Tributyltin chloride-utilizing bacteria from marine ecosystem of west coast of India

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Surface water samples were collected from Mumbai High oil-field area and Goa Shipyard Ltd and plated on nutrient agar and mineral salt medium (MSM) containing tributyltin chloride (TBTC) (0.4 mM). The total viable count of bacteria in the medium with TBTC ranged from 10 to 408 × 10^2 cfu/ml. The predominant bacterial colonies were isolated, purified and screened for utilization of TBTC as the sole source of carbon. Amongst these, five cultures showed prominent growth in MSM with 2 mM TBTC. Based on morphological, biochemical characteristics and phenogram, the isolates are grouped under *Pseudomonas* sp. and tentatively identified as *Pseudomonas stutzeri* [9(3A)], *Pseudomonas fluorescens* [3(4Sub)] and *Pseudomonas aeruginosa* (25W, 25B, 5Y_2).

AQUATIC uses of organotin compounds, particularly their incorporation as biocides in controlled-release of antifouling paints from ships pose serious detrimental impact on the coastal ecosystem, mainly the living biota[1]. On account of increased shipping activities, erosion and transport, tributyltin (TBT) compounds accumulate in harbour waters, higher organisms and sediments[2]. Among the TBT compounds, tributyltin chloride (TBTC) is most commonly used as an antifouling agent in marine paints[3]. Higher level of TBTC has been reported in marine and freshwater harbour areas which are primarily associated with boating activity[1,4]. It has also been reported that coastal waters of most Asian countries are worst affected by the persistent organic pollutants due to extensive use of these chemicals in paints and for agriculture purposes[5]. The sampling sites used in this study are potential sources of TBTC contamination, and include Goa Shipyard Ltd (GSL), Goa[6], one of the biggest shipyards in the west coast of India and Mumbai High oil-field area, 150 miles away from the west coast[7].

It is interesting to note that among organotin compounds, mono-, di-, and tetraorganotins are nearly non-toxic, whereas triorganotin compounds, whether aliphatic or aromatic are highly toxic[8]. Generally, trisubstituted organotins (R_3SnX) are more toxic than disubstituted (R_2SnX_2) and mono-substituted ($RSnX_3$) organotin compounds; the anion (X) apparently has little influence on toxicity[9]. The general order of toxicity to microorganisms increases with the number and chain length of organic groups bonded to the tin atom[10]. We report here the isolation and identification of marine bacteria from the west coast of India, which can utilize TBTC as the sole source of carbon.

Marine water samples were collected from Mumbai High oil field in the west coast of India during a cruise organized by Oil and Natural Gas Commission, Goa, India. Water samples were also collected at GSL from painting yards, vicinity of ships and surrounding ship-building areas. Sea-water samples were collected using Niskin sampler[11,12] in December 1999, from the two sites, in sterile polycarbonate bottles kept at 4°C and used within seven days of collection. Water samples were mechanically shaken prior to use and allowed to stand for 10 min to permit settling of heavy particles. A volume of 0.1 ml of water sample was plated on nutrient agar (NA) only, NA + 0.1 mM TBTC and mineral salt medium (MSM)[13] containing 0.1, 0.2, 0.3 and 0.4 mM TBTC respectively. Plates were incubated at room temperature and examined after 24 h, 48 h and one week for bacterial colony forming units (cfu ml^{-1}). Bacteria appearing on MSM agar + 0.4 mM TBTC were sub-cultured in MSM broth with increasing concentration of TBTC. Isolates which grew well on MSM broth + 2 mM TBTC were repeatedly subcultured and used in further studies.

The total viable count of all water samples obtained from Mumbai High oil field ranged from 23 × 10^2 to 408 × 10^2 cfu/ml when plated on NA. The viable count of the same sample on NA + 0.1 mM TBTC and MSM + 0.1 mM TBTC ranged from 8 × 10^2 to 85 × 10^2 cfu/ml and 18 to 697 cfu/ml respectively (Table 1). This indicated that 16% of natural bacterial population is resistant to 0.1 mM of TBTC as it utilizes this organotin biocide as the sole carbon source. However, when TBTC concentration was increased up to 0.4 mM in MSM agar medium, the viable count was considerably reduced.

