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Short Communication

Isolation and characterization of meta-toluic acid degrading marine bacterium

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The analysis of the sea water samples using sequential enrichment technique revealed a report of marine bacterium capable of degrading meta-toluic acid-a component of crude oil. An attempt to characterize the isolated culture using biochemical tests indicated the culture as a Gram- negative aerobic rod that was highly motile exhibiting biodegrading ability and was identified as *Pseudomonas spp.* strain GUI13. Further, a comparative analysis of the biochemical characters with the archae-type terrestrial soil bacterium indicated that the isolate required marked amounts of Sodium chloride (NaCl) in the medium to retain its viability. Substrate constant (K_s) of strain GUI13 with respect to meta-toluic acid was found to be eight times lower when compared to that of a terrestrial bacterium. A similar ratio was observed in case of Michaelis constants (K_m) for the key degradative enzyme, Catechol 2,3- dioxygenase, emphasizing the distinguishing feature of the marine bacteria that helps it to carry on the process of bio-transformations at very low concentrations of carbon, a unique condition that exists in the sea.

[**Key words**: Bio-degradation, marine pollution control, Substrate constant (K_s), Michaelis constants (K_m) values, crude oil, Tar balls, Catechol 2, 3-dioxygenase, and hazardous chemicals]

Introduction

The increasing use of petroleum for energy and for the production of a large number of chemicals has led to the introduction of an unlimited amount of crude oil and refined petroleum products into the biosphere. Worldwide attention has been particularly focused on the marine environment as oceans and coastlines have generally been the sites of oil spills. Technologies for cleaning up hazardous chemicals are often expensive, inappropriate for the site, or ineffective in handling complex mixtures of residual pollutants. Bio-technology using genetically engineered micro-organisms has been viewed as the most promising technology to solve this enormous problem [1].

Micro-organisms degrade the hazardous chemicals via non-toxic intermediates at very high speed in order to avoid dilution and extinction, by evolving specific catabolic degradative pathways and enzymes that rapidly turn over the available carbon compounds into cell material and energy. Genes for such pathways are generally clustered in shorter DNA regions, which are amenable for cloning[2]. Bio-transformation, therefore, is an attractive option because of the formation of only non-toxic residues such as CO_2 and water, which can get cycled within the biosphere. In many cases, these technologies are also less expensive and less disruptive than the options commonly used to remediate hazardous wastes such as excavation and incineration. A case in point is the classic genetic engineering of multi-plasmid micro-organism or degradation of fuel hydrocarbons [3,4].

In our earlier studies[5], we have reported the isolation of a series of marine bacteria biodegrading a number of crude oil components and have demonstrated their efficacy for clean up of tar balls in marine microcosms. Study on enzyme production by coastal micro-organisms was also the focus of our earlier studies[6,7,8]. The present study was undertaken to highlight specific differences between a marine micro-organism and terrestrial bacterium with respect to the degradation of meta-toluic acid – a toxic component of crude oil or tar balls that persists as a marine pollutant for long periods and is normally degraded by micro-organisms via a key intermediate, Catechol, to form cell material and carbon dioxide. The key enzyme for the bio-degradation of meta-toluic acid and catechol is Catechol 2, 3-dioxigenase[5].

A marine micro-organism (*Pseudomonas* species strain GUI13) was isolated and characterized up to its genus level and attempts were also made to study the dynamics of growth of the isolated strain on meta-toluic acid. A typical soil bacterium isolated independently from Japanese soils[4] was used in the

study for comparative analysis to identify the salient differences in bio-degradation of meta-toluic acid, including the key enzymatic activities involved in the bio-degradative pathway.

Seawater samples (500 ml) were collected from 1 m depth from different locations in the Arabian sea (Fig. 1) during February, 1994 onboard R.V. Gaveshani cruise (No.243), in sterile screw-cap bottles and filtered through 0.22 µm pore-size Millipore filters. The enrichment was initiated by transferring the Millipore filter itself into 50 ml of ASW medium[9] (pH 7.0) with 0.08 % sodium benzoate as the sole source of carbon. The flasks were rotated on environmental rotary shaker at room temperature (28°C) at 250 r.p.m. After 48 hours of obtaining substantial turbidity, aliquots of turbid culture were transferred to fresh ASW solid medium for four consecutive sub-cultures and the plates were incubated at room temperature (28°C). A loopful of this enriched culture was repeatedly inoculated in ASW agar and incubated at room temperature until isolation and purification of pure meta-toluic acid bio-degrading strains. The pure culture was morphologically and bio-chemically analyzed to enable its identification.

