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Screening, Identification and Biological Characterization of Highly Potent Tributyltin Chloride Resistant Marine Bacteria from West Coast of India (Goa)

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Abstract – Surface water and sediment samples were collected from various sampling sites surrounding the ship building industries in Goa region and plated on Zobell's marine agar, Nutrient agar and mineral salt medium (MSM) containing tributyltin chloride (TBTC) 0.1mM. The physico-chemical characteristics of the environmental samples were determined. The total viable counts of bacteria in the medium with TBTC ranged from 8 × 10^2 - 40 × 10^6 cfu/ml. The predominant bacterial colonies were isolated, purified and screened for TBTC tolerance studies. Amongst these, two bacterial strain tolerated 7mM TBTC and showed prominent growth in MSM with 5 mM TBTC. Based on morphological, biochemical, molecular characteristics and phenogram, the isolate was grouped under Alcaligenes sp.

Keywords: Tributyltin, PCR Amplification, Alcaligenes sp.2-6, Alcaligenes sp.swo.

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Tributyl tin (TBT) is an organotin compound most commonly used in marine anti-fouling paints. It is also used in lumber preservatives as an industrial biocide and a contaminant of organotin PVC stabilizers. It is highly toxic, bioaccumulative and persistent chemical that damages the immune and reproductive systems of aquatic life. Its use over the last 35 years as an anti-fouling paint applied to the hull of boats has spread TBT far and wide, making it an ubiquitous contaminant of coastal environments and can easily leach from such paints. It binds to suspended particulates and can accumulate several thousand folds in sediments and in the surface microlayer [15, 16]. The highest levels of TBT are found in and near harbours, vicinity of shipyards, and along busy waterways. 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 agriculture purposes. On account of increased shipping activities, erosion and transport, tributyltin (TBT) compounds accumulate in harbour waters, higher organisms and sediments. The sampling sites used in this study are potential sources of TBTC contamination, and considering Goa Shipyard Ltd (GSL), as one of the biggest shipyards in the west coast of India.

TBT quickly became a ubiquitous contaminant of coastal environments around the world. TBT hot spots are usually found around busy shipyards, near harbours and major commercial ports, given the release from heavy marine traffic and vessel maintenance in these areas. Because the primary use of TBT is as a biocide in ship paint, it is not surprising that shipyards, harbours and marinas are considered prime sources of TBT release to the marine ecosystem. Not only is TBT absorbed or consumed by aquatic species, it settles and accumulates in the sediments. TBT can cause lethal and sub-lethal effects on non-target organisms, e.g., imposex[11, 22] or intersex [7] in several gastropod species; malformation in oysters (*Crassostrea gigas*),[3] increased mortality and retardation of growth in larvae of blue mussels (*Mytilus edulis*)[8] and disappearance of clams (*Scrobicularia plana*). It is interesting to note that among organotin compounds, mono-, di-, and tetraorganotins are nearly nontoxic, whereas triorganotin compounds, whether aliphatic or aromatic are highly toxic. Generally, trisubstituted organotins (R_3SnX) are more toxic than disubstituted (R_2SnX_2) and mono-substituted ($RSnX_3$) organotin compounds. The general order of toxicity to microorganisms increases with the number and chain length of organic groups bonded to the tin atom[8, 28]. We report here the screening and identification of highly potent Tributyltin chloride resistant marine bacteria from west coast of India. Many workers have reported on organochlorine pesticides, [5] polychlorinated biphenyls (PCBs) [26] and trace metals [28] in the Indian marine environment. However, there has been no report on screening and characterization

of highly potent organotin tolerant strains in water and sediment from coastal environs.

Marine water samples were also collected from docking surface, paint yards, Ship walls, dry dock wall, vicinity of ships and waters surrounding ship building areas of Goa Shipyard Lts.,(GSL) and Western India Shipyard Ltd.,(WISL) in MPT harbour sites of west coast of India, Goa region. Seawater and sediment samples were collected using Niskin sampler from August-Oct, 2002 during monsoon season, from the two sites, using sterile polycarbonate bottles, kept at 4°C and used within one week of collection. The physicochemical characteristics of the water and sediment samples were determined in terms of temperature, pH, salinity, alkalinity, organic and inorganic (Nitrate, Nitrite and Phosphate) following the standard protocol[25]. 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 Zobell marine agar only taken as control , ZMB + 0.1 mM TBTC , mineral salt medium +0.1mM TBTC (MSM) and Nutrient agar + 0.1mM TBTC respectively. TBT stock solutions were prepared in ethanol and added to autoclaved medium after it had cooled to 50°C. Plates were incubated at room temperature and examined after 24 h; 48 h and one week for bacterial colony forming units (cfu ml^{-1}) and 30 colonies were randomly isolated from each plate and tested for tolerance to TBTCl. Bacteria appearing on MSM agar + 0.1 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 sub cultured and used in further studies.

