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Biochemistry of TBT-Degrading Marine Pseudomonads Isolated from Indian Coastal Waters

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Abstract Tributyltin (TBT) is a very effective biocide and an active ingredient in antifouling paints. Screening along the Indian coast yielded 49 bacterial isolates capable of TBT assimilation. The screening was done based on the ability of bacteria to grow in mineral salt medium (MSM) containing TBT as the sole source of carbon. All the isolates produced exopolysaccharides (biosurfactants) in the medium which aid in emulsification and thus ease bioavailability of TBT. Five isolates were identified as potent TBT degraders (namely, *Pseudomonas pseudoalcaligenes*, *Pseudomonas stutzeri*, *Pseudomonas mendocina*, *Pseudomonas putida*, and *Pseudomonas balearica*) based on their biomass production in MSM containing TBT as the sole source of carbon. In addition to evaluating the potential of individual bacterial strains, the study also focused on using a consortium of bacteria to explore their synergistic effect when grown on TBT. Further tests like growth profile, rhamnolipid secretion profile, extracellular protein secretion profile, and detection of siderophores were performed on these isolates when grown in MSM supplemented with 2 mM TBT concentration. Emulsification activity of the crude extracellular polysaccharides against kerosene was evaluated. It can be therefore inferred that TBT degradation by these marine pseudomonads is a two-step process: (a) dispersion of TBT in the aqueous phase and (b) tin–carbon bond cleavage by siderophores affecting debutylation of TBT. The consortium of bacteria may be effective in the treatment of TBT-contaminated waste water in dry docks.

Keywords Debutylation · *Pseudomonas putida* biotype A · Rhamnolipids · Siderophores · Tributyltin chloride (TBT)

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1 Introduction

Tributyltin (TBT) compounds are a subgroup of the trialkyl organotin family of compounds. They are used as biocides to control a broad spectrum of organisms. TBT is the active ingredient in antifouling paints applied to ship hulls and other submerged structures in the marine environment. TBT is extremely hazardous to some aquatic organisms because it is toxic at nanomolar concentrations in water (Blunden and Chapman 1986; Bangkedphol et al. 2009). It is an endocrine disruptor that can induce imposex in some female mollusks, caused by problems related to steroidogenesis (Horiguchi et al. 2006) and shell thickening in oysters (Bryan and Gibbs 1991). TBT

is a persistent organic pollutant that could pose a health threat to marine life and possibly humans due to biomagnification along the food chain (Bangkedphol et al. 2009).

European Union (EU) ban on the presence of TBT-based antifoulings on ships hulls in EU ports came into effect on 1 January 2008. Though there is a ban on application of TBT-based antifouling paints from September 2008 as per AFS convention (Rodríguez et al. 2010; Garg et al. 2011), the environmental risk will still persist as dry docking of ships for paint removal may release large amounts of TBT into near shore waters (Mukherjee et al. 2009; Garg et al. 2011). Waldock et al. (1988) estimated total input from cleaning a single vessel (naval frigate) at 100 g of freely available TBT but including TBT bound to paint chips (which might act as a long-term reservoir of butyltins) led to estimates of almost 1 kg TBT per vessel per cleaning operation.

The problem may be even bigger in developing countries where laws for waste disposal may not be very stringent. Although TBT is biodegradable in the water column, this process is not rapid enough to prevent the occurrence of elevated TBT levels in some areas. Gibson and Wilson (2003) evaluated the extent of imposex in *Thais orbita* 10 years after the partial ban on TBT was implemented in Australia and compared it with data collected before the ban. Their study revealed that though there was a decrease in the frequency of imposex occurrence in the coasts, no significant change in the frequency of imposex was observed in harbors and bays, indicating persistence of TBT. Widespread contamination of TBT is detectable along the coastal regions of many countries (Sudaryanto et al. 2002; Inoue et al. 2006; Kim and Kim 2008).

