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Potential Tributyltin Chloride Degrading Marine Bacterium *Alcaligene* sp. 2-6 Strain S3 from Goa, India

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Abstract – Tributyltin chloride (TBTC) is used as antifoulants in ship hulls, boats, fishing nets, marine installations, aquaculture cages and docks to prevent settling of barnacles, seaweeds and tubeworms, finds its way finally into marine environment, resulting in negative impact on non-target organisms. Keeping these facts we have isolated and characterized a potential TBTC degrader from the sediments of Western India Shipyard Ltd. (WISL) in Murmugao Port Trust (MPT) harbor at Vasco-da-gama in Goa. This isolate was selected among five of the total 48 isolates screened, identified as Alcaligenes sp. 2-6 (Strain S3), tolerated 7mM TBTC, showed good growth at 5mM TBTC in Zobell marine broth (ZMB) and Mineral salts medium (MSM). It also showed cross tolerance to Cd^{2+}, Hg^{2+} and As^{3+} ions, produced more exopolymer (EPS) and extracellular fluorescent pigment of phenazine type at 5mM TBTC. The TBTC degradation study revealed the presence of monobutyltin (MBT) within 45 days of incubation, acridine orange curing of bacterial cells revealed that genes conferring TBTC resistance and utilization are not plasmid borne. This isolate quickly adsorbs and slowly transforms TBTC, using dealkylation (debutylation) mechanism, in a stepwise fashion. Hence Alcaligenes sp. 2-6 (Strain S3), can be used in the bioremediation of TBTC contaminated sites of marine environment of Goa.

Key Words: Tributyltin Chloride, Debutylation, Alcaligenes sp. 2-6, Degradation, Phenazine, Exopolymer.

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INTRODUCATION

Tri-organotins have a very broad range of applications, their annual world production being close to 50,000 tons/year. They are most commonly used as PVC stabilizers, as fungicides, bactericides and insecticides and hence as preservatives for wood, leather, electrical equipments, textiles and paper (Bryan et al., 1986; Clarke et al., 1988; and Dowsan et al., 1993). Unfortunately, these compounds effects mechanism in mitochondria and chloroplasts and hence are toxic to planktonic and benthic organisms, examples being shell thickening and decrease of meat weight in Pacific oyster *Crossostrea gigas* and imposex in female dog whelk *Nucella lapillus* (Clarke et al., 1988; Hugget et al., 1992; and Dowsan et al., 1993). A variety of evidences suggest that biodegradation is the major breakdown pathway in sedimentary environment and majority of the studies have been done using microorganisms. While attempts to isolate bacteria able to utilize Tributyltin chloride as sole carbon source have not been successful, several Tributyltin chloride resistant marine bacteria have been isolated and characterized (Bryan et al., 1986; Clarke et al., 1988; Mac Donald and Trevors, 1988; Wurtz et al., 1991; Fukagawa et al., 1992; Suzuki et al., 1992; and Fukagawa et al., 1994). Regulations to control the use of these compounds were introduced including an environmental quality target of 20 ng per liter set for estuarine and coastal waters. Despite the regulations enforced to control their use as anti-foulants, tributyltins are still present at toxic levels in the water columns and sediments (Clarke et al., 1988). It is interesting to note that there are microorganisms predominating in sediments of decks and harbors and also colonizing antifouling paints containing high levels of TBTC (Clarke et al., 1988; and Fukagawa et al., 1994). Though bacterial strains from these niches are slow TBTC degraders, they may prove to be an excellent tool for bioremediation of marine sediments contaminated with organotins and other heavy metals. Hence it is important to isolate and screen these TBTC-resistant bacteria. This paper presents the screening and biological characterization of potential TBTC degrading marine bacteria from TBTC contaminated sites of Goa with reference to growth in different medium, tolerance to TBTC, cross tolerance to heavy metals, TBTC induced exopolymer (EPS) and pigment production, degradation of TBTC and relevance of plasmid to TBTC resistance.