The samples collected from various locations of Western India Shipyard Ltd.,(WISL) showed pH ranging from 7.8-8.04, temperature 28^0C-28.5^0C, salinity 28.40%-31.87%, alkalinity 2.26 meq.1^{-1} -2.38 meq.1^{-1}, organic content 178-368 mg/l, Phosphate 0.672-1.22 µmol.dm^{-3}l, Nitrate 2.98-4.14 µmol.dm^{-3}l and Nitrite 0.58-0.8204 µmol.dm^{-3}l. The water samples collected from varying locations of Goa Shipyard showed pH ranging from 7.8-7.9, temperature 27.4^0C-29^0C, salinity 28.03%-31.77%, alkalinity 2.28 -2.34 meq.1^{-1}, organic content 198-266 mg/l, Phosphate 0.644-1.14 µmol.dm^{-3}l, Nitrate 2.2.48-4.08 µmol.dm^{-3}l and Nitrite 0.78-0.8101 µmol.dm^{-3}l. The temperature, pH and salinity of water sample of specified sampling sites ranged from 27.4-29°C±2^0C, 7.8 - 8.0 pH, and 28.03-32.04% respectively (table-1). As these sampling sites are in coastal and continental shelf area of Arabian Sea, it is not surprising that these values are more or less the same. With reference to above physicochemical parameters of marine samples, the salinity of surface water of Arabian Sea varies between 34–37 % [33]. It has been reported that in surface water the concentration of nitrate was high at most of the places during monsoon and post monsoon. The high surface values of nitrate can be attributed to land run-off during monsoon. The phosphate and nitrate content of the water sample of Western India Shipyard Ltd., (WISL) was higher than Goa Shipyard Ltd (GSL) at Vadem. Whereas the nitrite content of Western India Shipyard Ltd.,(WISL) was similar to Goa Shipyard Ltd (GSL). This data (table-1) clearly indicated that nutrient content of estuarine water is higher than open sea. The Zuari estuarine network receives a large influx of fresh water from the rainfall; this in turn causes changes in the salinity, alkalinity, temperature, organic and inorganic nutrients. The distribution of nutrient elements in the marine and estuarine waters are controlled by constant circulation, mixing and other physical process, together along with biological, sedimentological and chemical processes[4]. The biogeochemical cycling of inorganic nutrient in the estuaries play a dominant role with compared to the marine waters. The biodiversity of organotin tolerant estuarine microflora largely depends upon above characteristics. Since organotins are present in these estuarine econiches in appreciable amount, interestingly, bacterial strains which can tolerate and degrade them get enriched. Therefore several TBTC degrading bacterial strains are abundant in these econiches (habitat) [35]. In addition to dissolved molecular nitrogen the sea water also contains low concentrations of inorganic and organic nitrogenous compounds. The main inorganic forms are nitrate (1-500 µg/L), nitrite (< 0.1- 50 µg /L) and ammonia (< 1-50 µg/L). Sea water also contains low concentrations of dissolved and particulate organic nitrogenous compounds which are associated with marine organisms as products of their metabolism and decay [29]. Likewise the levels of organic and inorganic nutrients (phosphate, nitrites and nitrates) increases. A correlation exists between dissolved oxygen and salinity, indicating that an increase in the salinity decreases the DO, as the solubility of DO is more in the fresh waters with compared to the saline waters[10]. During the present study, the levels of physico-chemical parameters in estuaries (GSL and WISL) showed the values as temperature (27.4^0C -29.0^0C ±2^0C); pH (7.8- 8.04); salinity (28.03 - 31.87 % in Estuaries and 33 - 34 % in marine waters); Alkalinity (2.28 - 2.48 meq.1^{-1} in estuaries and 2.08-2.17 meq.1^{-1} in marine waters); Organic content 178-368mg/lt; Phosphates (0.644 – 1.22 µmol.dm^{-3}l in estuaries and 0.45-0.64 µmol.dm^{-3}l in marine); nitrates (2.48-4.08 µmol.dm^{-3}l in estuaries and 2.64-3.05 µmol.dm^{-3}l in marine waters); nitrites (0.58 - 0.8204 µmol.dm^{-3}l in estuaries and 0.18-0.28 µmol.dm^{-3}l in marine waters) ; respectively (table-1).