The degradation of TBT in natural environment appears to be mainly controlled by microbial degradation, rather than physical and chemical action. Microorganisms capable of TBT uptake include certain fungi, viz., *Coniophora puteana*, *Trametes versicolor*, *Chaetomium globosum*, *Aureobacidium pullulans*, and *Cunninghamella elegans* (Barug 1981; Orsler and Holland 1982; Gadd et al. 1990), bacteria, e.g., *Alcaligenes faecalis*, *Flavobacterium* sp., and many *Pseudomonas species* (Visoottiviseth et al. 1994; Kawai et al. 1998; Inoue et al. 2000, 2003; Yamaoka 2003; Roy et al. 2004; Roy and Bhosle 2005; Stasinakis et al. 2005). Seligman et al. 1988 reported that microbial degradation was the primary process for TBT degradation in seawater and determined that the half-life for TBT was approximately 6–7 days. Since microbial degradation of TBT is the primary process in the environment, biological treatment has a high potential for TBT-contaminated wastewaters. Brandsch et al. (2001) found that biological degradation was much faster under aerobic conditions and, with increasing temperature, TBT was completely degraded (no measurable concentration at a detection limit of 1 mg/kg of dry weight) at temperature of 55°C (Kotrikla 2009).

The present study involves tests performed on potent TBT-degrading microbes and their consortium isolated from Indian coastal waters to understand the mechanism of TBT degradation. These microbes were identified as pseudomonads which produced rhamnolipid biosurfactants and siderophores in TBT-containing medium. Consortium of bacteria resulted in good growth, i.e., short lag phase in TBT than individual strains due to the synergistic effect of these isolates. Consortium secreted higher concentrations of extracellular proteins (ECP) and extracellular polysaccharides (EPS) consistently during the study period and could be employed in treatment plants with short retention times for TBT degradation.

Being a hydrophobic substrate, TBT uptake would depend on its dispersion/dissolution into the aqueous phase, which is brought about by surfactants/emulsifiers. Microorganisms are known to produce surfactants to increase the bioavailability of hydrophobic organic compounds (Balba et al. 2002; Urum et al. 2003). Microbes are also known to produce iron-chelating compounds called siderophores under iron-limiting conditions. In response to iron-deficient conditions, nearly all aerobic and facultative anaerobes have been found to produce Fe (III)-chelating siderophores to provide them with iron (Höfte 1993).

These molecules have very high affinity for iron and thus help in sequestering iron from the medium. Inoue et al. 2003 reported the role of pyoverdine (a fluorescent siderophore) secreted by *Pseudomonas chlororaphis* CNR15 in chelation of tin from triphenyltin (TPT). Several researchers have reported that siderophores are not only produced under conditions of iron limitation but also in the presence of heavy metals like mercury, zinc, etc. Sun et al. 2006 reported that *Pseudomonas aeruginosa* CGMCC 1.860 secreted

pyochelin into the medium even in the presence of 100 μM iron in TPT-containing medium. The supernatant from this culture exhibited a much higher capacity for TPT decomposition (40%) in contrast to resting cells which could degrade about 6% of TPT under similar conditions. In the debutylation of TBT and DBT by several strains of microorganisms, the MBT formed has been observed in solution rather than in the biomass (Errécalde et al. 1995).

2 Materials and Methods

2.1 Bacterial Strains

Marine pseudomonads isolated from Indian coastal waters and identified as potent TBT degraders (ability to grow in mineral salt medium (MSM) broth containing 2 mM TBT concentrations) namely *Pseudomonas pseudoalcaligenes* NIOT5, *Pseudomonas stutzeri* NIOT23, *Pseudomonas mendocina* NIOT15, *Pseudomonas putida* NIOT20, and *Pseudomonas balearica* NIOT44 were used for the present study. Bacteria were identified by fatty acid methyl esters analysis. A consortium of all these isolates was also formed after conducting plating experiments which confirmed that these isolates did not inhibit the growth of one another.

