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Isolation and Characterization of a marine bacterium belonging to the genus *Alkaligenes* capable of the complete mineralization of the dibenzothiophene

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Following enrichment on benzoate five bacterial isolates capable of aerobic growth on dibenzothiophene were obtained from sea water collected off the west coast of India near the city of Goa. Sampling location had been previously contaminated with furnace oil spilled from a tanker. Classified as marine bacteria by their requirement for and optimum growth on 3% NaCl, all the isolates were identified by morphological observations and biochemical tests as belonging to the genes *Alkaligenes*. A single isolates producing intense red pigmented colony when growing on dibenzothiophene was chosen for further studies and designated as Strain JR110. During catabolism of dibenzothiophene intermediates were observed after initial dissolution and complete mineralization was achieved. JR110, independently confirmed as belonging to the genus *Alkaligenes* by Im Tech, India was deposited in their culture collection as MTCC3317.

[Keywords: Biodegradation, bioremediation, marine pollution, xenobiotics, subtrate-solubilization, marine microbiology, sequential enrichment.]

Introduction

Biotechnology is viewed as an inexpensive and appropriate tool for detoxifying and bioremediating the components in contaminated environments ever since microorganisms were constructed in the laboratory to catabolize such xenobiotics[1-5]. The microbial biodegradative pathway genes are often clustered and thus are amenable to cloning[6]. In our earlier studies, we have reported the isolation of a series of marine bacteria degrading a number of crude oil components and have successfully demonstrated their efficacy for clean-up of tar balls in marine microcosms[5]. In the monsoon of 1995 a major devastation occurred along the coast of Goa on the western coast of India as a result of cyclone. A shipping vessel *M.V. Sea transporter* ran a ground off the Bay of Goa resulting spillage of large amounts of furnace oil from the ballast which contaminated large stretch of beaches. The present paper reports the isolation and characterization of a marine bacterium that has evolved to completely mineralize dibenzothiophene-a sulfur containing polycyclic aromatic hydrocarbon component of tar balls-from the site which was contaminated by tons of furnace oil spill in year 1995. Attempts have been made to follow up the changes undergone by dibenzothiophene in marine environment until its complete destruction by the marine bacterium belonging to the genus *Alkaligenes*.

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Materials and Methods

Marine microorganisms were cultured in the medium prepared in artificial sea water (ASW) of following composition formulated by Kester et al[8]: In 1 litre: Tris base-6.05 g; $MgSO_4$-12.32 g; KCl-0.74 g; $(NH_4)_2.HPO_4$-0.13 g; NaCl-17.52 g; $CaCl_2.2H_2O$-0.14g; pH adjusted to 7.4. Carbon substances whenever used were of 0.1% (w/v) concentration and solid medium contained 2.0% Agar-agar (Difco). Growth was determined by measuring the optical density at 600 nm on uv-visible spectrophotometer.

Deep sea sampling and sequential enrichment technique[7] for isolation of marine microorganisms

500 mL of deep sea samples were collected during the short scientific cruises on board small mechanized trawlers using a Niskin sampler mounted on a CTD device at a depth of 1 meter from desired preplanned locations around the oil spillage affected region. Each sample was filtered through 0.22 µm Millipore

membrane filter. The Millipore filter was aseptically transferred into 50 mL of ASW medium containing 0.1% (w/v) sodium benzoate in sterile Erlenmeyer flasks. The flasks were incubated at room temperature on the research vessel itself. On arrival of vessel to the laboratory, aliquots from only those flasks indicating substantial turbid growth were transferred to fresh ASW medium containing 0.1% (w/v) dibenzothiophene and subcultured until a pure culture colony could be isolated and purified on solid agar plate of ASW containing same concentration of dibenzothiophene. Morphological and biochemical tests were performed as described previously[9].

Creation of simulated condition of sea water

To create simulated conditions of sea, ASW medium containing 0.1% (w/v) dibenzothiophene was inoculated with marine strain and the medium was maintained at room temperature on a rotary shaker at 200 rpm (revolution per minute). 1 mL aliquots of growing culture were taken at regular intervals and growth determined along with the determination of residual concentration of dibenzothiophene per mL.

Determination of dibenzothiophene

The dibenzothiophene solutions in carbon tetrachloride show a maximum absorbance peak at 289 nm if scanned on uv-visible spectrophotometer. A standard curve was established with different concentrations of dibenzothiophene above 10 nanograms per mL. Pure, distilled carbon tetrachloride and dried over sodium sulfate lacking on absorbance peak at 289 nm served as a negative baseline control.