The total viable count of all water samples (table-2) obtained from Western India Shipyard Ltd., (WISL) was ranging from 24.6 x 10^6 - 42.3x10^6 when platted on ZMA without TBTC taken as control. But, when plated on Zobell marine agar+ 0.1mM TBTC and colony count made after 48 hrs of incubation ranged between 18.9 x 10^6 - 41.6 x 10^6 cfu/ml. It clearly indicates that approximately 9.9% of natural bacterial population is resistant to 0.1mM of TBTC as it utilizes TBTC as sole source of carbon (table-2). The viable count of the same samples on NA + 0.1mM TBTC was between 14.4 X 10^4 - 93.4 X 10^4 cfu/ml and on MSM + 0.1mM TBTC between 2.68 X 10^4 -8.89 X 10^4 cfu/ml

and on MSM + 0.1mM TBTC between 24 X 10^2 - 89 X 10^2 cfu/ml. It clearly indicates that approximately 14.5% of natural bacterial population is resistant to 0.1mM of TBTC as it utilizes TBTC as sole source of carbon (table-2). Viable count of water sample obtained from Goa Shipyard Ltd (GSL) ranged 40 x 10^6 - 50.8x10^6 when platted on ZMA without TBTC taken as control. But, when plated on Zobell marine agar+ 0.1mM TBTC and colony count made after 48 hrs of incubation ranged between 36.4 x 10^6 - 39.6 x 10^6 cfu/ml. Approximately 5.8% of natural bacterial population is resistant to 0.1mM TBTC in Zobell marine agar , The viable count of the same samples on NA + 0.1mM TBTC was between 64.3 X 10^4 - 68.9 X 10^4 cfu/ml and on MSM + 0.1mM TBTC between 58 X 10^2 - 66 X 10^2 cfu/ml. Approximately 13.66 % of natural bacterial population is resistant to 0.1mM TBTC in MSM. These studies have shown that bacterial flora of Western India Shipyard Ltd.,(WISL) are more resistant than Goa Shipyard Ltd (GSL) the reason is due to the fact that W.I.S.L receives both cargo and passenger ships for repair and construction, apart MPT Harbor houses many cargo and passenger ships at its various berths for weeks together which is the due reason for high level of tolerance. This is evident from similar findings which state that the antifoulant in ship paints, shipyards, harbours is considered to be the prime source of TBT in the marine ecosystem[20]. Western India Shipyard Ltd., (WISL) is the biggest of its kind in south Asia with modern ship repairing systems involved in the repair and construction of commercial ships. GSL is also one of the important harbours and shipbuilding yards of west coast of India, involved in the repairs and construction of commercial and naval ships, therefore organotin level are invariably high in surface water and sediments around W.I.S.L and GSL. Similar findings have also reported that coastal water near harbours is appreciably contaminated with TBTC[12, 19] and considerably high amount of organotin has already been reported in different coastal and harbour areas of the world. Most of the bacterial isolates failed to grow in the presence of higher concentration of TBTC (2 mM). Out of forty-eight isolates screened from 128 isolates that grew on MSMA+ 0.1mM TBTC (table-3) only one highly potent strain S3 that could tolerate upto 7mM TBTC and grew very well in 5mM TBTC was further characterized among other TBTC resistant isolates designated as S1, S2, Sn and Sp.

