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Isolation and biological characterization of highly potent Tributyltin chloride resistant bacteria Alcaligenes sp. swo (Strain Sd) from marine water of Goa, India

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ABSTRACT

Water samples were collected from various sampling sites surrounding the ship building industries in Goa and plated on Zobell marine agar (ZMA), Nutrient agar (NA) and Mineral salt medium agar (MSMA) containing 0.1 mM tributyltin chloride (TBTC). The physico-chemical characteristics of the water samples were determined. The total viable counts of bacteria ranged from 58×10^2 cfu/ml in MSMA+TBTC to 40.6×10^6 cfu/ml in ZMA+TBTC. This clearly indicates predominance of TBTC tolerant and degrading bacterial isolates in marine water of Goa. Although, several highly TBTC tolerant bacterial isolates were screened, one bacterial strain tolerated up to 7 mM TBTC and showed prominent growth in MSM supplemented upto 5mM TBTC. This clearly indicates that TBTC resistant bacterial isolate utilizes TBTC as a sole carbon source. Based on morphological, biochemical, and molecular characteristics (16S rDNA Sequence & taxonomic phenogram) this TBTC resistant isolate have been grouped in *Alcaligenes* sp. swo.

Keywords: Organotins, *Alcaligenes sp*. swo, Shipyard, Tributyltin, Carbon Source, 16 S ribosomal DNA.

1. Introduction

Tributyl tin (TBT) is an organotin compound most commonly used as a biocide in marine anti-fouling paints. It is also used as preservatives in paper, textile, leather and wood industries as a biocide, stabilizers and anti-yellowing agents in plastics, and as slimicides in cooling towers. It is highly toxic, bioaccumulative and a 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 and ships, marine cages, fishing nets and docks has spread TBT far and wide, making it a ubiquitous contaminant of coastal environments as a result of leaching due to hydrolysis from such paints. It binds to suspended particulates and can accumulate several thousand folds in sediments. (Cooney JJ. 1995, Cooney JJ, Wuertz S. 1989, Upal R, Dubey SK, Bhosle S. 2004). The highest levels of TBT are found near harbours and shipyards, along-with busy waterways. On account of increased shipping activities, erosion and transport, tributyltin (TBT) compounds accumulate in harbour water, higher organisms and sediments (Mar Pollu Bull 1992; 24:567, Dowson PH, Bubb J.M, Lester JN. 1993). Sampling sites chosen for this study were the Western India Shipyard Ltd., (W.I.S.L) in Murmagao Port Trust Harbour (MPT) and Goa Shipyard Ltd., (GSL), in Vasco-Da-Gama, the biggest shipyards in the west coast of India and hence a potential source of TBTC contamination. Many workers have reported on organochlorine pesticides, (Mar Pollu

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Bull 1992) polychlorinated biphenyls (PCBs) (Iwata H.1993) and trace metals (Mar Pollu Bull 1992) in the Indian marine environment. However, there has been no report on screening and characterization of highly potent organotin tolerant bacterial strains from coastal environment of Goa. We report here the isolation and biological characterization of Tributyltin chloride resistant bacteria *Alcaligenes sp.* swo from marine water of Goa, India.

2. Materials and methods

2.1 Collection of environmental samples

Marine water samples were collected from different suspected organotin polluted sites of Western India Shipping Ltd., (WISL) in Murmagao Port Trust (MPT), Goa Shipyard Ltd (GISL) in Vadem, local areas in Vasco-Da-Gama from West coast of India (Figure 1) by standard protocols (Cleary JJ, Stebbing ARD 1987, Diez S, Abols M, Bayona JM. 2000, de Mora SJ, Fowler SW, Cassi R. 2003). Water samples from these suspected organotin contaminated sites were taken by immersing 500 ml polycarbonate containers previously rinsed in 20% HNO_3 approximately 20 cm under the surface to prevent the inclusion of the surface micro-layer (Cleary JJ, Stebbing ARD.1987). The containers were stored at 4^0C. These collected samples were used within a week for physiochemical and bacteriological analysis. The containers with the samples were shaken manually and kept undisturbed for 10 minutes in order to allow the particulate matter to settle down. Approximate volume of upper layer was then taken for physiochemical and bacteriological analysis (Dowson PH, Bubb J.M, Lester JN. 1993).