Viable count of water samples obtained from GSL ranged between 32 × 10^2 and 383 × 10^2 cfu/ml when plated on NA only, but the viable count on MSM agar + 0.1 mM TBTC ranged from 198 to 765 cfu/ml (Table 1); 11.4% of natural bacterial isolates were resistant to 0.1 mM of TBTC in MSM and 66.8% of this TBTC-resistant population could grow up to 0.4 mM TBTC in MSM, utilizing it as the sole source of carbon.

The comparative study showed that bacterial isolates of GSL are more resistant than those of Mumbai High oil-field area. The extensive use of TBTC as an antifoulant in ship paints, shipyards and harbours is considered to be the prime source of TBTC in the marine ecosystem[14,15]. GSL is also one of the important shipbuilding yards of the west coast of India. Therefore, marine waters and sediment around the GSL are also contaminated with TBTC. Such contamination of TBT in coastal waters near harbours has been reported earlier[15,16].

Most of the bacterial isolates failed to grow in the presence of higher concentration of TBTC (2 mM). Out of forty-six isolates, only five cultures designated as

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Table 1. Total viable count of bacteria in marine water samples collected from various sites

Sampling site	Latitude	Longitude	NA (cfu × 10^2/ml) ± SE			MSMA (cfu/ml) ± SE		
Mumbai High oil field	–	–	NA only	NA + 0.1 mM TBTC	+ 0.1 mM TBTC	+ 0.2 mM TBTC	+ 0.3 mM TBTC	+ 0.4 mM TBTC
Heera	18°32.305N	72°15.921E	27 ± 2.8	24 ± 3.2	586 ± 76.5	558 ± 32.0	343 ± 52.0	250 ± 90.0
Neelam	18°42.458N	72°20.006E	74 ± 17.6	85 ± 17.6	697 ± 32.0	395 ± 64.0	305 ± 15.0	182 ± 10.7
Sagar Samrat	18°95.559N	72°02.579E	30 ± 7.4	23 ± 2.7	128 ± 28.0	102 ± 22.0	43 ± 2.3	12 ± 7.5
Bassin	19°12.083N	72°07.473E	57 ± 9.0	33 ± 8.1	78 ± 14.0	6 ± 4.0	–	–
Panna	19°17.969N	72°02.620E	46 ± 1.5	34 ± 7.5	55 ± 30.4	47 ± 18.0	5	–
Mukta	19°21.541N	71°52.298E	408 ± 62.8	61 ± 4.4	18 ± 8.0	–	–	–
SHP	19°17.206N	71°24.649E	45 ± 10.5	55 ± 5.3	114 ± 21	67 ± 8.5	17 ± 6.5	8 ± 2.5
ICP	19°21.100N	71°18.318E	23 ± 7.1	10 ± 1.8	325 ± 13.6	290 ± 60.0	265 ± 63	247 ± 52
NQ	19°34.090N	71°21.656E	93 ± 5.4	28 ± 5.5	277 ± 52	157 ± 52.5	148 ± 18	96 ± 3.3
BHN	19°32.548N	71°18.487E	142 ± 4.5	8 ± 2.6	169 ± 18	45 ± 20.0	36 ± 8.7	11 ± 9.0
BHS	19°21.366N	71°21.150E	46 ± 1.2	23 ± 6.0	231 ± 19	128 ± 42.0	110 ± 15	57 ± 3.7
Tapti	20°33.439N	72°01.142E	67 ± 2.0	47 ± 12.5	112 ± 33	44 ± 12	11 ± 1.0	10
GSL								
Close to ship	15°27.703N	73°49.985E	32 ± 6.7	26 ± 10.5	765 ± 12.5	728 ± 31.5	668 ± 28.0	488 ± 16.0
Ship wall	15°27.703N	73°49.985E	383 ± 31.0	74.5 ± 14.5	414 ± 118.0	374 ± 49.0	455 ± 14.5	200 ± 9.5
Painting yard	15°27.703N	73°49.985E	71 ± 8.6	38 ± 10.2	198 ± 12.0	95 ± 9.5	89 ± 9.0	60 ± 3.5
Near Fibre boat	15°27.706N	73°49.983E	263 ± 34.0	12.5 ± 2.5	609 ± 113.0	538 ± 115.0	506 ± 26.0	342 ± 53.3

