BIOLOGICAL CHARACTERIZATION OF TBTC(ORGANOTIN) RESISTANT BACTERIA FROM MARINE ENVIRONMENT OF WEST COAST OF INDIA

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BY

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Certificate

This is to certify that ***Mr. Upal Roy*** has worked on the thesis entitled **"Biological characterization of TBTC (organotin) resistant bacteria from marine environment of west coast of India"** under my supervision and guidance.

This thesis, being submitted to the Goa University, Taleigao Plateau, Goa, for the award of the degree of Doctor of Philosophy in Microbiology, is an original record of the work carried out by the candidate himself and has not been submitted for the award of any other degree or diploma of this or any other University in India or abroad.

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STATEMENT

I hereby state that this thesis for the Ph.D. degree on "**Biological characterization of TBTC (organotin) resistant bacteria from marine environment of west coast of India"** is my original contribution and that the thesis and any part of it has not been previously submitted for the award of any degree/diploma of any University or Institute. To the best of my knowledge, the present study is the first comprehensive work of its kind from this area.

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Upal

Dedicated

to my

Parents.

ABBREVIATIONS

α	alpha	**mM**	Milli molar
Abs	Absorbance	***m***	Meta
APS	Ammonium per sulfate	**µg**	Microgram
β	Beta	**µL**	Microlitre
b.p.	Boiling point	**µM**	Micromolar
°C	Degree celcius	**µ**	Micron
Ca^{2+}	Calcium ion	**NaCl**	Sodium chloride
Cd^{2+}	Cadmium ion	NH_4NO_3	Ammonium nitrate
cfu	Colony forming unit	NH_4Cl	Ammonium chloride
D/W	Double distilled Water	**NA**	Nutrient Agar
DBT	Dibutyltin chloride	**NaOH**	Sodium hydroxide
EDTA	Ethylene diamine -	**nm**	Nanometer
	- tetra acetic acid	**NMR**	Nuclear Magnetic Resonance
EPS	Exopolymeric substances	**NTG**	N-methyl-N-nitro-N-nitrosoguanidine
Fig.	Figure	**O.D.**	Optical Density
gm	Gram(s)	***o***	Ortho
GC	Gas chromatography	***p***	Para
hrs	Hour(s)	**PAGE**	Poly-acrylamide gel electrophoresis
HCl	Hydrochloric acid	**Rf**	Resolution factor
H_2SO_4	Sulphuric acid	**rpm**	Revolutions per minute
Hg^{2+}	Mercuric ion	**RT**	Room temperature
IR	Infra Red	**SDS**	Sodium dodecyl sulfate
K^+	Potassium ion	**sec.**	Second(s)
KDa	Kilo Dalton	**sp.**	Species
Kbps	Kilo base pairs	**TBT**	Tributyltin
KNO_3	Potassium nitrate	**TBTC**	Tributyltin chloride
L	Litre	**TEMED**	Tetra methyl ethylene diamine
LB	Luria Bertani Broth	**Thiol**	β- mercapto- ethanol
LA	Luria Bertani Agar	**TLC**	Thin layer chromatography
lbs	Pounds	**UV**	Ultra violet
M	Molar	**V**	Volts
mg	Milli gram(s)	**v/v**	Volume / Volume
MSM	Mineral Salt Medium	**w/v**	Weight / Volume
MSMA	Mineral Salt Medium Agar	Zn^{2+}	Zinc ion
Mg^{2+}	Magnesium ion	**ZMB**	Zobell Marine Broth
min	Minute(s)	**%**	Percentage
ml	Milli litre	λ	Lambda

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Chapter I

INTRODUCTION

1.1 Organotins in the environment.

Organotin compounds remained of purely scientific interest for a long time, since their discovery around 1850. Though the first mention of a practical application of organotin compounds was made in a patent taken out in 1943, which indicated their potential in antifouling systems (Tisdale, 1943), the commercial production started in 1960's. All organotin compounds are toxic, but the effect varies according to the number and type of organic moiety present (Table-1.1). Propyl and butyl groups bearing organotins are more toxic to fungi and bacteria (Evans and Smith, 1975). Extensive use of organotins worldwide provoked scientific interest on the toxic effect of organotin compounds on aquatic and terrestrial biota (Smith, 1978, 1980, 1998).

Table-1.1 Biocidal properties of organotin compounds

Organisms affected	R in R_3 SnX compound
Insects	Methyl
Mammals	Ethyl
Gram negative Bacteria	Propyl
Gram positive Bacteria	Butyl
Fungi	Butyl
Fish and Molluscs	Phenyl
Mites	Cyclo-Hexyl

Tributyltin (TBT) has been in use as a paint additive since 1970's to prevent bio-fouling on ship hulls, marine platforms and fishing nets. In the mid 1980s, researchers in France and United Kingdom confirmed that TBT present in antifouling paints is adversely affecting the non-target organisms. In the year 1942 France was the first country to ban the use of organotin based antifouling paints on boats less than 25m long (Alzieu et al. 1986, 1989). Similar regulations were also imposed in North America, Australia, New Zealand, South Africa, Hong Kong and most European countries since the late 1980s (Dowson et al. 1993, de Mora et al. 1995; de Mora., 1996; Minchin et al.