The following bacterial cultures were used in this study:

1. Marine *Pseudomonas* species strain GUI13 isolated and characterized in this study
2. Terrestrial soil *Pseudomonas putida* strain mt2 isolated from the Japanese soils by Prof. Sachie Inouye[4] spared for the present study.

A loopful of the activated culture was inoculated in 100 ml ASW medium containing a 0.08% meta-toluic acid as the sole sourec of carbon and the medium was incubated on the rotary shaker at room temperature.

The Indole production test[10] was carried out using peptone (Difco, 1.0g; sodium chloride 0.05 g; pH 7.2) dispensed in 5 ml volume in test tubes and autoclaved. The control tubes used were without any inoculum whereas the other test tubes were inoculated with overnight young cultures. For testing the degradation of aromatic substrates the cultures were inoculated in ASW medium containing 0.1% of each substrate. All the tubes were incubated on rotary shaker at room temperature and turbidity was detected after 48 hrs. After two days of growth 0.5 ml of Kovak's reagent was added to each tube. If deep red colour compound separated out in alcohol it indicated

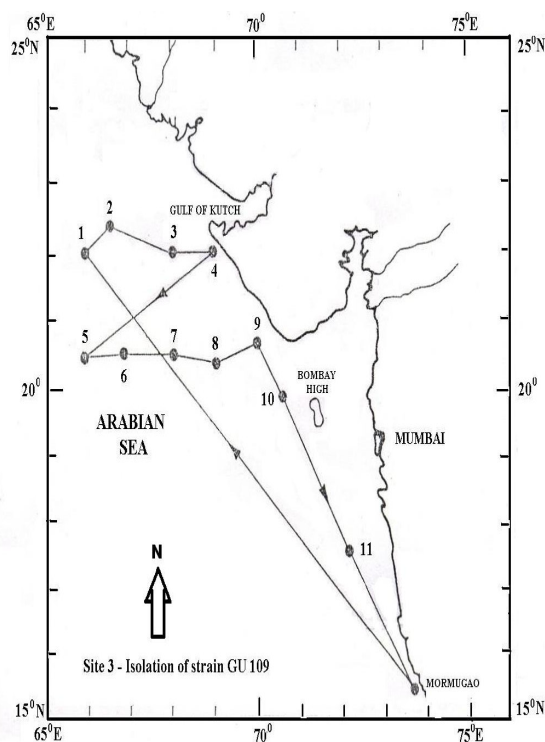

Fig. 1—Cruise track of R.V. Gaveshani (Cruise No. 243) showing sampling sites

the production of indole from peptone and were recorded as positive cultures.

The methyl red test[11] was carried out using the medium (K_2HPO_4 0.5g; peptone (Difco) 0.5g; distilled water 100 ml; pH 7.5; glusose separately filter sterilized was added at final concentration of 0.5%; 95% methyl red was separately made in distilled water and 5 drops were added to each tube of 5 ml and all tubes were incubated at room temperature for two days. Growth of the cultures in varying concentrations of meta-toluic acid was monitored at room temperature by measuring spectroscopically the increase in biomass at 550 nm in Shimatzu 240 spectrophotometer. Specific growth rates for the cultures in each concentration of meta-toluic acid were calculated by using the formula: $dx/dt = \mu x$; where x is biomass at any given time t, dx/dt is the slope of the growth curve, and μ is the specific growth rate. The substrate constant for (K_s) was calculated from the Lineweaver- Burk plot of reciprocals of specific growth rates activity against the reciprocals of substrate concentrations and expressed as moles of meta-toluic acid / ml.