SE, Standard error; NA, Nutrient agar; MSMA, Mineral salt medium agar.

3(4 Sub), 9(3A), 25B, 25W, $5Y_2$, showed good growth after 48 h of incubation under optimum condition of growth, i.e. pH 7.2 and salinity 2.5% at 180 rpm and at 28°C. On the basis of biochemical tests, all the five strains, 25W, 25B, 3(4 Sub), 9(3A), $5Y_2$ were identified according to *Bergey's Manual of Systemic Bacteriology*[17] (Table 2). Two standard strains such as *Pseudomonas mendocina* P_2d and *Sphingomonas paucimobilis* were used as standard culture for comparison. The characteristics of all the isolates and the phenogram[18] (Figure 1) showed 70% similarity among the isolates, which have been grouped as *Pseudomonas*. The biochemical characteristics of the isolates 9(3A) and 3(4 Sub) led to their tentative identification as *Pseudomonas stutzeri* and *Pseudomonas fluorescens* respectively. The other three isolates (25W, 25B, $5Y_2$) forming a cluster with 90% similarity, are tentatively identified as *Pseudomonas aeruginosa* USS25, *Pseudomonas aeruginosa* sp. 1 and *Pseudomonas aeruginosa* sp. 2 (Table 2).

Marine bacteria are known to be potent degraders of a variety of environmental pollutants[19], but little is known about the incidence of organotin resistance in natural microbial population, as well as the resistance mechanism with which microorganisms tolerate high levels of organotins. It has been reported that organotin compounds are toxic to both Gram-negative and Gram-positive bacteria, but triorganotins are more active towards the Gram-positive bacteria than Gram-negative bacteria[20]. Singh[21] and White *et al.*[22] have reported several organisms resistant to different organotin compounds, but bacteria utilizing TBTC as the sole source of carbon have not been reported so far[22]. Debutylation of TBT compounds to di- and mono-butyltins is known to take place in bacteria, algae and fungi, which provides a route for detoxification. In addition, microorganisms are also capable of accumulating TBTC, thus contributing to the removal of TBT from marine environment[9]. The high lipid solubility of organotins ensures the interaction of TBTC with intracellular sites by penetration through cell wall and cell membrane[9,23]. Although the degradation of organotins has been shown to be mediated by microorganisms, information is still limited in relation to the mechanism of degradation, tolerance mechanism of microbes and their relative significance, and also the role of anionic radicals in the degradation process in natural habitats[24,25]. Biotic processes have been demonstrated to be the most significant mechanisms for tributyltin degradation, both in soil as well as in freshwater, marine and estuarine environment[26,27].

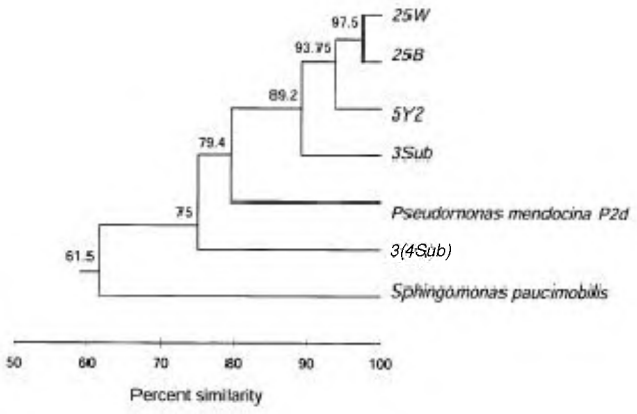

Figure 1. Phenogram showing similarity among different isolates.