1997; Evans, 1999; Champ 2000; 2001). Subsequently, worldwide monitoring programmes have shown reduced concentration of TBT in the water column, sediments and tissues of marine animals. The International Maritime Organisation (IMO) has repeatedly expressed concern about the harmful effects of the TBT based paints (Evans, 1999). It has also been shown that TBT may be responsible for the thickening of oysters and mussel shells as well as retardation of growth in various species of aquatic snails (Alzieu and Heral, 1984; Laughlin et al. 1986). Two widely published events in 1980s, such as the near-collapse of oyster farming in Arcachon bay, Western France and the demise of population of dogwhelk, *Nucella lapillus* at the central boating activity of Southwest England, have been attributed to severe TBT contamination. These alarming reports culminated in a number of surveys of TBT pollution worldwide and indicated that the problem was global (Maguire et al. 1982, 1986; Champ and Seligman, 1997; Evan 1999; Hoch, 2001). Tributyltin concentration in the aquatic environment have been monitored for many years at many locations throughout the world including the North Sea, Black Sea, Atlantic ocean, Pacific ocean and Japanese waters (Maguire, 1984, 2000; Cleary and Stebbing, 1987; Alzieu et al. 1989; Evans, 1999) (Table–1.2). The noticeable concentrations of organotins reported are 38 μg g-l TBT in Suva harbour, Fiji, 10.780 ng g-l Hexyl-tin in Vancouver, Canada, 518 ng g^{-l} TBT in Boston harbour, U.S.A., 400 ng g-l TBT in lake Lucrne, Switzerland and upto 380 ng g-l TBT in Puget sound, USA (Maguire et al. 1986; de Mora et al. 1995; de Mora, 1996). The International Maritime Organisation has already passed the resolution to ban the application of TBT-based antifouling paints on ships and boats and also proposed to establish a mechanism to prevent the potential future use of other harmful substances in antifouling systems (Champ, 2000). Triorganotins (TOT) such as tributyltin oxide (TBTO), tributyltin chloride (TBTC), triphenyltin chloride (TPTCl), tributyltin fluoride(TBTF), tributyltin hydroxide(TBTH), tributyltin naphthanate (TBTN) and tris (tributylstannyl) phosphate (TBTP) are very extensively used as biocides in antifouling paints on ship hulls, boats and docks, as slimicides in cooling towers, as fungicides, bactericides, insecticides, as

preservatives for wood, textiles, papers, leather and as stabilizing material in PVC pipes, electrical equipments and as catalyst for synthesis of polyurethane foam and silicon rubber (Clark et al. 1988; Fukagawa et al. 1992; Suzuki and Fukagawa, 1995). Trisubstituted organotins have wide ranging toxicological properties, and their biocidal uses have been reported to have detrimental environmental impacts (Inoue et. al. 2000). In UK under the Control of Pollution Act-1974, the retail sale of organotin paints was restricted to co-polymer paints containing <7.5% tin and free association paints containing <2.5% tin in the dry film (Dowson et al. 1993). During the 90s United States of America alone produced 10,000 metric tonnes of organotin compounds each year (Boopathy and Daniels, 1991). Recent estimates show that the annual world production of organotin may be close to 50,000 tonnes per year (Inoue et al. 2000).Commercial ships, in particular, consume about 75% of total tributyltin used in antifouling paints (Atireklap et al. 1997). In Suva Harbour, Fiji, the water blasting of relatively big vessels has caused severe contamination of near shore sediments and shellfish. A British survey revealed that unregulated dry dock practices clearly result in the release of large quantities of TBT in marine environment (de Mora et al. 1995). Non-point sources of environmental exposure include discard and sanitary landfill disposal of plastic and direct release of biocides to aquatic or marine environment. Other dissipative uses of organotins, which pose potential risk to human include PVC food wrappings, bottles and rigid potable water pipes, whereas long term human health hazards due to low level exposure to organotins are not known. Toxic metal cycling in the environment including biomethylation of inorganic tin by naturally occuring bacteria is also of immense concern (Craig, 1982; Hallas et. al. 1982a). *In situ* measurement of tributyltin based antifouling paint leachates have shown that tributyltin is the principal compound released in water. It has been shown evidently that different forms of the tributyltins such as hydroxide, chloride, and various carboxylate forms are released in aqueous environment from different types of paints as a result of leaching (Clark et al. 1988).

1.2 Chemistry of Tributyltin (organotin) compounds.

In view of the diversity of organotins used industrially, knowledge of their environmental chemistry is of fundamental importance and some aspects have already been reviewed (Craig, 1982). The organotin compounds which are used in antifouling paints are already listed in Table–1.3. It is interesting to note that alkyls tend to be more toxic than aryls and triorganotins are more toxic than di-, mono or tetra-organotins. Generally, toxicity of the organotin is influenced more by the alkyl substituents than the anionic substituent. Progressive introduction of organic groups to the tin atom in any member of the R_3SnX_{4-n} series produces maximal biological activity against all species, when n=3, for the triorganotin compounds, R_3SnX (Smith, 1978, 1980; Blunden et al. 1984; Singh, 1987). Generally trisubstituted (R_3SnX) organotins, where R=Butyl/Phenyl are highly toxic than di- and monosubstituted organotin compounds and the anion (X) has little influence on the toxicity (Pain and Cooney, 1998; Gadd, 2000). It is interesting to note that they could provide antifouling cover for five or more years and have bee acclaimed widely as the most effective antifoulants ever devised. TBT in such paints is chemically bonded in a copolymer resin system via an organotin-ester linkage but there is a slow and controlled release of the biocide, as the link get hydrolysed when sea water comes in contact with paint's surface (Evans, 1999).