2.2 Physiochemical characterization

The physicochemical characteristics of the water samples (Table-1) were determined in terms of temperature, pH, salinity, alkalinity, organic and inorganic (Nitrate, Nitrite and Phosphate) content following standard protocol (Grasshoff K. 1983, Koroleff F.1983).

2.3 Determination of viable count

Marine water samples stored at 4^0C were kept at room temperature in the lab for 1 hour. The samples were then kept over rotary shaker for 10 minutes at 180 rpm before use. 0.1 ml of each sample was serially diluted in 2% saline up to 10^{-6} dilution. 0.1ml of the serially diluted sample was spread plated on Zobell marine agar (ZMA), Nutrient agar (NA) and Mineral salt medium agar (MSMA) containing 0.1mM TBTC and a ZMA plate without TBTC as a control. The plates were incubated for 24hrs, 48hrs, 72hrs and for a week at room temperature and then the viability was determined in terms of colony forming units/ml (cfu/ml) (Table-2).

2.4 Screening of TBT resistant bacterial isolates

The bacterial isolates which grew in MSM agar with 0.1mM TBTC were sub-cultured continuously on MSM agar plates with varying concentrations of TBTC ranging from 0.5mM-7.0mM. Out of these, 3 isolates showing varying range of tolerance to TBTC viz., 2mM, 3mM and 5mM, were selected for further characterization.

2.5 Maintenance of TBTC resistant bacterial isolate

The bacterial isolates, which grew on Mineral Salts Medium Agar (MSMA) supplemented with 2.0mM of TBTC, were repeatedly sub-cultured in MSM agar with increasing concentration of TBTC ranging from 0.5mM-7.0mM. These TBTC resistant isolates were then maintained on ZMA plates; ZM Broth and MSM Agar plates supplemented with 2mM, 3mM and 5mM TBTC at 28ºC and were designated as S1, S2 and Sd for experimental convenience.

2.6 Identification of TBTC resistant bacterial isolates

Biochemical tests for all the three TBTC tolerant strains, S1, S2, and Sd (Table-3) were carried out according to Cruickshank et al. (1972) and Hi-media rapid biochemical identification test kit (Hi 24 Enterobacteriaceae Identification Kit-KB003). Based on the results, the isolates were tentatively identified as per *Bergey's Manual of Systematic Bacteriology* (Krieg NR, Holt JG. 1984). Subsequently, the most potent TBT resistant isolate Sd was identified by 16S rDNA amplification and sequencing (Data not shown). The 16S rDNA data was further analysed by NCBI-BLAST homology search (Altschul SF.1990) to identify correctly. Taxonomic Phenogram was prepared using Clustal-X- analysis programme (Figure 2).

3. Results and Discussion

The physiochemical characteristics of the water samples collected from various locations in this study showed more or less similar results (Table-1). As these sampling sites are in the coastal and continental shelf area of the Arabian Sea, it is not surprising that these values are more or less the same (Subramanyam NS.2000). The Phosphate, Nitrite, Nitrate and Organic content was high at most places of Western India Shipyard Ltd., (WISL) than Goa Shipyard Ltd (GSL) due to higher anthropogenic activities. These data's (table-1) clearly indicates that nutrient content of estuarine water is higher than open sea. Since organotins are present in these estuarine econiches in appreciable amount, bacterial strains which can tolerate and degrade them get enriched (Wuertz S.1991).