MATERIALS AND METHOD

Isolation and Identification of the isolate – Sediment samples were collected from different sampling sites of Western India Shipyard Ltd. (WISL) in Murmugao Port Trust (MPT) harbor at Vasco-da-gama in Goa, followiing standard procedures (Stebbibgs, 1985). Samples were serially diluted and plated on Zobell Marine agar with 0.1 mM TBTC, five selected isolates designated as S1, S2, S3, Sd and Sp were sub-cultured and maintained in Zobell Marine Broth (ZMB) and MSM broth with 0.1mM TBT and identified by biochemical tests following *Bergey's manual of Systematic Bacteriology* (Krieg and Holt, 1984). The identity of the most potent strain S3 was confirmed by 16s rDNA sequencing and homology search through NCBI-BLAST (Alschul et al., 1990).

Study of TBTC tolerance limit: The isolate was grown in MSM broth supplemented with varying concentrations of TBTC (1.0-10.0mM TBTC). Growth was measured in terms of absorbance at 600 nm and total protein content estimated after 24 hrs incubation at 28^0C respectively by Lowry's method (Lowry et al., 1954).

Study of growth pattern in MSM+TBTC and ZMB+TBTC: 5% overnight grown culture was inoculated in 100 ml of flasks containing Minimal salts medium (MSM) with 5mM TBTC, ZMB with 5mM TBTC and only ZMB and MSM was taken as control. Growth was determined in terms of absorbance at 600nm after every two hours till the stationary phase was reached.

Study of cross tolerance to heavy metals (Hg, Cd and As): All the heavy metals used were of analytical grade, stock solutions of heavy metals viz. $HgCl_2$, $CdCl_2$ and As_2O_3 (10 mM), were prepared in deionized double distilled water and membrane filtered (0.22 µm, Millipore) into sterile glass vials. Metal tolerance was determined by growing the potent isolate with increasing concentrations of test metals (0.5 mM – 5 mM) in Luria Bertani broth (100 mL). 5 mL of the culture broth was withdrawn at 2 hrs interval for growth measurements turbidometrically..

Study of exopolymer production under TBTC stress: EPS production by the isolate was studied in Mineral salts medium and Zobell marine broth supplemented with 5mM TBTC. The exopolymer was recovered from the culture supernatant by using a cold ethanol precipitation–dialysis procedure (Bhosle et al. 1995).

Study of pigment production under TBTC stress:

Overnight grown culture in MSM broth with 5mM TBTC was centrifuged, pigmented cells were then sonicated (pulse of 15 seconds for two minutes) using ice jacket in acetone and centrifuged to collect clear supernatant (pigment extract). Pigment extracted was then excited between 181 to 780 nm and the emission range was recorded at 181 nm (Gaber, 1973).

Study of TBTC degradation by Spectrophotometry and Thin Layer Chromatography: 5% overnight grown culture was inoculated in 200ml of MSM with 5mM TBTC incubated on a rotator shaker (180 rpm at room temperature). After every 24 hours 5 ml of the sample was withdrawn, centrifuged at 8000rpm for 5minute still 45 days. The cell supernatant and chloroform extract of the cell pellet was scanned between 190-700 nm. The chloroform extract was concentrated using nitrogen gas and analyzed by thin layer chromatography (TLC) (Hamilton and Hamilton, 1987) using petroleum ether (40^0C -60^0C) and glacial acetic acid in the ratio of (9.5:0.5) as solvent system.

Isolation and analysis of relevance of plasmid to TBTC resistance: Plasmid was isolated from overnight grown cells in Luria Bertani broth by Alkaline lysis method (Birnbom and Dolly, 1979). Cell suspension containing 10^6 cells were inoculated with increasing concentrations of Acridine orange (10 -100µg/ml) and incubated at 37°C for 24 hours in the dark. Growth was measured as absorbance at 600 nm, culture from the tube showing 50% growth was appropriately diluted and spread plated on Luria Bertani agar. Colonies obtained were checked for ability to grow on TBTC-MSM agar. The same colonies were then checked for presence of plasmid in order to correlate loss / retention of TBTC resistance with loss of plasmids.