Morphologically the isolate was circular, cream, entire, opaque, raised, motile, gram-negative, sticky, short rods. Biochemical characteristics carried out according to *Bergey's Manual of Systemic Bacteriology* [27] revealed it to be catalase positive, oxidase positive, facultative aerobe, MR positive, Indole positive, casein and Tween 80 hydrolysis positive, nitrate reduction positive, sugar utilization (glucose, arabinose, xylose, galactose, cellubiose, melibiose, trehalose and saccharose) positive. It was tentatively identified to be an *Alcaligenes sp.* Its identity was confirmed by 16S rDNA gene amplification by polymerase chain reaction (PCR) as previously described by[18]. 16S rDNA was amplified, amplification was achieved using primers corresponding to position 8–28 (Eubacterial forward primer, F' 341 -CCT ACG GGA GGC AGC AG and reverse primer R' 1387- GCC CGG GAA CGT ATT CAC CG of *Escherichia coli* 16S rRNA sequence. Phylogenetic analyses using the BLAST program showed that strains S3 belonged to the gamma subdivision of the phylum Proteobacteria and that it was closely related to the genus *Alkaligenes* sp.2-6. The DNA sequence was determined using the dideoxy chain termination method[32]. A total of 1000 bp was sequenced. The sequence was compared with other bacteria available in GenBank (http://www.ncbi.nlm.nih.gov.)[1]. The sequence was then aligned with available 16S rRNA reference sequences. Similarities and alignments were obtained using the Basic Local Alignment Search Tool (BLAST) [2] algorithm to identify known sequences with a high degree of similarity. Two strains such as *Pseudomonas aeruginosa* USS25W and *Pseudomonas aeruginosa* PA01 were used as standard cultures for comparison. The characteristics of all the isolates and the phenogram (Figure 2) showed similarity among the isolates, which have been grouped as *Alkaligenes* sp. The biochemical characteristics of the isolate S3 led to the tentative identification as *Alkaligenes* sp. (fig. 2). Phenogram showing similarity among different isolates.

The comparative study showed that bacterial isolates of WISL are more resistant than GSL. This may be due to the fact that MPT harbour being one among the oldest port of India (since 1934), receives many cargo and passenger vessels at its various berths, these ships dock for weeks, during which the TBT may be leached to the marine environment due to hydrolysis as early reported by[14]. WISL with its latest ship repairing systems is the only one of its kind in the west coast of India. Like wise GSL (commissioned in 1957) involves repairing and construction of Naval ships. In these process, the old paint that is being blasted out before applying the new antifouling paint ends up in to the marine environment, where TBT leaches into the marine environment as reported earlier[14]. 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.

Chau and co-workers[12] demonstrated that heavy contamination of coastal waters by TBT was associated with major commercial harbours. For example, the range of butyltin concentrations in five sediment samples collected from Hamilton Harbour was from 63 to 294 ng Sn g-1. 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[30, 31] 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[31]. 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[13]. The high lipid solubility of organotins ensures the interaction of TBTC with intracellular sites by penetration through cell wall and cell membrane [13]. 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[17, 23, 24]. 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[6, 19].

Ship repair activities involve removal of the old paint from the hull and application of a new coating. High amounts of TBT can be released due to wastewater discharge, wind transportation of dust and paint and mismanagement of waste. In areas where high TBT input had occurred, or is still occurring, the sediment acts as a TBT reservoir. There is a serious risk of re-introduction of this TBT to the aquatic ecosystem if the sediment is remobilized (e.g. dredging). Organotins are thus pollutants of anthropogenic origin. They make their way from a variety of industrial and agricultural sources into aquatic ecosystems, where they can be concentrated up to 10,000-fold in the surface microlayer and up to 4,000 times in oily sediments[14, 16]. Ecotoxicological effects of organotins include morphological and reproductive aberrations and metabolic disruption in a variety of nontarget organisms, including shellfish and finfish. They can be bioaccumulated in microorganisms, which are at the base of the food web, and from there into higher organisms.

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Table-1: Physicochemical characteristics of environmental samples