The total viable count of all water samples (Table-2) obtained from Western India Shipyard Ltd., (WISL) and Goa Shipyard Ltd., (GSL) on different media viz., ZMA, NA and MSMA (Table-2) indicates that approximately 18.5% of natural bacterial population is resistant to 0.1mM of TBTC. Since these isolates could grow on MSMA supplemented with TBTC, utilizing it as a sole source of carbon. These studies have shown that bacterial flora of Western India Shipyard Ltd., (WISL) are more resistant than Goa Shipyard Ltd., (GSL) due to higher shipping activities. As evident from similar findings which state that the anti-foulants in ship paints, shipyards, harbours is considered to be the prime source of TBT in the marine ecosystem (de Mora SJ, Pelltier E. 1997). Western India Shipyard Ltd., (WISL) and Goa Shipyard Ltd., (GSL) are the biggest shipyards in west coast of India with modern ship repairing systems involved in the repair and construction of commercial and naval ships. Therefore organotin levels are invariably high around W.I.S.L and GSL. Similar findings have been already reported, where the coastal water near harbour areas of the world is appreciably contaminated with TBTC (Chau YK.1997, de Mora SJ, Stewart C, Phillips D. 1995). Most of the bacterial isolates failed to grow in the presence of higher concentration of TBTC (2 mM). As the aim of the study was to screen for a bacterial isolate which has inherent capability to resist and degrade TBTC, all the bacterial isolates were sub-cultured with increasing concentration of TBTC (0.1mM to 7mM). Out of 112 isolates growing in 2mM TBTC, 3 isolates showed consistent good growth in presence of 2mM, 3mM and 5mM TBTC in MSM broth after 48hr incubation with the optimum condition for growth i.e.

temperature at 28^0C, pH 7.8-8.0 and 2.8-3.1% salinity designated as S1,S2 and Sd were screened, selected, and purified for further studies. On the basis of their growth on MSM agar with increasing concentrations of TBTC (2mM-5mM). Highly potent strain Sd growing in the presence of 5mM TBTC was chosen for further studies. This TBTC utilizing isolate was then maintained on MSMA, ZMA and ZMB with 5mM TBTC and incubated at 28ºC, which is an ambient temperature for marine /estuarine samples. Most of the bacterial isolates could not grow in presence of higher concentration of TBTC (2mM) due to cellular toxicity and inhibitory effect on metabolic process and viability of bacterial strains (Pettibone GW, Cooney JJ. 1986). Although Singh (1987) and White, et al. (1999) have reported the range of microbial resistance up to 0.07mM for different organotin compounds, but bacteria utilizing TBTC as sole source of carbon has not been reported so far. Debutylation of TBT compounds to di and mono-butyltins is known to occur in bacteria, algae and fungi, which provides one route for detoxification of tributyltin. In addition, microorganisms are capable of accumulating TBT compounds, which is another mechanism of removal of TBT from marine environment (Gadd G.M. 2000). It has already been reported that TBTC tolerant bacteria are present in sea-water (Fukagawa et al. 1994) and *Pseudomonas aeruginosa* can degrade tributyltin oxide at 2.5 ppm level (Suzuki S, Fuagawa T, Takma K. 1992). Although a few researchers have reported degradation of TBT by environmental microorganisms, isolation of TBT utilizing bacteria has not been accomplished so far (Suzuki S, Fukagawa T. 1995). Further more, not much is known on TBTC degradation rates under ambient environmental conditions in marine coastal waters and it is expected that the fate of TBTC will be dependent on direct biological degradation by TBTC tolerant bacteria present in the ambient marine environment (Dubey SK, and Roy U. 2003, Seligman PF, Valkris AO, Lee RF. 1986).