Table 1.3 Ingredients of antifouling paints

Trialkyltins	**Triaryltin**
Bis(tributyltin)oxide	Triphenyltin hydroxide
Bis(tributyltin)sulfide	
Tributyltin acetate	
Tributyltin acrylate	
Tributyltin fluoride	**Monocyltin**
Tributyltin naphthenate	Monocyltin tris iso-ocyltin mercaptoacetate
Tributyltin resinate	
Tributyltin methacrylate	
Bis-(tributyltin)adipase	**Dialkyltin**
Tricyclohexyltin hydroxide	Dibutyltin dilaurate
Tributyltin chloride	

Source: Kuch, 1986

1.3 Biological activity of organotins

While tin in its inorganic form is considered to be less toxic, the toxicological pattern of the organotin compounds is complex (Hoch, 2001). Tributyltin, tripropyltin and triphenyltin are highly effective biocides against several marine fouling organisms including snails, barnacles, sea weeds, bacteria and fungi where as it affects the different energy and growth related pathways in different organisms (Table-1.4). In general, organotin toxicity to microbes decreases in the following order: $R_3SnX > R_2SnX_2 > RSnX_3 > R_4Sn$. Since, microorganisms accumulate organotin in the cell wall envelope by a non energy requiring process, organotins such as tripropyl, tributyl and triphenyltin seem to be highly toxic to bacteria and fungi (Cooney and Wuertz, 1989; Laurence et al. 1989; Cooney, 1995). It is very interesting to note that increased total surface area and lipid solubility of the tri-substituted tin correlates well with the toxic effect observed and confirms that triorganotins exert toxicity through their interaction with membrane lipids. It has been reported that organotin compounds are toxic to both Gram negative and Gram positive bacteria but tri-organotins are more active towards Gram positive bacteria than towards Gram negative bacteria. Among the trialkyltin series the most active compounds inhibiting growth of the Gram positive species at 0.1 mg/l, belong to the type R_3SnX. Gram -positive bacteria are less sensitive to tri-ethyl and tripropyltin acetate or chloride than Gram-negative bacteria whose growth is inhibited at concentration of 20-50 μg/ml. Tri-butyltin chloride or acetate had a strong growth inhibitory effect on Gram-positive bacteria than on Gram negative bacteria (Yamada et al. 1978, 1979).

Table-1.4 Toxic effect of TBT on microbes

Process affected	Organisms/ Organelles	Inhibitory concentration
Respiration	Bacteria	0.04-1.7μM
Photosynthesis	Cyanobacteria	1 μM
Nitrogen fixation	*Anabaena cylindrica*	1 μM
Primary productivity	Microalgae	0.55-1.7 μM
Growth	Microalgae	0.17-8.4 μM
Energy linked reaction	*E. coli*	0.15->50 μM
Growth/ Metabolism	Fungi	0.28-3.3 μM
Growth/ Metabolism	Bacteria	0.33-16 μM
Photoophosphorylation and ATP Synthesis	Chloroplast	0.56-5 μM
ATPase activity on plasma membrane	*Neurospora crassa*	0.06 μM
ATPase activity on Mitochondria	*Neurospora crassa*	0.01 μM

Source: Kuch, 1986.

TBT is a membrane active lipophilic compound known to exhibit the same inhibitory mechanisms in bacteria as seen in mitochondria and chloroplasts by acting as an ionophore facilitating halide-hydroxyl ion exchange by interfering with the energy transduction apparatus. In addition, TBT can inhibit a variety of energy linked reactions in *Escherichia coli,* including growth, solute transport, biosynthesis of macromolecules and activity of transhydrogenase (Singh, 1987). Boopathy and Daniels (1991) have also tested toxic effects of several organotins and tin chloride on the methanogenic bacteria, *Methanococcus thermolithotrophus, Methanococcus deltae* and *Methanosarcina barkeri* 227. These methanogens were strongly inhibited by triethyltin, tripropyltin and monophenyltin generally below 0.05 mM level. Less inhibition was observed for TBT at 0.1 mM but there was complete inhibition of growth at 1mM concentration. Virtually all organotin toxicological studies have been conducted using aerobic microorganisms viz. bacteria and yeast (Hallas and Cooney, 1981b; Hallas et al., 1982b; Pettibone and Cooney, 1988; Cooney et al. 1989; Cooney and Wuertz, 1989; Laurence et al. 1989; White et al. 1999). In addition, biocidal effects of organotins against other marine fouling organisms viz. algae (*Enteromorpha, Ectocarpus* and *Ulothrix*), barnacles, tubeworms and shrimps have also been studied (Skinner, 1971; Christie, 1972; Mawatari, 1972; Phillip, 1973).

1.4 Tributyltin resistant bacteria.

Several reports have been documented on isolation and characterisation of TBT resistant bacteria from soil, marine and estuarine environment (Barug and Vonk, 1980; Barug, 1981; Hallas and Cooney, 1981a; McDonald and Trevors, 1988; Wuertz et al. 1991; Fukagawa et al. 1992; Suzuki et al. 1992; Pain and Cooney, 1998). The isolation and characterization of TBT resistant marine bacterium, *Alteromonas sp.* M-l is the first record of its kind. It is interesting to note that addition of TBT to the natural sea water specifically enriched TBT tolerant bacteria (Suzuki et al. 1992; Fukagawa et al. 1994). These resistant bacteria could tolerate high levels of TBT biocides due to their inherent capability to (i) transform them into less toxic compounds viz. di- and mono- butyltin by dealkylation mechanism or (ii) exclusion /efflux of these toxicants out side the cell mediated by membrane proteins or iii) degradation / metabolic utilization of them as carbon sources mediated by enzymes or iv) bioaccumulation of the biocide without breakdown using metallothionein like proteins (Blair et.al. 1982; Fukagawa et al. 1994).