Sampling site	Ph	Temperature	Salinity	Alkalinity	Organic content	Inorganic content		
						Phosphate	Nitrate	Nitrite
WISL								
OVER BERTH	8.0	28 °C	31.87 %	2.30 (meq.l⁻¹	288mg/l	1.12μmol.dm⁻³	3.04μmol.dm⁻³	0.02μmol.dm⁻³
BERTH WALL	8.0	28 °C	31.87 %	2.30 (meq.l⁻¹	368mg/l	1.02μmol.dm⁻³	3.05μmol.dm⁻³	0.04μmol.dm⁻³
NEAR SHIP	7.8	28.4 °C	30.46 %	2.30 (meq.l⁻¹	176mg/l	0.672μmol.dm⁻³	2.98μmol.dm⁻³	0.58μmol.dm⁻³
SEDIMENT	8.04	28.3 °C	32.84 %	2.40 (meq.l⁻¹	196mg/l	1.22μmol.dm⁻³	3.78μmol.dm⁻³	0.79μmol.dm⁻³
PAINT YARD	8.0	28 °C	31.87 %	2.26 (meq.l⁻¹	296mg/l	0.978μmol.dm⁻³	4.18μmol.dm⁻³	0.8284μmol.dm⁻³
GSL								
SEDIMENT	7.9	27.6 °C	31.77%	2.34 (meq.l⁻¹	332mg/l	1.48μmol.dm⁻³	2.48μmol.dm⁻³	0.0181μmol.dm⁻³
BERTH WALL	7.9	27.4 °C	31.77%	2.34 (meq.l⁻¹	218mg/l	0.678μmol.dm⁻³	2.48μmol.dm⁻³	0.08μmol.dm⁻³
NEAR SHIP	7.8	29 °C	29.83%	2.29 (meq.l⁻¹	198mg/l	0.648μmol.dm⁻³	5.28μmol.dm⁻³	0.78μmol.dm⁻³
PAINT YARD	7.9	28 °C	31.65%	2.28 (meq.l⁻¹	284mg/l	1.13μmol.dm⁻³	4.08μmol.dm⁻³	0.01μmol.dm⁻³

Table-2: Viable count of bacteria in water samples.

Sampling site	Geographical positions		Viable counts (cfux10⁴cells/ml) ± se in ZMA		Viable counts (cfux10⁴cells/ml) ± se in NA	Viable counts (cfux10⁴cells/ml) ± se in MSMA
W.I.S.L	Latitude	Longitude	0MM TBTC	0.1MM TBTC	0.1MM TBTC	0.1MM TBTC
OVER BERTH	15°27'620"N	73°49'842"E	28.6±.3	41.6±.1	49.6±1	30±1
BERTH WALL	15°27'620"N	73°49'842"E	34.6±.6	38.9±.2	43.4±2	41±2
SHIP WALL (Barnacles)	15°27'620"N	73°49'842"E	38.5±.5	40.6±.2	79±.9	53±1
SEDIMENT	15°27'620"N	73°49'842"E	24.6±.6	38.9±.2	44±.6	39±.2
NEAR SHIP	15°27'620"N	73°49'842"E	42.3±.3	48.4±1	76.8±.4	71±.4
PAINT YARD	15°27'620"N	73°49'842"E	31.0	50.4±.4	84.2±.5	54±.4
G.S.L						
SEDIMENT	15°27'780"N	73°49'985"E	58.8±.8	76.4±.3	84.3±.8	66±1
NEAR SHIP	15°27'780"N	73°49'985"E	41.6±.6	57.6±.4	65.0±.9	58±1
PAINT YARD	15°27'780"N	73°49'985"E	46±.2	59.6±.5	68.9±1.2	64.7±.6

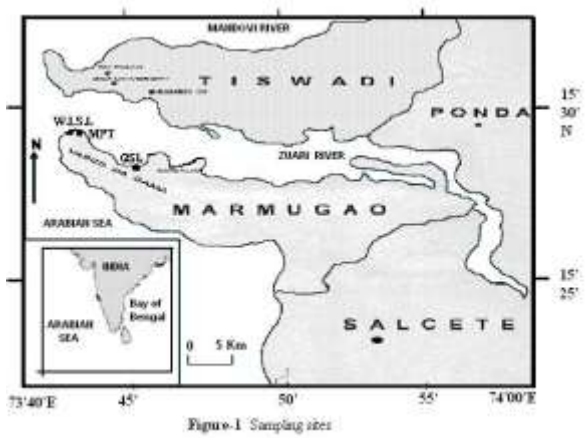

Figure-1 Sampling sites

Figure-1. Map showing sampling sites for collection of Tributyltin tolerant marine bacterial isolates.

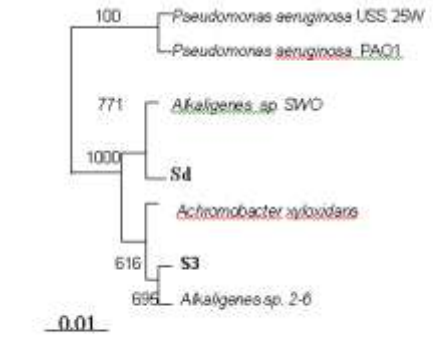

Figure-2 Taxonomic Phenogram of highly potent Tributyltin chloride resistant marine Bacterial isolates S3 and Sd

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