Initially, colony characters of all three bacterial isolates (S1, S2 and Sd) revealed that S2 and Sd were cream coloured colony which turned to brown after 48hrs of incubation, the other bacterial isolate S1 showed yellow pigmentation. The Gram's characters of all three isolates showed that these bacterial isolates were Gram's negative S1 was short rods, S2 curved rods and Sd long rods. Biochemical tests (Table-3) for all three strains were initially done according to Cruickshank et al. (1972). On the basis of biochemical and carbohydrate fermentation tests all the strains were tentatively identified according to *Bergey's Manual of Systematic Bacteriology* (Krieg NR, Holt JG. 1984), as *Flavobacterium balustinum* (strain S1), *Vibrio harveryi* (strain S2) and *Alcaligenes sp,* (strain Sd) The identity of the most potent strains Sd was confirmed by 16S rDNA gene amplification by polymerase chain reaction (PCR). 16S rDNA was amplified, using 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 (Altschul SF.1997) showed that strain Sd belonged to the gamma subdivision of the phylum Proteobacteria and that it was closely related to the genus *Alcaligenes sp.,*swo. The DNA sequence was determined using the dideoxy chain termination method (Sanger F, Nicklen S, Coulso AR. Proc. Natl. 1977). A total of 1000 bp was sequenced; the sequence was compared with other bacteria available in GenBank (Altschul SF.1990). The sequence was then aligned with available 16S rRNA reference sequences. Similarities and alignments were obtained using the Basic Local Alignment Search Tool (BLAST) (Altschul SF.1997) 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 for comparison. The characteristics of all the isolates and the taxonomic phenogram (Figure 2) showed similarity among the isolate, which have been grouped as *Alkaligenes* sp. swo. (figure 2).

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 ‰	2.38 meq.1^{-1}	288mg/l	1.12µmol. dm^{-3}l	3.84µmol. dm^{-3}l	0.82µmol. dm^{-3}l
Berth wall	8.0	28 °C	31.87 ‰	2.36 meq.1^{-1}	368mg/l	1.02µmol. dm^{-3}l	3.85µmol. dm^{-3}l	0.84µmol. dm^{-3}l
Near ship	7.8	28.4 °C	28.40 ‰	2.36 meq.1^{-1}	178mg/l	0.672µmol .dm^{-3}l	2.98µmol. dm^{-3}l	0.58µmol. dm^{-3}l
GSL								
Berth wall	7.9	27.4 °C	31.77 ‰	2.34 meq.1^{-1}	218mg/l	0.678µmol .dm^{-3}l	2.48µmol. dm^{-3}l	0.80µmol. dm^{-3}l
Near ship	7.8	29 °C	28.03 ‰	2.28 meq.1^{-1}	198mg/l	0.644µmol .dm^{-3}l	3.28µmol. dm^{-3}l	0.78µmol. dm^{-3}l

Table 2: Viable count of bacteria in environmental samples

Sampling site	**Geographical positions**		**Viable counts (10^6cfu/ml) ± SE in ZMA**		**Viable counts (10^4cfu/ml) ± SE in NA**	**Viable counts (10^2cfu/ml) ± SE in MSMA**
W.I.S.L	**Latitude**	**Longitude**	**0mM TBTC**	**0.1mM TBTC**	**0.1mM TBTC**	**0.1mM TBTC**
Over berth	15º27'628'N	73º49'842'E	28.6±.3	41.6±.1	89.6±.1	80±.2
Berth wall	15º27'628'N	73º49'842'E	34.6±.6	38.9±.2	93.4±.2	81±.2
Near ship	15º27'628'N	73º49'842'E	42.3±.3	28.4±.1	76.8±.4	71±.4
G.S.L	**Latitude**	**Longitude**	**0mM TBTC**	**0.1mM TBTC**	**0.1mM TBTC**	**0.1mM TBTC**
Near ship	15º27'703'N	73º49'985'E	41.6±.6	37.6±.4	65.8±.8	58±.1
Berth wall	15º27'628'N	73º49'842'E	38.5±.5	40.6±.2	79±.9	73±.1

Table 3: Morphological and Biochemical characteristics of all TBTC tolerant bacterial isolates