Determination of rate of dissolution of dibenzothiophene in aquatic medium

1 g of dibenzothiophene was placed in clean sintered glass funnel of 5 mL capacity. 4 ml of ASW medium was over layed on the dibenzothiophene and the medium was stirred regularly over the sintered glass in the funnel which was attached to an Erlen Mayer flask with side arm leading to a vacuum pump. The filtrate from sintered glass funnel was regularly collected at every 60 mins in the flask by applying the vacuum and the amount of dibenzothiophene in the aqueous solution was determined by extracting with equal volume of carbon tetrachloride and measuring the OD at 289 nm and dissolved amount of dibenzothiophene in µg per mL.

Preparation of cultures cells preactivated with Na-benzoate

For activation of the culture cells with Na-benzoate a pure colony of the culture was inoculated in 100 mL of ASW medium containing Sodium benzoate 0.1% (w/v) and incubated on rotary shaker at room temperature. After 48 hrs the turbid culture was centrifuged at 5000 rpm at 4°C. The pallet was suspended in 10 mL of ASW and the suspension was used as benzoate activated culture.

Detection of the intermediary transformation catabolites of dibenzothiophene in bacterial culture

Whole culture broths along with cell free supernatants were acidified at various time intervals during the process of growth of culture in ASW medium containing dibenzothiophene and extracted with 3 volumes of dried and distilled ethyl acetate. The ethyl acetate extract was dried over anhydrous sodium sulfate and evaporated to dryness in vacuum. The residual mixture was redissolved in minimum amount of ethyl acetate and the solutions were applied onto preparative silica gel G ready-made to use TLC plates and the chromatograms were developed in a solvent system of chloroform and methanol (100:5). After locating the fluorescent pots under uv light, the silica gel surface over the spot was carefully seraped out and mixed with methanol or chloroform to elute out the resolved compounds. Authentic compounds from sigma chemical co. USA were used to compare the resolved chromatograms.

Results

Isolation and characterization of marine bacteria utilizing dibenzothiophene.

The programme of isolation of the degraders of oil components was under taken six month after the disaster and the samples were taken from the restricted areas around the spillage site. 500 mL of Samples were processed by sequential enrichment technique .

The sequential enrichment technique on Sodium benzoate followed by dibenzothiophene resulted in the production of an orange to red coloration in the tube containing ASW medium with dibenzothiophene within two weeks. Aliquotes from these tubes when plated on ASW agar containing dibenzothiophene revealed a number of dibenzothiophene-degraders which were numbered in the sequence of their isolation and characterized further for their

morphology and biochemical phenotypes (Table 1). Out of five dibenzothiophene-utilizing culture strains, isolate strain JR110 which showed intense red pigmented colony on dibenzothiophene agar was picked up and further purified on the same medium until an isolated pin-pointed dark red colony was obtained. All the strains listed in the Table 1 grew optimally on the growth media when supplied with up to 3% NaCl, without which their viability was lost after 2 to 3 subcultures. Morphologically the strain JR110 was an aerobic, gram negative and highly motile coccobacillus. A battery of biochemical tests indicated that the strain JR110 belonged to the genus Alkaligenes according to the Bergey's manual of determinative bacteriology[10]. Strain JR110 was deposited at the IMTECH Chandigardh, India as MTCC 3317. The morphological and biochemical characteristics and identification carried out at IMTECH corroborated with those described in Table 1.

Dissolution of dibenzothiophene in the sea water medium

To follow up the fate of dibenzothiophene in the sea, the marine isolate strain JR110 was grown in the artificial sea water medium which simulates the condition in the sea. Rate of dissolution of dibenzothiophene were as estimated. . To determine the extent of dissolution initiated by the marine strain

