin harbor, South East Coast of India (Lat. 08°44'N; Long. 78°13'E). Further analysis of the sample was carried out at Centre of Advanced Study in Marine Biology, Annamalai University, Parangipettai, India. Bushnell Haas agar (g L^{-1} of $MgSO_4$-0.2, $CaCl_2$-0.2, KH_2PO_4-1.0, K_2HPO_4-1.0, NH_4NO_3-1.0, $FeCl_3$-0.05 and agar-15 g) supplemented with 0.1% crude oil was used for the isolation of bacteria.

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The strains were identified to species level by following Bergy's manual of determinative bacteriology (Buchanan *et al*., 1974).

Estimation of crudr oil degradation: *B. megaterium*, *C. kutscheri*, *L. delbrueckii* and *P. aeruginosa* were cultured in 250 mL culture flasks containing 50 mL of mineral salt medium (g L^{-1} NH_4Cl 0.5, K_2HPO_4 0.5, NaH_2PO_4 0.5, pH 7.5, natural seawater 750 mL and 250 mL of distilled water) supplemented with 0.1% of crude oil (Juwarkar and Khirsagar, 1991) and the cultures were maintained in shaker at 150 rpm for 7 days at 30°C. After 7 days, cell free culture broth was extracted with three volume of toluene and extract was made up to 10 mL and the OD was measured at 420 nm. The percentage of degradation was calculated from the standard curve. The standard graph was obtained with different concentration of crude oil in toluene (Rahaman *et al*., 2002). The experiment was carried out in duplicate and the mean values were expressed.

Isolation of plasmids: Strains were grown in Luria-Bertani (LB) broth and cells were pelleted by centrifugation at 5000 rpm for 15 min at 4°C. Plasmids from the cells were isolated using alkaline lysis method (Sambrook and Russel, 2001). Isolated plasmids were stored in TE buffer until analysis. 0.8% of agarose gel was casted by dissolving 0.8 g of agarose in 100 mL of 1X TAE buffer and the plasmid DNA was loaded along with the loading buffer into the wells. Electrophoresis was carried out with 50 mA of current and the DNA bands were viewed under UV trans-illuminator. Molecular weight of the isolated plasmids was determined by using Total Lab Software. ECOR1 Hind-III double digest was used as molecular weight marker.

Plasmid curing: The role of plasmids in biodegradation process was confirmed by curing the plasmids with acridine orange at a concentration of 500 μg mL^{-1} which was added to the culture broth and incubated for 12 h (Fujji *et al*., 1997). The plasmid cured strains were screened for biodegradation.

RESULTS AND DISCUSSION

Biochemical and physiological characteristics of the strains are given in Table 1. The existence of hydrocarbonoclastic bacteria in the marine environment was already documented by the researchers (Bhosle and Mavinkurve, 1980; Baruah and Deka, 1995) supported the findings of the present study on isolation of HCB from marine environment. Hydrocarbon pollution in a particular area may increase the fraction of hydrocarbon-utilizing microorganisms and it may also increase the capacity of the microbial community to degrade hydrocarbons (Leahy *et al*., 1990). It was evidenced by the results obtained in the present study on biodegradation of crude oil. *P. aeruginosa* showed maximum degradation of crude oil (85.15%), followed by *B. megaterium* (78.5%), *C. kutscheri* (76.4%) and *L. delbrueckii* (71.6%). Similar results were obtained with bacterial strains namely *Rhodococcus rhodochrous* KUCC 8801 (93.1%), *Rhodococcus* sp. ISO1 (81.1%), *Acinotebacter calcoaceticus* IRO7 (91.2%) and *Pseudomonas putida* IR32 (47.6%) with 5 days of incubation (Sorkhoh *et al*., 1990) also complemented the results of the present study on biodegradation.