Although little is known about the resistance mechanism with which microorganisms tolerate this biocide (Wuertz et al. 1991), several organotin resistant bacteria have been reported which includes *Escherichia coli, Pseudomonas fluorescens, P. aeruginosa, Proteus mirabilis, Serratia marcescens* and *Alkaligenes faecalis* which are Gram negative and *Staphylococcus aureus, S. epidemidis, Bacillus subtilis, Mycobacterium phlei* and *Vibrio sp.,* which are Gram positive (Wuertz et al. 1991; Fukagawa et al. 1994; Suzuki and Fukagawa, 1995; Gadd, 2000) (Table-1.5).

1.5 Degradation of Tributyltin by abiotic and biotic factors.

Organotin degradation involves sequential removal (dealkylation) of alkyl groups from the tin atom which generally results in a toxicity reduction (Blair et al. 1982; Cooney, 1988; Cooney, 1995). This can be achieved by biotic and abiotic factors with UV and chemical cleavage being the most important abiotic factors in aquatic and terrestrial ecosystems (Barug, 1981; Blunden and Chapman, 1983). Although the

degradation of organotins has been shown to be mediated by microorganisms, information is still severely limited in relation to mechanism of degradation, tolerance mechanism of microbes and their relative significance and also the role of anionic radicals in degradation process in natural habitats (Cooney, 1988; Gadd, 1993, 2000). Biotic processes have been demonstrated to be the most significant mechanisms for tributyltin degradation both in soil as well as in fresh water, marine and estuarine environment (Barug, 1981; Dowson et al. 1996). Rate of TBT degradation may be influenced by several biotic and abiotic factors, such as nature and density of microbial population, TBT solubility, dissolved suspended organic matter, pH, salinity, temperature and light.

1.5.1 Abiotic factors.

The biogeochemical cycle of organotin clearly shows that bioaccumulation, biomethylation and photolytic degradation are major processes involved in organotin transformation in nature, but knowledge on environmental fate of TBT in coastal water is still limited (Fig-1.1). This fact stimulated research interest on the aspect of biodegradation and bioaccumulation of TBT in water columns and sediments, by microorganisms and also by higher marine organisms. Environmental surveys from different locations throughout the world have shown that tributyltin is present in three main compartments of the aquatic ecosystem, the surface microlayer, the water column and the surface layer of the bottom sediments (Clark et al. 1988). In aquatic environment tributyltin and other organotins accumulate on the surface microlayer, in sediments, and on suspended particulate. Binding of tin compounds to sediments varies greatly with the sediments and tin species, and binding is influenced by salinity, pH, and amount of particulate matter (Cooney, 1988). The bio-availability of organotins to microorganisms is a key determinant for uptake, bioaccumulation and toxicity, which depends on the chemical speciation of organotin in aquatic milliue (Chaumery and Michel, 2001).

Therefore, environmental variables viz. temperature, pH and ionic composition are most important parameters governing bioavailability as well as degradation of organotins.

The result of these studies indicates that TBT can be degraded rapidly in marine water column to di-butyltin and mono-butyltin with a half life of several days. TBT degradation by photolysis alone proceeds slowly with a half-life of > 89days (Wuertz et al. 1991). Half life of TBT from a clean water site (0.03μg/L of TBT) were 9 and 19 days for light and dark treatments respectively (Seligman et al. 1986), but photolysis probably is not a significant breakdown process for TBT (Clark et al. 1988). In case of TBT present in sediments, a first order multi-step kinetic model of the sequential degradation of TBT to form DBT, MBT and Sn (IV) has been proposed which indicated that the half life of TBT, DBT and MBT was 2.1, 1.9 and 1.1 years respectively (Sarradin et al. 1995).The principal degradation product in all experiments was di-butyltin with lesser amounts of mono-butyltin. Complete mineralization of TBT measured by the formation of $^{14}C0_2$, proceeded slowly with a half-life of 50-75 days (Table-1.6). Sheldon (1978) has reported that ^{14}C labelled TBTO, TBTF and TPTF in soil was degraded faster in aerobic conditions than anaerobic conditions. However persistence does not necessarily equate to a compound being toxic, because it may not be bio available (Evans, 1999). Interaction of microorganisms with organotin is significantly influenced by environmental conditions. In aquatic ecosystems, both pH and salinity can determine organotins speciation/ bio-availability and therefore, biological activity. In one study K^+ release was used as an index of toxicity, as both the rate and the extent of K^+ release was affected by salinity. Increased NaCl concentration reduced the toxic effect of TBT, with the possible effects being due to Na^+ and Cl^- moieties, as well as possible osmotic responses of the organisms which included changes in intracellular compatible solute and membrane composition (Cooney et al. 1989). These environmental factors may also alter selectively the resistance of micro-organisms in polluted aquatic systems (White et al. 1999). Biological and chemical degradation of TBT in marine and freshwater sediments has been reported to be slow

(Wuertz et al. 1991), as the half life of TBT in marine water has been found to be about a week, whereas in sediments it was about 2.5 years (Atireklap et al. 1997). This clearly indicates that sorption of TBT in the silty sediments strongly reduced the bioavailability of the biocide to microorganisms (Stronkhors et al. 1999). Because of the low water solubility, TBT preferably binds to suspended organic matter released from marine sediments. It is interesting to note that the extent of binding to bottom sediments varies with location, organic matter content and particle size (Laughlin et al. 1986).