RESULT AND DISCUSSION

Isolation, Screening and Identification of the isolate: A creamy isolate screened among five of the total 48 isolates was identified to be *Alcaligenes* sp.2-6 strain S3 (Gen Bank Accession No. EU428755), by standard morphological, physiological, and biochemical tests following *Bergey's Manual of Systematic Bacteriology* (Table 1.1) and molecular characteristics (16S rDNA Sequence & taxonomic phenogram) (Krieg and Holt, 1984; and Dauga, and Grimont, 1991).

Growth, TBTC tolerance limits and Cross Tolerance to Heavy Metals: The isolate tolerated upto 7mM TBTC and showed prominent growth at 5mM TBTC in both Zobell marine broth (ZMB) and Mineral salts medium .(MSM) with a lag of 2 hrs and 4 hrs respectively, the log phase extended upto 8 hrs, after which the stationery phase was attained, which remained till 24 hrs probably the time taken to acclimatize and utilize TBTC as a sole carbon source (Fig 1.1 & 1.2). It is really very interesting to note that the isolate showing high level of resistance to TBTC, was also cross tolerant to some heavy metals with LD_{50} values of 1.5mM to $HgCl_2$, 1.0mM to $CdCl_2$ and 2.5mM to As_2O_3 indicating the presence of plasmid mediated heavy metal efflux pump mechanism (Fig 1.3) (Summers and Silver, 1978; Silver and Laddaga, 1990; Jain and Ali, 2000) (Table 1.2).

Study of exopolymer production under TBTC stress: The isolate produced more amount of exopolymer (EPS) in nutrient rich media (ZMB) than (MSM) containing TBTC and control without TBTC such as 38

µg/mL in ZMB only, 98.3µg/mL in ZMB+5mM TBTC, and 46.8µg/mL in MSM+5mM TBTC. The EPS was acidic in nature with neutral sugars, proteins, uronic acids and methyl pentoses with good emulsification activity (Rosenberg et al., 1999) (Fig-1.4).

Study of pigment production under TBTC stress: The isolate showed an enhanced production of extracellular yellow-green fluorescent pigment in the presence of 5mM TBTC with several weak peaks and an intense peak at 462 nm corresponding to phenazine methosulfate. The spectroflurometric scan of the culture broth confirmed the presence of phenazine pigment, as its colour got intensified upon addition of concentrated HCl. The enhanced production of the pigment may be attributed to TBTC cometabolism as reported in *Pseudomonas Chlororaphis* (Gaber, 1973) (Fig 1.5).

Study of TBTC degradation by Spectrophotometry and Thin Layer Chromatography: The spectrophotometric scan of the cell supernatant and chloroform extract of the cell pellet obtained after 1, 2, 3 weeks and 45th day of incubation revealed the presence of DBT and MBT with an absorption maxima of 263 nm and 280nm respectively (Fig.1.6). These observations confirms that, the isolate accumulates TBTC prior to degrade as suggested by Page (1988) that higher bioaccumulation can help in degradation. The TLC profile of the chloroform extract of the cell pellet obtained on 45th day revealed the presence of both DBT and MBT with Rf value of 0.8 (Solvent front -15±2, TBTC -12.5±1.5) and 0.94 (Solvent front - 15±2, Product - 14.1±2) respectively, corresponding with standard values (Orsler and Holland, 1982) (Fig 1.7). The above observation reveals that though TBTC forms a MSM+TBTC complex in the medium, as the culture grows, it takes up the complex on the cell surface because of lipophilic nature of the cell surface. This study demonstrates that outer membrane of bacterial cell plays an important role in TBTC biodegradation in aquatic system, but limited number of these microorganisms has been identified till now. It has been reported that two *Chlorella* species can metabolize TBT to the less toxic species DBT. The microalgae species *Skeletoneam costatum* is capable of degrading TBT and some bacteria like *Pseudomonas aeruginosa*, *Pseudomonas putida* and *Alcaligenes faecalis* are able to degrade organotin compounds (Hoch, 2001). Hence this clearly shows that TBTC is degraded to MBT by *Alcaligenes* sp.2-6 strain S3.