2.2 Media

MSM broth [in grams per liter—$FeSO_4$ 0.06, K_2HPO_4 12.6, KH_2PO_4 3.64, NH_4NO_3 2.0, $MgSO_4{\cdot}H_2O$ 0.2, $MnSO_4{\cdot}7H_2O$ 0.0012, $Na_2MoO_4{\cdot}2H_2O$ 0.0012, $CaCl_2{\cdot}2H_20$ 0.15, pH 7.4] as described by Mahtani and Mavinkurve (1979) containing 2 mM TBT (TBT stock solution was prepared as a 100 mM solution of TBT in 0.2 μm filtered ethyl alcohol). All media components used were procured from Hi-Media and TBT from Sigma-Aldrich. Zobell marine broth (Hi-Media) was used for inoculum preparation.

2.3 Experiment

The five potent TBT degraders and their consortium were inoculated into MSM broth containing 2 mM TBT. Inoculum was prepared by harvesting cells grown in ZMB and resuspending them in phosphate-buffered saline (pH 7.2) to obtain optical density of 1 at 600 nm. A control containing 20 ml/L ethanol in MSM was employed for each isolate. Culture flasks were incubated in a rotary shaker at 25°C and 150 rpm for 5 days. All experiments were carried out in triplicate and mean values recorded. Cell-free supernatants were obtained by centrifugation of cultures at 10,000 rpm for 10 min at 4°C. Cell density (colony forming units (CFU) per milliliter), rhamnolipid, and ECP estimations were carried out after 6, 24, 48, 72, and 96 h of incubation. Qualitative estimation of siderophores was performed after 96 h of incubation.

2.4 Growth Profile

The growth profile of the cultures was measured as CFU per milliliter by plate count method by plating appropriate dilutions of culture onto Zobell marine agar at different time intervals.

2.5 Rhamnolipid Extraction and Estimation

Rhamnolipid concentration was determined by the orcinol assay as described by Pamp and Tolker-Nielsen (2007). Culture supernatants were extracted twice with diethyl ether, and the organic fractions evaporated. Rhamnolipids were reconstituted in double-distilled water and diluted tenfold using orcinol reagent (60% sulfuric acid/1.6% orcinol, 1.5:1). The mixture was heated at 80°C for 30 min and read spectrophotometrically at 421 nm after cooling to room temperature. Concentrations were calculated from a standard curve prepared with L-rhamnose and expressed as rhamnose equivalents.

2.6 Emulsification Activity of Crude EPS

Extracellular polysaccharides (EPS) were extracted by ethanol precipitation of 50 ml of cell-free culture supernatants at −20°C and subsequently cleaned up by dialysis (using Pierce Snake Skin Membrane) against double-distilled water. The partially purified EPS was reconstituted in 10 ml of distilled water. Emulsification indices (E_{24}) for ethanol extracted EPS were calculated against kerosene after a 24-h standing time by the formula E_{24}=Volume of emulsion/Total volume of mixture. Uninoculated medium (MSM with TBT) was used as control. The density of emulsion was measured spectrophotometrically as absorbance at 600 nm.

2.7 ECP Purification and Estimation

Protein purification was carried out by trichloroacetic acid precipitation method. Protein estimation was done using the bicinchoninic acid assay (Protein Estimation Kit, Bangalore Genei) using bovine serum albumin obtained from Sigma as standard.

2.8 CAS Assay for Detection of Siderophores

Siderophore production was checked by Liquid CAS assay (Schwyn and Neilands 1987). Cell-free supernatants were diluted 1:1 with the CAS reagent (comprising CAS, 2×10^{-4} M; $FeCl_3$, 2×10^{-5} M; Cetrimide; 1.6×10^{-3}; PIPES, 1×10^{-4}; pH adjusted to 5.8 by adding HCl) and vortexed. Absorbance was read at 630 nm after 1 h of incubation at room temperature. Uninoculated medium served as negative control and desferrioxamine (Desferal, Novartis Pharmaceuticals) as the positive control. All glassware used in the experiment were washed after soaking in 6 N HCl for 24 h.