Abiotic degradation processes have also been put forward as the possible pathways for removal of TBT from soil sediments and water columns, as the Sn-C bond could be broken by four different abiotic processes, viz. UV irradiation, chemical cleavage, gamma irradiation and thermal cleavage (Sheldon, 1975). Because gamma irradiation rarely occurs and the Sn-C bond is stable up to 200°C, gamma irradiation and thermal cleavage have a negligible effect on the environmental breakdown of TBT. Only the near UV spectrum (300-350 nm) is likely to cause direct photolysis of tributyltin, and due to low transmittance of UV light, this breakdown process is expected to occur only in the upper few centimetres of the water column (Clark et al. 1988).

Numerous studies undertaken on the fate of TBT have indicated that it degrades by stepwise debutylation mechanism to the less toxic dibutyltin (DBT) and monobutyltin (MBT) which have also been detected in the aquatic environment (Dowson, et al. 1993; Gadd, 2000). Maureen and Willingham (1996) have reported that TBT degradation process may be explained as a sequential loss of an alkyl group from TBT to form non-toxic inorganic tin ultimately in the following manner: R_3Sn^+ -> R_2Sn^{2+}-> RSn^{3+} -> Sn(IV). Complicating the issue of organotin persistence, is the possibility of other degradation pathways for tributyltin species including a number of possible redistribution reactions catalysed by environmental molecules such as amines, sulfides or other reactants. The possibility of environmental methylation of butyltins has been raised by a recent report of the presence of mixed butylmethyltin species in sediments, presumably arising by biological methylation of anthropogenic butyltin in the aquatic environment.

A few of the possible reactions of Sn-C includes:

(i) $2Bu_3Sn^+ \rightarrow Bu_2Sn^{2+} + Bu_4Sn$

(ii) $Bu_2Sn^{2+} + Bu_3Sn^+ \rightarrow BuSn^{3+} + Bu_4Sn$

(iii) $Bu_3Sn^+ + Me^- \rightarrow Bu_3MeSn$

At present the source of methyl carbanion is unknown, but it may be due to redistribution and biogenesis of methyltin species (Matthias et al. 1986a).

In aquatic ecosystem both pH and salinity determine organotin speciation and therefore it's reactivity (White. et al. 1999). Maximum toxicity to microorganism occurred at pH 6.5 for Bu_3SnCl, $BuSnCl_3$, Ph_3SnCl and at pH 5.0 the toxicity of Bu_2SnCl_2 was maximum . Toxicity decreased above and below these pH values. At an initial pH of 5.2, 0.3μM TBTC prolonged the lag phase of *Aureobasidium pullulans*, which was followed by an exponential phase of similar rate to the control culture. When the pH was reduced to 4.0, the same concentration resulted in complete growth inhibition (White et al. 1999). Speciation of various triorganotins (TOT) in aqueous solution has been investigated. In natural water, these compounds are present predominantly as neutral TOT-OH species or as TOT^+ cation depending on the pH value. At pH< 4 the predominant species is the cation Me_2Sn^+, while under environmental conditions (pH 6-8) the species mainly found is $Me_2\ Sn(OH)_2$.

The distribution of tributyltin species also depends on pH and salinity (Hoch, 2001). The aqueous solubility of four organotin compounds, such as tributyltin chloride (TBTC), Bis tributyltin oxide (TBTO), Triphenyltin chloride (TPTC) and bis (triphenyltin) oxide (TPTO) was determined at various salinity, pH and temperature (Inaba et al. 1995). The optimum degradation of TPT in sea water was at pH 7-8.5 (Yamaoka et al. 2001).

Blair et al. (1982) did not find evidence of TBT metabolism by tin resistant bacteria isolated from Chesapeak Bay, Canada although the organisms accumulated tin.

Later on TBT biodegradation was observed in sample collected during winter and incubated at winter temperature, but sample collected during summer, degraded TBT to di and monobutyltin. Exposure to incandescent light during incubation stimulated biodegradation, suggesting that photosynthetic bacteria may be involved in biodegradation. The four year TBT degradation study of Brest Naval harbour showed that formation of TBT degradation product i.e. DBT varies with change of water temperature (Chaumery et al. 2001). Yamaoka et al (2001) have reported that *Pseudomonas chlororaphis* can degrade triphenyltin in sea water with increasing temperature from 4°C to 37°C.

The solubility of organotin compounds decreased with increasing salinity (Inaba et al. 1995). External NaCl also influences organotin toxicity. Interactions between Bu_3SnCl and microbial biomass decreases with increasing salt concentration (Avery et al., 1993). Microbial uptake of Bu_3SnCl was reduced at salt concentration corresponding with that of sea water (~ 0.5M NaCl) (White et al. 1999). The presence of NaCl can alter toxicity in three ways (i) Na^+ can reduce interaction of the organotin with the cell surface by competing for binding sites or interacting with the compound itself (Cooney et al. 1989), (ii) the membrane – lipid composition may be altered, making the cells more resistant to membrane active compounds (Cooney et al. 1989). (iii) Cl^- can inhibit the solubility of tributyltin compounds by association with the cation to form covalent organotin chloride (Blunden et al. 1984). Clearly , the effects of organotin contamination varies in freshwater and marine environments and the level of Na^+ or Cl^- ions is considered in toxicity studies (White et al. 1999). The toxicity of butyltin was reduced by salinity levels approximately to sea water conditions which emphasizes the significance of environmental factors in determining organotin toxicity. A reduction of salinity in the medium also increased monobutyltin chloride toxicity possibly as a consequence of increased availability of the hydrated tributyltin cation i.e. $[Bu_3Sn(H_2O)_2]^+$ (Gadd, 2000).