It has been reported that TBTC-tolerant bacteria are present in sea water[28] and some organisms such as *P. aeruginosa* can degrade tributyltin oxide when the compound is present at a concentration of 2.5 ppm[29]. Although a few researchers have reported degradation of TBTC by environmental microorganisms, isolation of TBTC utilizing

Table 2. Morphological and biochemical characterization of bacterial isolates obtained from water sample

Characteristics	*Pseudomonas mendocina*	*Sphingomonas macrogaltabidus*	3(4 Sub)	9(3A)	25W	25B	5(Y_2)
Morphology of organism	Short rods	Coccobacilli	Short rods	Short rods	Coccobacilli	Short rods	Short rods
Gram's stain	–	–	–	–	–	–	–
Motility	+	+	+	+	+	+	+
Catalase activity	+	+	+	+	+	+	+
Oxidase activity	+	+	+	+	+	+	+
HL media (O/F)	O	O	O	O	O	O	O
VP test	+	–	–	–	–	–	–
Indole	–	–	–	+	–	–	–
MR	–	–	–	+	–	–	–
Utilization of glucose, lactose, galactose, sucrose, xylose, arabinose, mannose, salicin, raffinose	+	+	+	+	+	+	+
Utilization of mannitol, inositol, sorbitol, rhamnose	–	–	+	+	+	+	+
Utilization of fructose, maltose	–	+	+	+	+	+	+
Utilization of alanine, isoleucine	+	ND	+	+	+	+	+
Casein hydrolysis	+	–	+	–	+	+	+
Arginine hydrolysis	+	–	+	–	+	+	+
Gelatin hydrolysis	–	–	+	–	–	–	–
Tween 80 hydrolysis	+	+	+	+	+	+	+
Growth on TSI media	+	–	+	+	+	+	+
Growth on Mc Conkeys agar	+	–	+	+	+	+	+
Starch hydrolysis	–	+	–	+	+	+	+
Urease activity	+	–	+	+	+	+	+
Fluorescent pigment production	–	–	–	–	+	+	+
Nitrate reduction	–	–	–	+	–	–	+
Yellow pigment on King B agar	–	+	–	–	–	–	–
Green pigment on King B agar	–	–	+	–	+	+	+
Lysine decarboxylase	–	–	–	–	–	–	–
Arginine decarboxylase	–	–	–	–	–	–	–
Ornithine decarboxylase	–	–	–	–	–	–	–
Bioluminescence	–	–	–	–	–	–	–
Growth on cetrimide agar	+	–	+	–	+	+	+
Pigment on cetrimide agar	Brown	–	–	–	Green	Dark green	–
Thiosulphate citrate bile sucrose agar	–	–	+	–	+	+	+
Eosin methylene blue agar	–	–	–	–	–	–	–
Citrate utilization	+	–	+	+	+	+	+
H_2S production	–	–	–	–	–	–	–
PHB production	–	+	–	–	–	–	–
Growth at 4°C	–	–	–	–	–	–	–
Growth at 37°C	+	+	+	+	+	+	+
Growth at 43°C	+	–	+	+	+	+	+
Growth at pH 3.6	–	–	–	–	–	–	–
Tentitively identified	–	–	*P. Fluorescens*	*P. stutzeri*	*P. aerugi-nosa* USS25	*P. aerugi-nosa* sp. 1	*P. aerugi-nosa* sp. 2

P, *Pseudomonas*; (+), Positive; (–), Negative; O, Oxidative; ND, Not done.

bacteria has not been successful so far[9,30]. Further, not much is known on TBTC degradation rates under ambient environmental conditions in marine coastal waters[4]. It is expected that the fate of TBTC will be dependent on direct biological degradation by bacteria. The cultures isolated during the present study from marine ecosystem, show the ability to utilize TBTC as the sole source of carbon even up to 2 mM level. Further biochemical and molecular biological studies on these isolates with reference to TBTC biotransformation/utilization are in progress.