Characteristics	Bacterial strain S1	Bacterial strain S2	Bacterial strain Sd
Colony Morphology	Circular, yellow, entire, opaque, raised, non-motile, gram-negative, short rods.	Circular, cream, entire, opaque, raised, motile, gram-negative, curved rods.	Circular, cream, entire, opaque, raised, motile, gram-negative, sticky, long rods.
Gram's stain	gram–ve	gram–ve	gram–ve
Motility	-	+	+
Catalase activity	-	+	+
Oxidase activity	-	+	+
HL Media (A/FA)	FA	FA	FA
V.P test (Acetoin)	ND	-	-
MR	ND	+	+
Indole	+	+	+
Utilization of Glucose	+	+	+
Utilization of Sucrose	-	+	(d)
Utilization of Arabinose	-	-	+
Utilization of Mannose	-	+	(d)
Utilization of Mannitol	-	+	(d)
Utilization of Galactose	-	+	+
Utilization of Lactose	-	-	-
Utilization of Salicin	+	-	-
Utilization of Raffinose	-	-	-
Utilization of Maltose	+	-	-
Utilization of Xylose	ND	ND	+
Utilization of Adonitol	ND	ND	-
Utilization of Cellubiose	ND	ND	+
Utilization of Melibiose	ND	ND	+
Utilization of Saccharose	ND	ND	+
Utilization of Trehalose	ND	ND	+
Casein hydrolysis	+	+	+
Gelatin hydrolysis	+	+	+
Starch hydrolysis	-	+	-
Tween 80 hydrolysis	+	+	+
Growth on TSI Media	ND	-	+
Growth on MaConkeys agar	+	+	+
Growth on NA	+	+	+
Growth on ZMA	+	+	+
Growth on LBA	+	+	+
Growth on MSMA	+	+	+
Urease activity	+	-	-
Fluorescent pigment production	-	+	+

Nitrate reduction	-	+	+
Lysine Decarboxylase	ND	+	-
Ornithine Decarboxylase	-	+	-
Thiosulphate Citrate Bile Sucrose Agar	-	+	-
Citrate utilization	-	+	+
H_2S production	ND	-	+
Tentative Identification	*Flavobacterium* sp.	*Vibrio* sp.	*Alcaligenes* sp.
16 S rDNA sequence	ND	ND	*Alcaligenes* sp. swo Accession number: genbank EU401448
(+)= Positive, (-)=Negative, (d)= Doubtful, O= Oxidative, A= Aerobe, FA= Facultative aerobe and N.D= Not Done			