Influence of media constituents on apparent organotin toxicity has been previously reviewed (Cooney and Wuertz, 1989). Jonas et al (1984) have reported that the medium composition would be expected to alter the physiochemical equilibrium of the metal species compared to natural water. The selectivity of the nutrient medium depends on the microbial community that can be cultured under the chosen nutrient medium. In other observation, Serine and hydroxyflavone enhanced inorganic tin toxicity, while gelatin and humic acids increased resistance of the estuarine microorganisms. Complexation of tin with the smaller molecules may facilitate transport across the membrane, while larger molecules may be excluded on a size basis. When $NaNO_3$ and KNO_3 were substituted for NaCl and KCl as the inorganic salts, a three fold increase in cell viability was reported (Hallas et al. 1982b). Inhibition of nitrification by four heterotrophic bacteria such as two *Bacillus sp*, an unidentified Gram-positive rod, and a *Pseudomonas sp.*, occurred at nanomolar levels of butyltin. In these organisms nitrification is independent of growth and is assumed to follow the pathway ($NH_4^+ \rightarrow NH_2OH \rightarrow NO_2^- \rightarrow NO_3^-$). For each of the four organisms, TBT inhibited growth, NH_4^+ uptake and accumulation of NH_2OH and NO_2^- . Effect on NH_4^+ uptake were deemed to be as a result of general toxicity and not due to direct inhibition of process steps. DBT inhibited NH_4^+ uptake and accumulation of NH_2OH and NO_2^- at the concentration, which did not inhibit cell growth (White et al. 1999). Though each organism has its own pattern of response to the three butyltin, suggesting that the organisms do not carry out nitrogen metabolism in identical ways and /or that they respond differently to these butyltins. It may account that TBT's interference with nitrification event is because of its disruption of cell function in prokaryotes and eukaryotes (Miller & Cooney 1994). Characterization of organotin as metal or organocompounds in the environment, and prediction of uptake mechanisms depends on speciation (White et al. 1999). Types of exchangeable cation, pH values, salinity and the mineralogical and chemical composition of the adsorption material are important parameters controlling the adsorption behaviour of organotin compounds (Hoch, 2001).

1.5.2 Biotic factors.

There are very few reports on biodegradation of TBT which is mediated by microorganisms viz. bacteria, fungi, cyanobacteria and green algae in terrestrial and aquatic environment (Sheldon, 1978; Barug and Vonk, 1980; Cooney, 1988; Gadd 1993, 2000). Barug (1981) has reported that Gram negative bacteria viz. *Pesudomonas aeruginosa, Alkaligenes faecalis* and fungi viz. *Tramatis versicolor* and *Chaetomium globosum* could degrade tributyltin oxide via dealkylation process. Pure cultures of wood rotting fungi, *Coniophora puteana* and *Coriolus versicolor* can also degrade this biocide to form di- and mono-butyltin derivatives (Henshaw et. al. 1978). It is interesting to note that some of *Pseudomonas* sp. have even been reported to bioaccumulate tributyltin up to 2% of its dry weight (Blair et al. 1982; Gadd, 2000). It has also been reported by Barug (1981) that several other Gram negative bacteria also possess capability to accumulate tributyltin oxide without its breakdown. The high lipid solubility of organotin ensures cell penetration and association with intracellular sites, while cell wall components also play an important role (Gadd, 2000). It is evident that the site of action of organotins may be both at the cytoplasmic membrane and intracellular level. Consequently, it is not known whether cell surface adsorption and accumulation within the cell, or both is a prerequisite for toxicity. TBT biosorption studies in fungi, cyanobacteria and microalgae indicate that cell surface binding alone occurred in these organisms, while studies on the effect of TBT on certain bacterial strains indicated that it can also interact with cytosolic enzymes (White et al. 1999). The elimination of such hydrophobic compounds is facilitated by their biotransformation to water soluble polar compounds. Thus metabolism of a compound generally reduces persistence, increases removal or elimination and results in a reduction of toxicity. Therefore, microbial degradation is probably the most predominant process for the breakdown of TBT in near shore waters with dibutyltin as the major degradation product (Page, 1989).