1. Kan-Atireklap, S., Tanabe, S. and Sanguansin, J., *Mar. Pollut. Bull.*, 1997, **34**, 894–899.
2. Cardwell, R. D. and Sheldon, A. W., Proceedings of the Symposium on Oceans (ed. Champ, M. A.), 1986, pp. 1127–1129.
3. Fukagawa, T., Suzuki, S., Fukagawa, K., Suzuki, T. and Takama, K., *FEMS Microbiol. Lett.*, 1992, **93**, 83–86.
4. Seligman, P. F., Valkirs, A. O. and Lee, R. F., *Environ. Sci. Technol.*, 1986, **20**, 1229–1235.
5. Monirith, I. *et al., Mar. Pollut. Bull.*, 2003, **46**, 281–300.
6. Alam, S. M., Khan, A. K. and Nagabhushanam, R., *Marine Biodeterioration, Advanced Techniques Applicable to the Indian Ocean* (eds Thomson, M. F., Sarojini, R. and Nagabhushanam, R.), Oxford and IBH, 1988, pp. 539–550.
7. Rawte, T., Ph D thesis., Goa University, 2002.
8. Kochkin, D. A., Proceedings of the 4th International Congress on Marine Corrosion and Fouling, 1976, pp. 281–284.
9. Gadd, G. M., *Sci. Total. Environ.*, 2000, **21**, 119–127.
10. Cooney, J. J., *J. Ind. Microbiol.*, 1988, **3**, 195–204.
11. Tamburini, C., Garcin, J., Ragot, M. and Bianchi, A., Deep Sea Research Part–II: Topical Studies in Oceanography, 2002, vol. 49, pp. 2109–2123.
12. Sardessai, S. and de Souza, S. N., Deep Sea Research Part–II:

Topical Studies in Oceanography, 2001, vol. 48, pp. 3353–3361.
13. Mahtani, S. and Mavinkurve, S., *J. Ferment. Technol.*, 1979, **57**, 529–533.
14. De Mora, S. J. and Pelltier, E., *Environ. Technol.*, 1997, **18**, 1169–1177.
15. Chau, Y. K., Maguire, R. J., Brown, M., Yang, F. and Batchelar, S. P., *Water Qual. Res. J. (Canada)*, 1997, **32**, 453– 521.
16. De Mora, S. J., Stewart, C. and Phillips, D., *Mar. Pollut. Bull.*, 1995, **30**, 50–57.
17. Krieg, N. R. and Holt, J. G., *Bergey's Manual of Systemic Bacteriology*, The Williams & Wilkins Co, Baltimore, USA, 1984, vol. 1.
18. Goodfellow, M., Numerical taxonomy. In *CRC Hand Book of Microbiology, Vol-1. Bacteria* (eds Laskin, A. I. and Lechevalier, A. H.), CRC Press, Ohio, USA, 1977, 2nd edn, pp. 579–596.
19. Barkay, T. and Pritchard, H., *Microb. Sci.*, 1988, **5**, 165–169.
20. Dubey, S. K. and Roy, U., *Appl. Organometal. Chem.*, 2003, **17**, 1–6.
21. Singh, K., *Frontiers in Applied Microbiology* (eds Mukherjee, K. G., Singh, V. P. and Garg, K. L.), Publication Print House, New Delhi, 1987, vol. II, pp. 297–316.
22. White, J. S., Tobin, J. M. and Cooney J. J., *Can. J. Microbiol.*, 1999, **45**, 541–554.
23. Gray, B. H., Porvaznik, M., Flemming, C. and Lee, L. H., *Toxicology*, 1987, **47**, 35–47.
24. Wuertz, S., Miller, C. E., Pfister, R. M. and Cooney, J. J., *Appl. Environ. Microbiol.*, 1991, **57**, 2783–2789.
25. Gadd, G. M., *FEMS Microbiol. Rev.*, 1993, **11**, 297–316.
26. Barug, D., *Chemosphere*, 1981, **10**, 1145–1154.
27. Dowson, P. H., Bubb, J. M. and Lester, J. N., *Estuarine Coastal Shelf. Sci.*, 1996, **42**, 551–562.
28. Fukagawa, T., Konno, S., Takama, K. and Suzuki, S., *J. Mar. Biotechnol.*, 1994, **1**, 211–214.
29. Suzuki, S., Fukagawa, T. and Takama, K., *Appl. Environ. Microbiol.*, 1992, **58**, 3410–3412.
30. Suzuki, S. and T. Fukagawa., *J. Ind. Microbiol.*, 1995, **14**, 154–158.