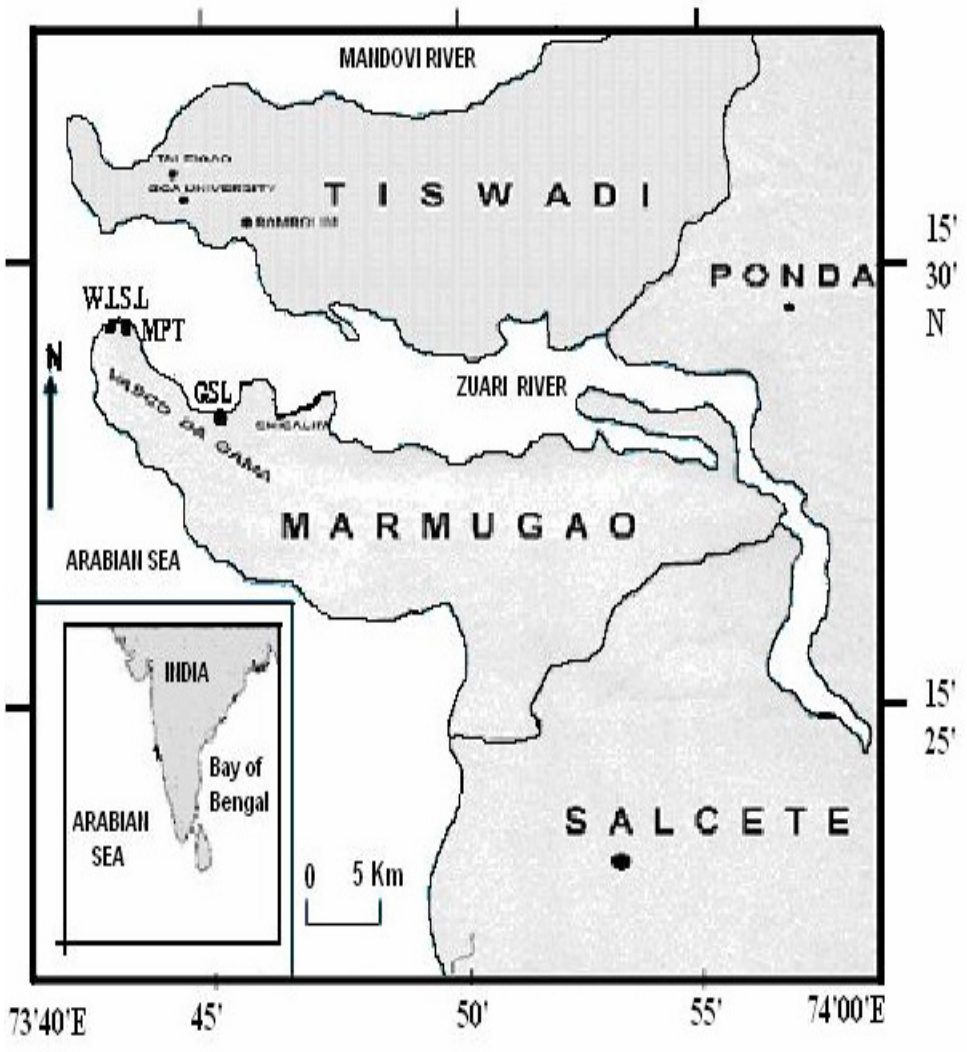

Figure 1: Sampling sites

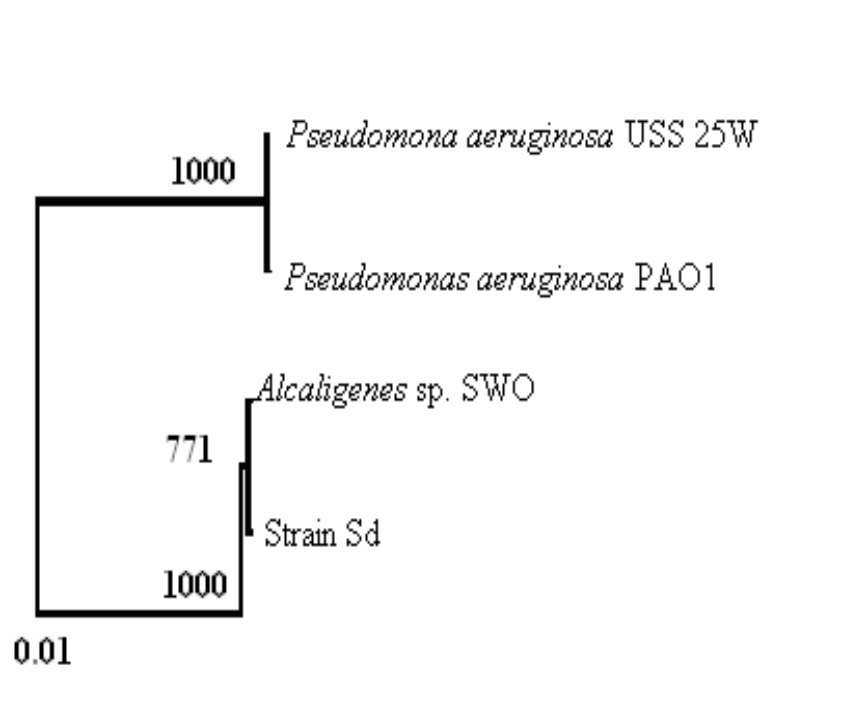

Figure 2: Taxonomic phenogram of highly potent Tributyltin chloride resistant marine bacterial isolate *Alcaligens sp.* SWO Strain sd.