1.6 Heavy metal resistance in TBTC resistant bacteria.

Among the 19 heavy metals arsenic, cadmium, mercury and lead have no known essential biological function and are extremely toxic to microorganisms. Residual effect of most of these heavy metals on aquatic biota are long lasting and highly deleterious as they are not easily eliminated from these ecosystems by natural degradative processes. These metals tend to accumulate in sediments and move up in the aquatic food chain, ultimately reaching to human being, in whom they produce chronic and acute ailments (De, et al. 2003). At higher concentration heavy metal ions form unspecific complex compounds in the cell, which leads to toxic effects. Some heavy metal cations e.g. Hg^{+}, Cd^{+} form strong toxic complexes, which makes them too dangerous for any physiological function. Even physiologically important trace elements like Zn^{2+}, Ni^{2+} and especially Cu^{2+} are toxic at higher concentration (Nies, 1999). Depending on their concentration in sea water four classes of heavy metals can be easily differentiated as possible trace elements: frequent elements with concentration between 100 nM and 1μM (Fe, Zn, Mo), elements with concentrations between 10nM and 100nM (Ni, Cu, As, Mn, Sn ,U), rare elements (Co, Ce, Ag, Sb) and finally elements just below the 1nM level (Cd, Cr, W, Ga, Zr, Th, Hg, Pb) (Nies, 1999). Many laboratory strains as well as naturally occurring microorganisms have capability to degrade and assimilate a wide range of toxic organic compounds to simple harmless compounds such as water and carbon di oxide. Also living or dead microbial biomass can be used to bioremediate waste-water contaminated with toxic metals (Dubey and Rai,1987; Dubey and Rai,1990a; Dubey et al., 1993). Most cells solve this problem by using two types of uptake system for heavy metal ions, one is fast, non-specific and since it is used for a variety of substrates, constitutively expressed. The second type of uptake system has a high substrate specificity, is slower and often uses ATP hydrolysis for energy, sometimes in addition to the chemo-osmotic gradient, and these expensive uptake systems are only induced by the cells as and when needed such as, starvation or a special metabolic situation (Nies & Silver, 1995). Virtually all bio-molecule have high affinity to toxic metals and radionucleides. Several mechanisms

by which metals interact with microbial cell walls and envelopes are well established. However, some biomolecules function specifically to bind metals and are induced by their presence. These are metallothioneins or metalloproteins produced by microbes and have got possible involvement in metal detoxification and metal ions homeostasis (O'Halloran, 1993). These metalloproteins play structural and catalytic roles in gene expression. They exert metal responsive control of genes involved in respiration metabolism and metal specific homeostasis, such as iron uptake and storage, copper efflux and mercury detoxification. The metallo-thioneins are small cystine rich proteins that bind heavy metals. It is interesting to mention that metallo-thionines are present in all vertebrates, invertebrates, plants and even lower eukaryotes such as yeast and prokaryotes such as *Vibrio alginolyticus*, cyanobacteria and *Pseudomonas putida* (Higham et al. 1984; Turner and Robinson, 1995; Pazirandeh, et al. 1995, 1998). They play very important role in various biological / metabolic process, including toxic metal detoxification. Other molecules with significant metal binding abilities, like fungal melanins, may be overproduced as a result of exposure to sub-lethal concentration of heavy metals and interference with normal metabolism. The cell wall of bacteria also has several metal binding components which contribute to the biosorption process. The carboxyl group of the peptidoglycan is the main metal binding site in the cell walls of Gram positive bacteria, with phosphate groups contributing significantly in Gram negative micro-organisms (Gadd and White, 1993). For example, Organomercurials may be detoxified by microbial enzyme, organomercurial lyase , the resulting Hg^{2+} then being reduced to Hg^0 by mercuric reductase enzyme. Microbial dealkylation of organometallic compounds such as organotins can result in the formation of ionic species which could possibly be removed using biosorptive biomolecules like metalloproteins (Gadd and White, 1993).

Pain et al. 1998 have reported that most of the TBT resistant bacteria are also resistant to six heavy metals (Hg, Cd, Zn, Sn, Cu, Pb) which suggest that resistance to heavy metals may be associated with resistance to organotins. *Pseudomonas ambigua*

and *Pseudomonas fluorescens* are highly resistant to chromate which is plasmid mediated (Ohtake et al. 1987). Fukagawa et al. (1994) have reported 11 bacterial strain which are resistant to TBT and methyl mercury. Wuertz et al.(1991) have reported that the bacteria isolated from fresh water and estuarine environment are resistant to Zinc as well as TBT. Usually the TBTC tolerant strains also show cross tolerance to Me-Hg (Suzuki et al. 1992). It may be possible that genes conferring metal resistance are mostly plasmid borne whereas genes conferring organotin (TBTC) resistance are located on chromosomal genome (Fukagawa et al. 1993, Suzuki et al. 1994)

1.7 Antibiotic resistance of TBTC resistant bacteria.

Bacterial isolate obtained from nature possess multiple antibiotic resistance which is not surprising. It is very clear that multiple metal resistance (Hg, Zn, Cd, Pb, As etc) and antibiotics resistance (Penicillin, Ampicillin, Streptomycin, Chloromycin etc.) are wide spread among TBTC resistant micro-organisms isolated from both estuarine and freshwater sites. In this case both the antibiotic and heavy metal resistance may be plasmid mediated (Wuertz et al. 1991). It is known that bacterial isolates screened from toxic chemical waste more frequently contain plasmids and demonstrate resistance to antimicrobial agent. Bacteria isolated from Barceloneta Regional Treatment plant, Barceloneta, Puerto Rico are resistant to penicillin, erythromycin, nalidixic acid, ampicillin, m-cresol, quinine along with bis tributyltin oxide and also possess plasmid (Baya et al. 1986).

(Penicillin, Streptomycin, Spectinomycin)

All TBT resistant bacterial isolates were resistant to three antibiotics, such as *Flavobacterium* sp strain OWC-7 and *Pseudomonas* sp strain NOWC-1 were resistant to several antibiotics tested along with TBTC resistance. On the contrary, some of the bacterial strains such as *Bacillus sp.* strain MC-24, *Proteus sp* strain MC-26 and *Proteus sp* strain MC-29 do not show any resistance to any antibiotic though they are resistant to organotin (Wuertz et al. 1991).