Table 1—Morphological and biochemical characteristics of marine isolates*

Tests	strain 107	strain 108	Strain 109	Strain 110	Strain 111
Colony Morphology					
Margin	Entire	Cnvex	Entire		
Elvation		Convex		Convex	Convex
Colour	Cream	Cream	Cream	Translucent	Translucent
Size	2mm	1mm	1-2mm	1-2mm	1-2mm
Shape	consistancy	circular irregular	Circular butyrous	Circular butyrous	Oval
Gram Reaction of the Cell		Gram-ve rod	Gram-ve rod	Gram-ve rod	Gram-ve
Motality	Motile	Highly motile	Motile	Motile	Motile
Biochemical tests					
Indole production methylred test	-	-	-	-	-
Voges Proskauer citrate-utilization test	-	-	-	-	-
Urea hydrolysis test	-	-	-	+	-
Casein hydrolysis test	-	-	-	+	-
Starch hydrolysis test	+	+	+	+	-
Tween 80 hydrolys	+	+	-	+	+
Catalase test	+	+	+	+	+
Gelain liquefication test	+	+	+	+	+
Arginine dihydrolase oxidase test		+	+	+	+
Degradation of aromatic substances					
Benzooate	+	+	+	+	
Nepthalene					
Phenantherene				+	
Fluorene				+	
Pyrene		-		+	
Anthracene		-		+	
Pyridine		-	-	+	
Indole		-	+	-	-
Quinoline				+	
Thionepthalene	-	-	-		-
Dibenzothiophene	+	+	+	+	+
Degradation of intermediates of Dibenzothiophene					
Dibenzothiophene sulfone	-	-	-	+	-
Pigment formation on Dibenzothiophene medium	-	-	-	Red pigment	-

* +, Positive growth; -, no growth.

JR110, 0.1 mL of the culture suspension that was pre-activated on sodium benzoate was added to 5 mL of ASW in sintered glass funnel and the dissolved dibenzothiophene was similarly determined. Fig. 1 shows the rates of dissolution of dibenzothiophene in ASW and ASW spiked with sodium benzoate-pre-activated cell-suspensions of marine Alkaligenes species Strain JR110.

Dynamics of biodegradation of dibenzothiophene in simulated marine environments

Mineralization is the complete degradation of dibenzothiophene from the growth medium. The simulated conditions of sea were created . ASW medium containing 0.1% dibenzothiophene (w/v) was inoculated with marine Strain JR110 and the medium was maintained at room temperature on a rotary shaker at 200 rpm (revolution per minute). 1 mL of aliquots of growing culture were taken at regular interval and the growth was determined along with the determination of residual concentration of dibenzothiophene per mL. Figure 2 shows that the depletion of residual dibenzothiophene from the sea water medium was concomitant with the growth of marine Strain JR110; and growth was initiated when the dissolved dibenzothiophene was at its maximum level (Fig. 1). Gas chromatographic profile of the ethyl acetate extracts of the cell-free culture-supernatant indicated that the mineralization of dibenzothiophene was accompanied by formation of distinct metabolites showing peak different from that of dibenzothiophene. Confirmation of the intermediate product formation could be evident from the uv-visible scans of the coloured cell-free supernatants, performed at different time intervals during growth of the culture on dibenzothiophene. Thin layer chromatograms of the ethyl acetate extracts from culture supernatants during the lag phase of growth revealed the presence of 4 metabolites, one of which co-migrated with the authentic compound, dibenzothiophene sulfone, however none of the metabolites could be detected during the late