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Mass multiplication of AM inoculum: Effect of plant growth-promoting rhizobacteria and yeast in rapid culturing of *Glomus mosseae*

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The efficiency of plant-growth-promoting rhizobacteria (PGPR), viz. *Azospirillum* sp., *Azotobacter chroococcum*, *Pseudomonas fluorescens*, *Pseudomonas striata* and yeast, viz. *Saccharomyces cerevisiae* was evaluated for maximization of *Glomus mosseae* (Nicol. and Gerd.) Gerd. and Trappe root colonization and spore number in the root zone of Rhodes grass (*Chloris gayana* Kunth). The pot culture experiment was carried out under polyhouse condition and observations were recorded at 45 days and 90 days of plant growth. The PGPR considerably enhanced mycorrhizal colonization compared to yeast, with *Azospirillum* sp. being the most efficient. They not only stimulated AM development, but also accelerated the root growth.

ARBUSCULAR mycorrhizal (AM) fungi are obligate symbionts and are grown in association with living tissues[1]. Several culture techniques based on this constraint are applicable for commercial scale production of the inoculum. The most widely used is pot culture, where the fungi are usually maintained and multiplied in conjunction with suitable host plant roots[2]. Related approaches, viz. soilless culture, hydroponic culture, aeroponic culture, and axenic root organ culture techniques are well reviewed[3]. These are two-member (plant and fungus) systems, technically feasible and hold commercial potential. But importantly, all of them involve extended culture periods of several months, making AM inoculants relatively expensive to produce[4]. As such, development of rapid and more efficient culture systems remains an important challenge for commercialization. Synergistic effects of AM fungi and plant-growth-promoting rhizobacteria (PGPR)[5] and yeast[6] on root colonization and subsequent sporulation have been documented. The term PGPR is now applied to a wide spectrum of strains that have, in common, the ability to promote the growth of plants following inoculation onto seeds and subterranean plant parts[7]. The present investigation has been undertaken with a view to explore the possible use of PGPR and yeast to maximize AM fungal root colonization and sporulation in a short period. Uninoculated culture media and cell-free supernatants of the respective organisms were also included to assess the potential of the microbial whole cell.

A culture of *Glomus mosseae* (Nicol. and Gred.) Gred. and Trappe[8] was obtained from Native Plant Institute, Salt Lake City, Utah, USA and maintained as a pure stock-plant culture in pots containing sterilized soil and sand (3 : 1)[9] using Rhodes grass (*Chloris gayana* Kunth) as a host for four months. Spores were collected by wet-sieving and decanting the root zone soil from ten different pots and were mixed to form a composite sample[10]. Healthy pale yellow-brown coloured spores were selected with the aid of stereomicroscope and were surface sterilized in chloramine-T (2% w/v) for 20 min and rinsed 3–4 times in sterile deionized water[11].

The test microbes included were two diazotrophic and two non-N_2-fixing bacterial strains of PGPR and a yeast. Two diazotrophic bacteria, viz. *Azospirillum* sp. R.v.zae grown on Okon's broth[12] as modified by Lakshmi Kumari *et al.*[13] and *Azotobacter chroococcum* M5 grown on Jensen's N-free broth[14] isolated from sporocarp of the ectomycorrhizal fungus (*Rhizopogan vinicolor*) and rhizosphere

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