3 Results and Discussion

3.1 Growth Profile

Since the medium contains TBT as the sole carbon source, any growth in the medium indicates assimilation of TBT into the growing bacterial cells. The control flasks did not show any bacterial counts beyond 6 h for lack of carbon source. The growth profile of the isolates and their consortium is given in Fig. 1. The rate of growth of *P. putida* and *P. stutzeri* was higher at 0.22 and 0.20 CFU/ml per hour respectively than the consortium (0.162 CFU/ml per hour).

P. mendocina and *P. balearica* had a growth rate of 0.135 and 0.09 CFU/ml per hour, respectively, for the first 72 h. *P. pseudoalcaligenes* had a growth rate of 0.10 CFU/ml per hour for first 48 h after which the cells slowly entered stationary phase. All isolates showed a marked decrease in the growth rate after 72 h except *P. mendocina* which maintained the same rate of growth for 96 h. The consortium of bacteria showed a marked decline in growth rate after 72 h (0.01 CFU/ml/h) which was half the rate of *P. balearica*, the slowest growing organism in the study, implying that the consortium had a shorter life cycle, i.e., it had a shorter lag phase (as is evident by the higher cell counts at 6 h) and an early stationary phase when compared to individual isolates. *P. mendocina* showed intermediate growth in the medium. *P. pseudoalcaligenes and P. balearica* showed poor growth in the medium. After 96 h of incubation, the fast growing cells of *P. putida*, *P. stutzeri*, and the consortium show adhesion to glass walls of culture flask and auto aggregation, making further study of growth profile impossible.

3.2 Rhamnolipid Secretion Profile

During incubation, TBT morphology went through three apparent changes. Upon addition of TBT, the medium turned milky. In the first few hours that followed, TBT appeared as a scum over the aqueous medium or stuck on the glass walls of the culture flask. Later, TBT dispersed into the medium, making it more transparent albeit with a yellow tinge. This

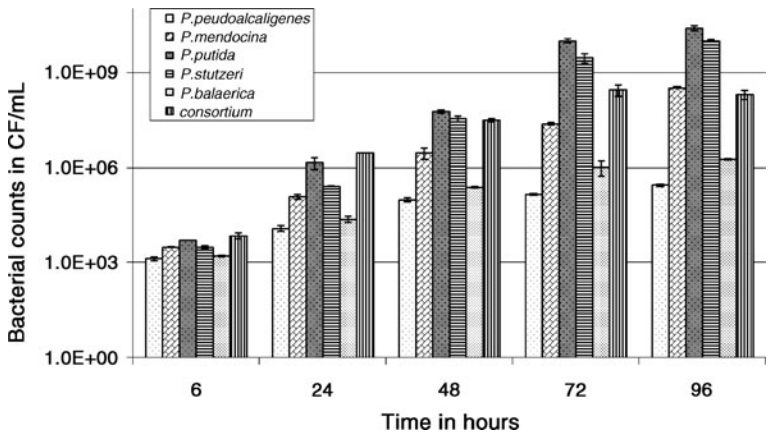

Fig. 1 Growth profiles of the different isolates and their consortium

phenomenon is closely related to the emulsification of TBT by rhamnolipids.

Rhamnolipid secretion profile (Fig. 2) for a given organism shows that rhamnolipid concentration increases with the growth of the organism in the medium; however, higher rhamnolipid secretion does not indicate better growth. For instance, while *P. putida* shows very good growth in the medium (10^{10} CFU/ml), it secretes only moderate levels of rhamnolipid (1,900 μg/ml) after 96 h of incubation, whereas *P. mendocina* and consortium of bacteria which show moderate growth (10^{8} CFU/ml) secrete higher levels of rhamnolipid (approx. 3,000 μg/ml) in the medium under similar conditions.