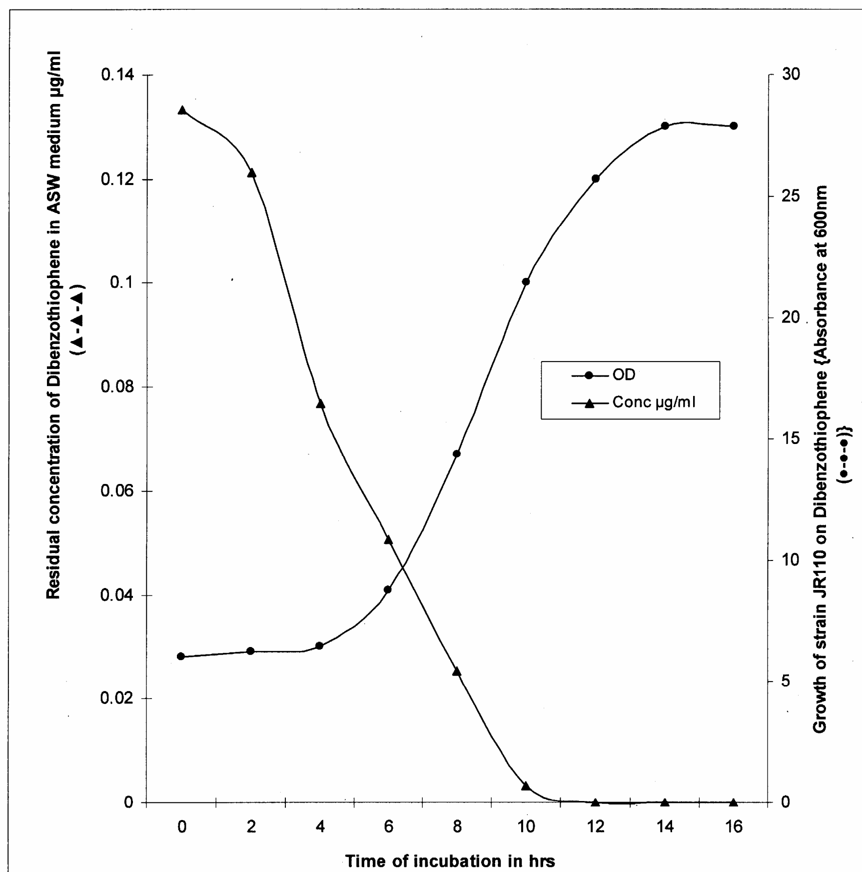

Fig. 1—Rate of dissolution of Dibenzothiophene in ASW (▲-▲-▲), dissolution intiated by isolate Alkaligenes species Strain JR110; (●-●-●), indigenous dissolution.

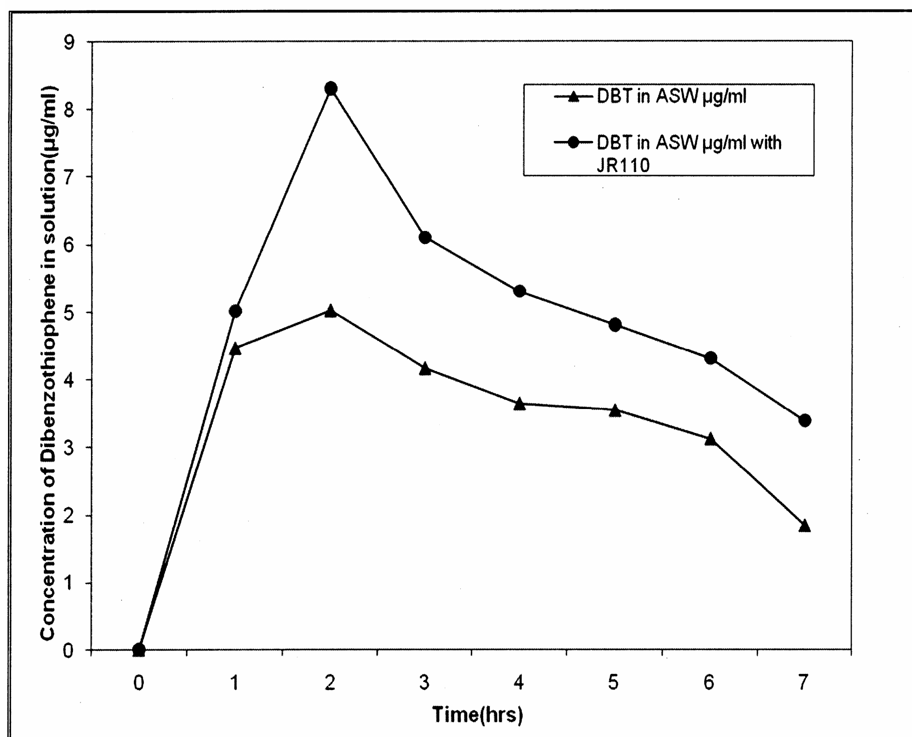

Fig. 2—Dynamics of mineralization of Dibenzothiophene by Alkaligenes species Strain JR110. (▲-▲-▲), concentration of residual dibenzothiophene in ASW medium; (•-•-•), growth profile of the Strain JR110.

stationary phase of growth indicating clearly their transient formation in culture before dibenzothiophene was completely mineralized.

Discussion

Dibenzothiophene is a polycyclic aromatic hydrocarbon consisting of 2 benzene rings fused to a central sulfur-embedded thiophene ring and is a prominent member of the heavier asphalthene, NSO fraction of crude oil. Thus, eluding from this compound resists the destructive physical and chemical weathering pressure exerted in the environments, Alkaligenes species strain JR110 isolated in this study is one of the isolates that clearly demonstrated complete mineralization of dibenzothiophene first, by initiating the dissolution and followed by formation of its transient intermediary metabolites. The absorbance peaks in the uv-visible spectra at 281 nm, 390 nm 480 nm and at 485 nm represent characteristic absorption maxima of 3-hydroy-2-formyl benzothiophene and trans-4-[2-[(3-hydroxy)-benzothiophene]-2-oxo-3-butanic acid (HBTOB) as described by Kodama *et al.*[11] in which he has recognized two major types of pathways for dibenzothiophene metabolism in bacteria. It is quite evident that the intermediates formed by strain JR110 are similar to those detected by Kodama et al[11]. Ready utilization of DBT sulfone (Table 1) and the disappearance of the intermediate compound during the late stationary phase of growth provide unequivocal proof that DBT sulfone and HBTOB the transient intermediary products in the mineralization of dibenzothiophene. Fig. 3 summarizes the predicted degradative pathway of dibenzothiophene by strain JR110. Utilization of dibenzothiophene sulfone as sole carbon source by Strain JR110 (Table 1.) proves that it is one of the transient intermediary products during the mineralization of dibenzothiophene. The Strain JR110 plays a remarkable potential for its application for bioremediation of crude oil-contaminated marine environments because it has with all the characteristics attributable to a typical marine bacterium defined by Macleod[12] .