For preparation of crude extracts containing Catechol 2, 3- dioxygenase enzyme, 100 ml of culture broth was centrifuged at 6000 r.p.m. at 4°C for 20

Table 1—Morphological and bio-chemical characteristics of bio-degrable micro-organisms

No.	Tests-Colony Morphology	*Marine Pseudomonas sp* strain GUI13	*Soil Pseudomonas putida* mt2
		Results	
1	Margin	Entire	
2	Elevation	Convex	Elevated
3	Colour	Cream	Translucent
4	Size	1-2mm	I mm
5	Consistency	Butyrous	Butyrous
6	Shape	Oval	Circular
7	Gram reaction of the cell	Gram negative rod	Gram negative rod
8	Motility	Highly motile	Motile
Biochemical tests			
9	Indole production		-
10	Methyl red test		-
11	Voges Proskauer test		-
Substrate utilisation tests			
12	Citrate utilisation test	-	
13	Urea hydrolysis	-	-
14	Casein hydrolysis	+	+
15	Starch hydrolysis	+	+
16	Tween 80 hydrolysis	+	+
17	Catalase test	+	+
18	Gelatin hydrolysis	+	-
19	Oxidase	+	+
Biodegradation of aromatic substrates			
20	Benzoate	+	+
21	Toluic acid	+	+
22	Xylene	+	+
23	Substrate constant (K_s) with respect to meta-toluic acid (m moles of meta-toluic acid/ ml)	0.006	0.048
24	K_m, value for the enzyme Catechol 2, 3-dioxygenase (n moles of pyrocatechol/ ml)	0.007	0.056

minutes. The pellet was resuspended in 50 ml of 50 mM phosphate buffer, pH 7.5 and sonicated at 4°C using Vibracell sonicator. The sonicated suspension was then centrifuged at 10,000 r.p.m at 4°C. The supernatant was used as a crude extract of the enzyme for analysis containing Catechol 2,3-dioxygenase enzyme.

For analysis of Catechol 2, 3- dioxygenase[8], the reaction mixture contained pyrocatechol in a total volume of 1ml 50 mM phosphate buffer, (pH 7.4) and after addition of 60 µl of crude extract containing the enzyme, the absorbance at 375 nm was measured in quartz cuvette with a 1.0 cm light path. One unit of activity was defined as the amount of enzyme required to form 1 µm of the product per minute under the conditions of the analysis. Specific activity was expressed as the unit of activity of enzyme per mg of protein in the reaction mixture. The Michaelis constant (K_m value) was calculated from the Lineweaver-Burk plot of reciprocals of specific activities against the reciprocals of substrate concentrations and expressed as n moles of pyrocatechol / ml.

The sequential enrichment technique resulted in the formation of a turbid growth on ASW containing sodium benzoate as sole source of carbon. Aliquots of this culture worked out to be good source of inocula to seed the ASW medium containing a more complex compound, meta-toluic acid. A pure culture isolated on meta-toluic acid and purified further on meta-toluic acid and sodium benzoate was Gram- negative, aerobic rod, highly motile, that conformed many characteristics of reported bio-degrading soil bacteria[12] *Pseudomonas,* hence was identified as *Pseudomonas* sp. strain GUI13 based on th*e* scheme of Bergey's manual of systematic bacteriology[13]. Since the culture was unique in degrading meta-toluic acid the culture was designated as strain GUI13.

Our results for the requirements of the marine culture strain GUI13 indicate that it grows optimally on all growth media when supplied with up to 3% NaCl. MacLeod[12] believes that this unique dependence on NaCl and ability to live in the sea is the only characteristic that distinguishes marine micro-organisms from other bacteria. Strain GU I13 however, showed very high bio-degradation activity even at low concentrations of meta-toluic acid when associated with requisite concentration of NaCl. The substrate constant (K_S), which is the indicator of bio-degradation, is 8-fold lower for the marine micro-organism, strain GUI13 than its terrestrial counterpart *(Pseudomonas putida mt2;* Table 1) which was borrowed from Japanese scientists. These differences are attributed to the degradative enzymes, which are distinctly faster in transforming the toxic compounds by marine micro-organisms as reflected also in the Km value of key enzyme catechol 2,3-Dioxygenase towards its respective substrate.

In conclusion, this study demonstrated that there are continuous degradations of toxic components of

crude oil in Northern Arabian Sea and by using suitable enrichment technique it is possible to isolate pure marine culture degrading the toxic components. The important strength of this work lies in showing the unequivocal evidence that the rates of degradation of meta-toluic acid is faster than that of a soil bacterium. This count makes the marine micro-organisms to be viewed as better candidates in bio-remediation processes.

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