Rhamnolipids cause release of lipopolysaccharides from bacterial cell membrane resulting in increased cell surface hydrophobicity (Al-Tahhan et al. 2000) for effective cell–substrate interaction. Rhamnolipids are biosurfactants and help disperse polar substrates into the medium. Though the dispersion of hydrophobic substrates in the aqueous phase is vital for its uptake by the microbes, the secretion of biosurfactants in huge amounts is not the only criteria for judging the emulsifying capacity of bacteria. Experiments involving washed cell suspensions showed that in *P. putida* and *P. stutzeri*, a considerable amount of emulsifying capacity is retained on the cell surface itself (data not shown).

3.3 Emulsification Activity of Crude EPS

The crude EPS from the various culture supernatants reveal that the nature of emulsion formed by different cultures varies in the density of emulsion (Table 1). While slight changes were observed in the emulsion volumes for a half-hour and 24-h standing time for samples forming loose emulsions, the emulsion volumes remained largely unchanged for samples forming compact emulsions indicating that compact emulsions are more stable than loose ones. Though the amount of EPS produced by *P. putida* is only moderate, the emulsification capacity is high. This is because the emulsifier from this bacterium is more efficient than those secreted by other bacteria.

3.4 Extracellular Protein Secretion

The extracellular protein (ECP) secretion in the medium does not follow the same trend as EPS production by the microbes (Fig. 3). Consortium reached high ECP levels in the medium (192 μg/ml) at 48 h and maintained high levels throughout the study period. In the case of *P. stutzeri*, the ECP concentration in the medium increased until it reached 162 μg/ml at 48 h and then slowly declined. *P. putida* showed maximum ECP in the medium at 96 h (191 μg/ml) followed by a slight decline. This may be attributed to the irreversible change in the protein structure after the catalysis of TBT into its lesser toxic form. While consortium of bacteria produced high levels of proteins steadily throughout the study period, *P. pseudoalcaligenes* and *P .balearica* produced only low levels of protein in the medium. Higher protein secretion in the medium correlated with better growth of the organisms. The exact role of

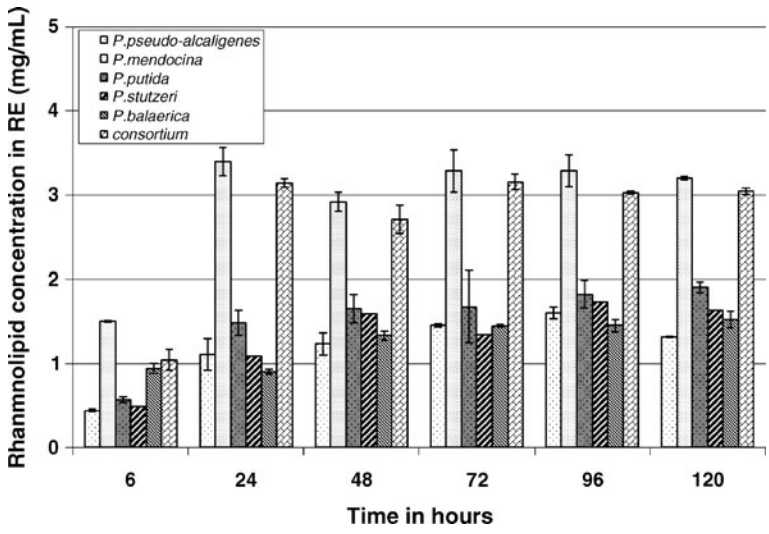

Fig. 2 Rhamnolipid secretion profiles of the different isolates and their consortium

Table 1 Emulsification activity of EPS

Bacterial isolate	Emulsification indices (E_{24}) at 25°C	Density of emulsion (OD_{600})	Nature of emulsion
P. psuedoalcaligenes	0.175±0.025	0.32±0.51	Loose
P. mendocina	0.30±0.013	0.451±0.63	Loose
P. putida	0.645±0.017	0.695±0.7	Very compact
P. stutzeri	0.485±0.032	0.564±0.3	Compact
P. balearica	0.284±0.02	0.507±0.9	Compact
Consortium	0.355±021	0.347±0.32	Loose

ECP in the degradation of TBT though needs to be evaluated.