Conclusion

Present study demonstrates that there is continuous evolution of degradation processes of xenobiotic components of crude oil in marine environment of west coast of India in response to the exposure of the sea to any toxic xenobiotics damage. It also demonstrates that it is possible to isolate the marine microorganisms evolved to mineralize xenobiotics by

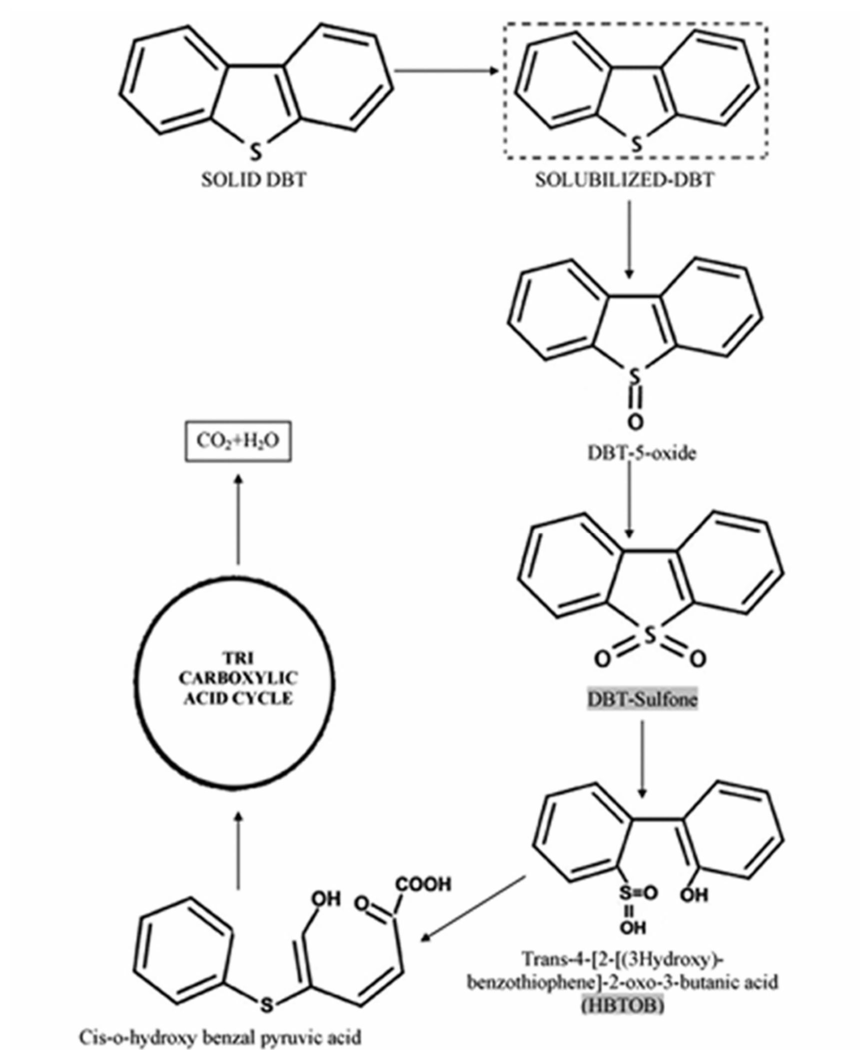

Fig. 3—Predicated degradation pathway of DBT in Alkaligenes species JR110. Names of the products framed in grey blocks are identified in this study.

using appropriate enrichment such as sequential enrichment technique used in this study. The intermediary metabolites of benzoate formed by the residual benzoate utilizing pathway in the gram negative and marine bacteria are believed to exert a positive pressure on the degradative pathway to activate enzymes for mineralization of dibenzothiophene leading to the success of sequential enrichment technique in enriching the requisite bacteria.

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