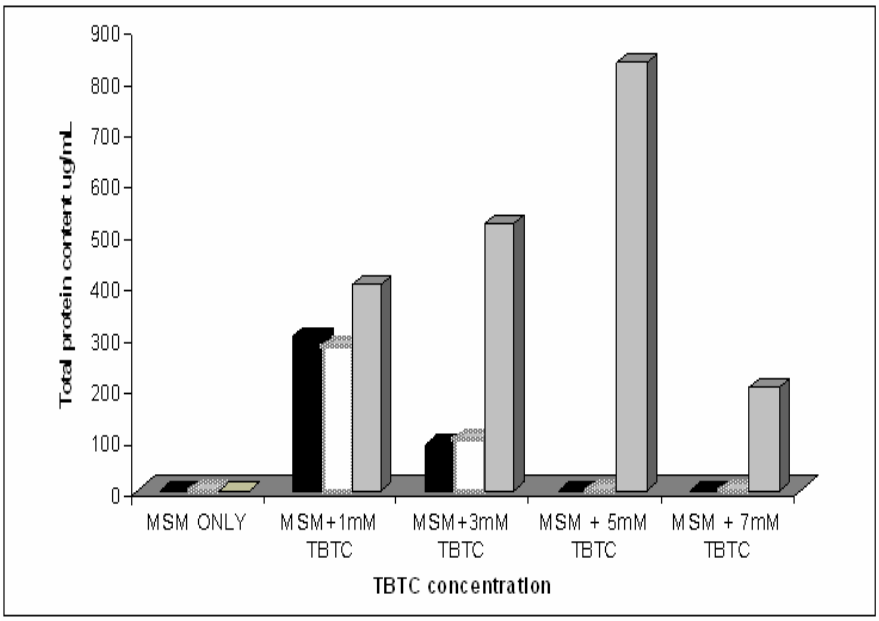

Figure 3: TBTC tolerance limits of marine bacterial isolates

S1 , **S2** , **Sd**

Abbreviations

(+)= Positive, (-)=Negative, (d)= Doubtful, O= Oxidative, A= Aerobe, FA= Facultative aerobe and N.D= Not Done, cfu= Colony forming units, ml=Milliliter, TBTC=Tributyltin Chloride, NA= Nutrient agar, ZMA= Zobell Marine Agar, MSMA=Mineral Salt Medium

Agar, LBA= Luria Bertani Agar, HL= Hug leifson, MR=Methyl Red, V.P=Vogues Proskuer, WISL=Western India Shipping Limited, GSL=Goa Shipping Limited, ‰= $meq.1^{-1}$ = mg/l =µmol.dm^{-3}l=

The selected three bacterial isolates S1, S2 and Sd were primarily screened on the basis of TBTC utilization as a sole carbon source. Most of the isolate which initially grew on MSM agar supplemented with 2mM TBTC, lost their viability on the same medium after repeated subculturing, but very few of them could survive at higher concentration of TBTC i.e. 2mM-5mM. The selected three isolates consistently grew on MSM agar supplemented with 2mM (S1&S2), and 5mm TBTC (Sd) with in 48hrs. In subsequent higher concentration of TBTC i.e., 6mM, 7mM etc., isolates did not grow (Figure 3).

This study clearly showed that, the bacterial isolates of WISL are more resistant than GSL. This may be due to heavy ship traffic and also MPT harbour is one among the oldest port of India (since 1934), receiving cargo and passenger vessels at its various berths, which dock for weeks, during which TBT leaches to the marine environment due to hydrolysis as earlier reported (de Mora SJ, Stewart C, Phillips D. 1995) moreover 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. During this process, old paint is being blasted out, which finally ends up in to the marine environment where gradually TBT leaches (de Mora SJ, Stewart C, Phillips D. 1995).

Chau and co-workers, (1997) have demonstrated that heavy contamination of coastal waters by TBT was associated with major commercial harbours and ship repair activities involving removal of the old paint from the hull and application of a new coating which could release TBT as wastewater discharge. 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 (Cooney JJ. 1988, Gadd GM. 1993, Gadd G.M. 2000). 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 (Barug D.1981, de Mora SJ, Stewart C, Phillips D. 1995). 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 (Cleary JJ, Stebbing ARD.1987, Cooney JJ. 1995). 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. Though microorganisms have been shown to bioremediate heavy metal and aromatic hydrocarbon of polluted sites, but bioremediation of organotin contaminated sites mediated by microbes is far away from real large scale commercial process, since very little work has been done to explore the exact biochemical mechanism of organotin biodegradation and genes involved in the process. The highly potent isolates identified as *Alcaligenes sp.* swo (Strain Sd) tolerating up to 7mM TBTC and growing very well in 5mM TBTC, could be really very promising in the bioremediation of organotin contaminated marine and estuarine environment of Goa in west coast of India.

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