Isolation and analysis of relevance of plasmid to TBTC resistance: The plasmid profile of the isolate showed a super coiled high molecular weight plasmid. The isolate grown in their LD_{50} values of acridine orange were plated on TBTC containing plates. Loss of plasmid and concurrent presence of TBTC resistance in colonies of the isolate confirms that TBTC resistance genes are not plasmid borne (Fig 1.8). This confirmed that the tolerance and degrading ability of the isolate was not plasmid mediated, but the genetic determinants would be present on the chromosomal genome.

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Tables:

Table 1.1 : List of morphological and biochemical characteristics of the isolate

Sl. No.	Feature	Observation
1	Colony Morphology	Circular, cream, entire, opaque, raised, motile, gram-negative, sticky, short rods.
2	Gram's stain	gram–ve
3	Motility	+
4	Catalase activity	+
5	Oxidase activity	+
6	HL Media (A/FA)	A
7	MR	+
8	Utilization of Glucose	+
9	Gelatin hydrolysis	-
10	Starch hydrolysis	-
11	Tween 80 hydrolysis	+
12	Growth on TSI Media	+
13	Growth on MaConkeys agar	+
14	Growth on NA	+
15	Growth on ZMA	+
16	Growth on MSMA	+
17	Urease activity	-
18	Fluorescent pigment production	+
19	Nitrate reduction	+
20	Thiosulphate Citrate Bile Sucrose Agar	-
21	Citrate utilization	+
22	H_2S production	+
23	Tentative Identification	*Alcaligenes sp.*

24	16 S rDNA sequence	*Alcaligenes sp.2-6*

Table 1.2: Optimum concentration of TBTC

TBTC conc.	500µM	1mM	3Mm	5mM	7mM
Growth	+	++	++	+++	++

Figures:

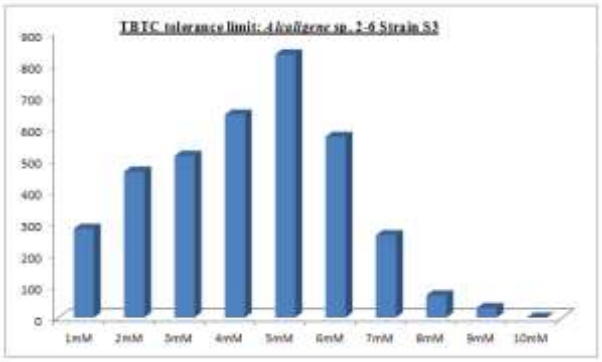


Fig. 1.1

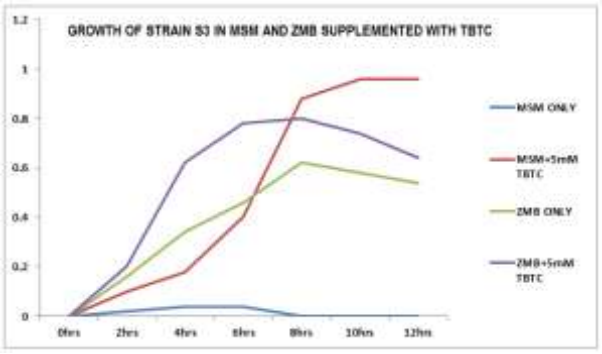


Fig .1.2

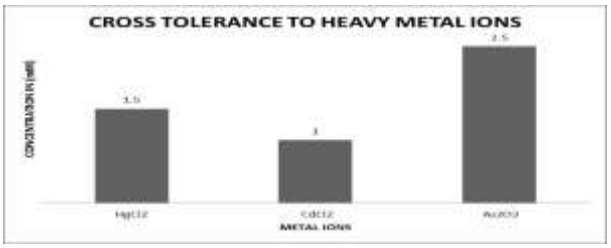


Fig. 1.3

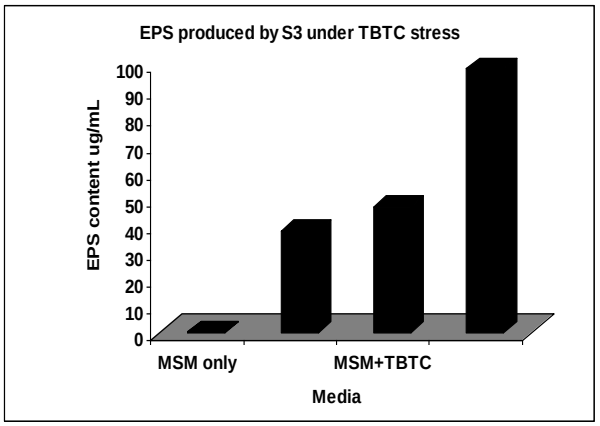


Fig. 1.4

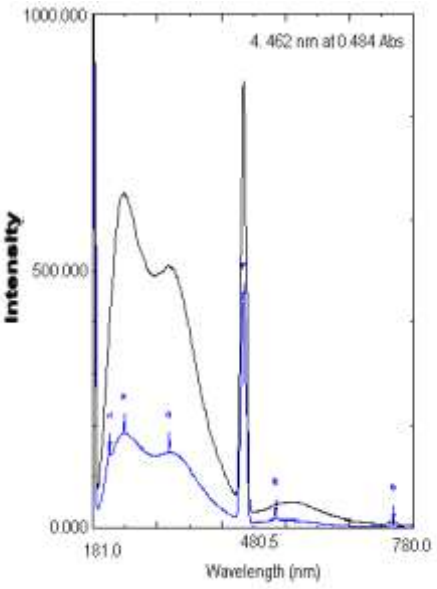
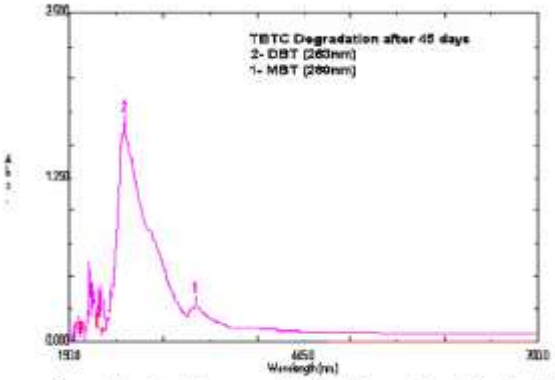


Fig. 1.5 Diagram of pigment scan



Spectrohotometric scan of chloroform extract of cell pellet of S3 grown in MSM+5mM TBTC after 45 days of incubation

Fig.1.6

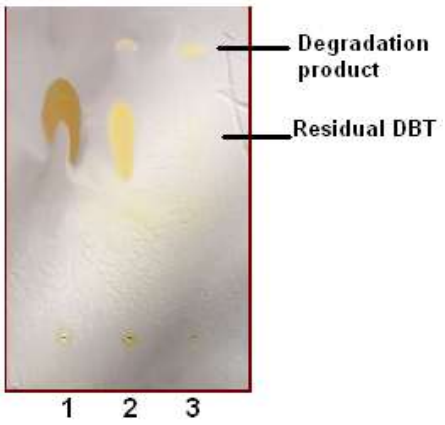


Fig. 1.7 Photography of TLC

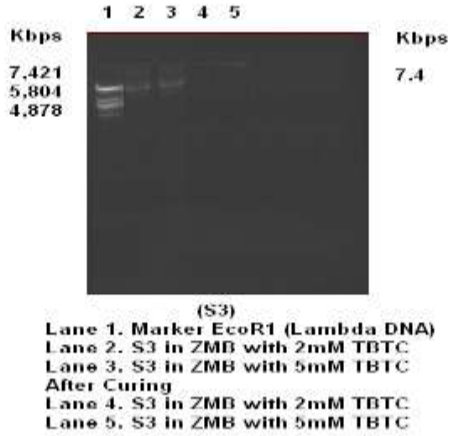


Fig. 1.8

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