3.5 CAS Assay for Detection of Siderophores

The CAS assay solution changed from blue to yellow due to chelation of iron from CAS reagent indicating the presence of siderophores in the extracellular supernatants from these marine pseudomonads.

The species producing pyoverdines belong to *Pseudomonas* RNA homology group I and include the species *P. aeruginosa*, *Pseudomonas fluorescens*, *P. chlororaphis*, *P. putida*, *Pseudomonas tolaasii*, and *Pseudomonas syringae* (Palleromi, 1984). In this study, *P. putida* NIOT20 produced pyoverdines which was confirmed by thin layer chromatography (picture not shown). Inoue et al. 2003 reported TPT degradation by a pyoverdine-secreting pseudomonad *P. chlororaphis* CNR15 isolated in Japan. They also confirmed both TBT and TPT degradation activities in culture supernatants of certain bacteria in which no pyoverdine was detected by IEF analysis; suggesting that the degradation reaction of organotin compounds is likely to occur by chelation of tin by certain types of siderophores. Additional tests need to be performed to ascertain the chemical nature of siderophores produced by these bacteria.

4 Conclusion

The biochemical behavior of five potent microorganisms when subjected to TBT stress was studied in order to evaluate the TBT-degrading potential of the individual isolates and their consortium. Of the five potent microorganisms, *P. putida* was found to grow best in TBT-containing medium (2 mM concentrations). Though *P. putida* reached the highest cell density, the consortium attained its maximum growth earlier than any individual isolate (at 72 h). The consortium was seen to secrete high amounts of exopolymers, i.e., rhamnolipids and ECP throughout the study period. As the field application of any microbe for TBT degradation will depend on its rate

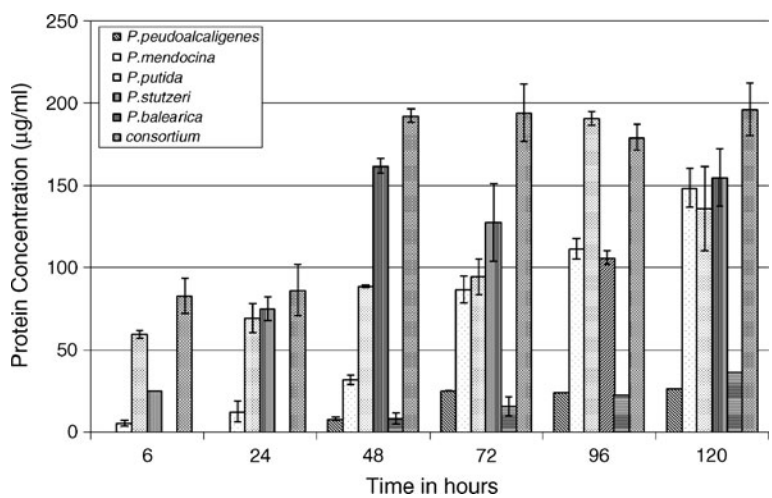

Fig. 3 ECP secretion profiles of the different isolates and their consortium

of TBT uptake, an organism that attains high cell densities with lesser incubation times would be a better candidate so as to design a treatment plant for lower retention times. The consortium of bacteria appears to be a better candidate for the treatment of TBT wastewaters. All the isolates secreted siderophores in the present study indicating that siderophores are not only produced under iron-limiting conditions but also under TBT stress as they act as biocatalysts in the breakdown of TBT.

The good growth of the consortium may be due to the complementary effect of organisms on each other. ECP production by *P. putida* and *P. stutzeri* in the early growth phase is likely to aid in reducing the lag in growth of the other bacteria in the consortium. The moderately high and steady secretion of proteins by the consortium may imply a steady degradation of TBT by the bacteria. Presently, studies using consortium have been carried out at ambient temperature (24°C to 29°C). The effectiveness of growth conditions of the consortium for its possible use in TBT degradation under field conditions needs to be carried out. The evaluation of degradation capacity of the pure culture of *P. putida* and that of the consortium also need to be carried out to better understand the biochemistry of TBT degradation.

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