Table 1: Biochemical and physiological characteristics of hydrocarbonoclastic bacteria

Test	*B. megaterium*	*C. kutscheri*	*L. delbrueckii*	*P. aeruginosa*
Gram reaction	+	+	+	-
Cell shape	Rod	Rod	Rod	Rod
Spore formation	+	-	-	-
Catalase	-	+	-	+
Motility				
Hydrolysis of				
Starch	+	-		+
Gelatin		-		+
Fat	+			+
Carbohydrate utilization				
Adonitol				-
Arabinose	+		-	-
Cellobiose			-	-
Fructose			+	+
Galactose				-
Glucose			+	-
Mannitol	+		-	+
Mannose			+	-
Rhamnose			-	-
Ribose			-	+
Sorbitol			-	-
Sucorse		+	-	-
Trehalose			-	-
Xylose	+		-	-
Dnitrification	+	-		+

+ Positive result, -: Negative result

Results obtained in the plasmid analysis revealed that, *P. aeruginosa* was found to harbor two plasmids with molecular weight of 4.2 and 3.8 kb. The other bacterial strains namely *L. delbrueckii*, *C. kutscheri* and *B. megaterium* were found to have single plasmid with respective molecular weight of 3.8, 4.2 and 4.1kb (Fig. 1). Presence of catabolic genes responsible for the degradation of naphthalene in plasmid of *P. putida* was reported by Park *et al*. (2003). This finding supported the present study on plasmid mediated degradation of crude oil. Plasmids that have been found to harbor genes encoding the transformation of environmental pollutants are known as catabolic plasmids. From single step reaction to multi step pathways, plasmids appear to be versatile mean for microorganisms to gain metabolic

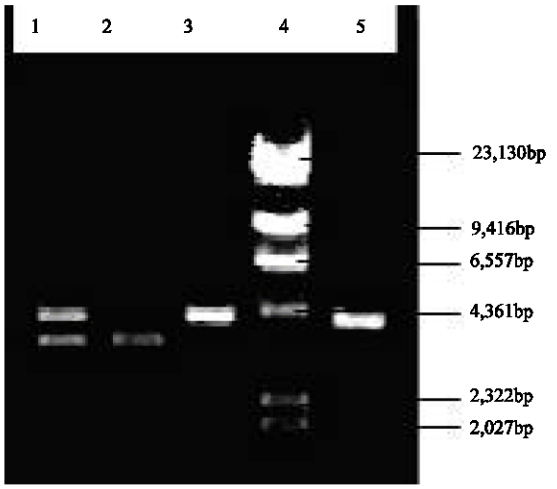

Fig. 1: Plasmids isolated from four species of HCB, Lane 1. *Pseudomonas aeruginosa*, Lane 2. *Lactobacillus delbrueckii*, Lane 3. *Corynebacterium kutscheri*, Lane 4. λ DNA/Hind III marker, Lane 5. *Bacillus megaterium*

capacities for the exploitation of otherwise unavailable resources (Anthony *et al*., 2000). The incidence of plasmids in oil degrading bacteria had been already reported by many researchers. According to Devereux and Sizemore (1982), plasmids were detected in 21% of the strains isolated on crude oil and 17% on polynuclear aromatic hydrocarbons. They also observed multiple plasmids in 50% of the plasmid containing strains are similar to the two plasmid observed in *P. aeruginosa* in the present study. Bacteria isolated from oil polluted environments have previously been shown to be more effective in degrading crude oil than bacteria from unpolluted environments (Colwell *et al*., 1973) and plasmid frequency increased in various hydrocarbon-contaminated environments (Burton *et al*., 1982; Day *et al*., 1988; Hada and Sizemore, 1981; Ogunseitan *et al*., 1987; Schutt, 1989). Small plasmid was obtained from *Pseudomonas* strain with a molecular weight 3.2 MDa in sediments from Campeche Bank (Leahy *et al*., 1990) which complemented the present result on plasmids with molecular weight of 3.8 to 4.2 kb in oil degrading bacteria.

The total loss of biodegradation activity after plasmid curing over emphasizes the role of plasmids in catabolic activity of hydrocarbon biodegradation. Rheinwald *et al.* (1973) reported the loss of isobutyrate degradation after plasmid curing in *Pseudomonas putida*. Stuart-Keil *et al.* (1998) observed the loss of naphthalene degradation after plasmid curing in marine heterotrophic bacterial population. These findings supported the results of present study on loss of biodegradation ability after plasmid curing.

In Conclusion, results obtained in the present study on biodegradation of crude oil inferred the possibility of applying these four hydrocarbonoclastic bacteria or their products (biosurfactants) for environmental cleaning of oil spill. Loss of biodegradation activity after plasmid curing indicated that, the genes responsible for biodegradation of crude oil may be plasmid mediated.

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