1.8 Biosorption and Bioaccumulation of Tributyltin compounds.

The term "biosorption "is used to encompass uptake by whole biomass (living or dead) via physio-chemical mechanisms such as adsorption or ion exchange, where living biomass is used, metabolic uptake mechanism may also contribute to the process. As a result of metal toxicity , living cell may be inactivated , therefore most living cell system exploited to date have been used to decontaminate effluent containing metal at subtoxic concentrations (Gadd and White, 1993). A large proportion of organotin contamination are found to be associated with the clay fraction for particulate matter , indicating that adsorption and concentration onto this fraction is an important control mechanism concerning distribution and fate of organotins in the environment. The adsorption behaviour of organotin species is important in determining the transport process as well as their bioavailability which are more likely to be released in the sea water or directly ingested into the food chain component by bioaccumulation. The sequence of adsorption affinity of butyltin compounds on hydrous Fe oxide (MBT > TBT > DBT) suggested that MBT is most likely to remain in an estuarine sediment while DBT exclusively remains in solution. The strongly toxic TBT is likely to be present in the water column as well as in the sediment (Hoch, 2001).

The mechanisms responsible for biosorption includes (a) Van der Waal's forces wherein uncharged atoms or molecules are loosely bound in the matrix by electrostatic attraction, (b) Ionic bond between a metal cation, and an anionic reactive group of the biosorbent, (c) Crystallization of metals at the surface of the cell which is slower process but one that often produces higher rate enrichment, (d) electrostatic attraction or matrix entrapment, which can result in adsorption of precipitates on the cell envelopes (Voleski, 1994). As organic compounds, organotins will also exhibit lipophilic interaction with cellular membranes. Uptake of lipophlic organic metal complexes by membrane diffusion mechanisms may occur in addition to or in place of the facilitated uptake of the metal ion. As hydrophobic, nonpolar species, organometals may dissolve into the

membrane and enter the cytosol. Passive uptake of uncharged lipophilic chloride complexes is the principal accumulation route of both methyl-mercury and inorganic mercury in phytoplankton. Organotin may act as cationic metal ions, i.e., having a positive ionic charge and as organic compounds in solution. Cell surfaces are predominantly anionic because of the presence of ionized group such as carboxylate, hydroxyl and phosphate in the cell wall polymers. Such groups act as ligands, binding metals to the cell surface rather than penetrating them (Avery et al. 1993; White et al. 1999). Biosorption studies on fungus , cyanobacteria, and microalgae indicated that cell surface binding alone occurred in these organisms, while studies on the effects of TBT on certain microbial enzyme indicated that in some bacteria TBT interacts with cytosolic enzymes also (White et al. 1999).

Biosorption of triorganotin compounds by cyanobacteria, *Synechocystis* PCC 6803 and *Plectonema boryanum* and the microalga, *Chlorella emersonii* increased with molecular mass of the organotins, the order being triphenyltin > tributyltin > tripropyltin > trimethyltin > triethyltin. Cyanobacterial tributyltin biosorption was complete in 5min with no subsequent accumulation. In contrast a second phase of uptake in *C. emersonii* resulted in an approximate 2.4 fold increase in cellular TBTC between 5min and 2hr. Comparatively, over 50% of the total TBTC biosorption by *Aureobasidium pullulans* occurred almost instantaneously (Avery et al. 1993). Furthermore, accumulation of TBTC by a *Pseudomonas sp* isolate to 2% of the dry cell weight, was not influenced by the metabolic activity of the cells and was attributed to adsorption at the cells surface (Blair et al. 1982).

Greater biosorptive capacity of the pigmented strain was attributed to the presence of melanin. Previous studies which attributed to inorganic metal indicates that melanin-pigmented chlamydospores accumulate greater amounts of metal than hyaline cell types (White et al. 1999).Characterization of organotin as metal or organometalic-compounds in the environment, and prediction of uptake mechanisms depends on speciation (White et al. 1999). Types of exchangeable cations, pH values, salinity and the

mineralogical and chemical composition of the adsorption material are important parameters controlling the adsorption behaviour of organotin compounds (Hoch. 2001). Salinity has little effect on the sorption of TBT on the sediments while the partitioning coefficient between pore water and surface water decreased with increasing salinity. The pH has significant effect on sorption on the sediments. It indicated that TBT sorption on the sediments followed the process of TBT partitioning into pore water after TBT rapidly disappeared from the overlaying water (Ma et al. 2000).

1.9 Biochemical basis of tributyltin resistance in bacteria.

1.9.1 Effect of tributyltin on exopolymer production.

Microbial exopolysaccharides have gained wide commercial importance because they offer advantages over plant and sea weed derived marine polysaccharides (Ashtaputre et al. 1995a). Microorganisms offer more attractive alternative as they can be grown under controlled condition and they greatly extend the range of available polymers because of their unique properties (Ashtaputre et al., 1995b). Bacteria have devised complex regulatory circuits controlling exopolymer synthesis at the level of gene expression (Vandevivre et al. 1993). A wide range of bacteria from clinical and environmental habitat, is known to produce complex and diverse exopolysaccharides (EPS), occurring as capsular polysaccharides intimately associated with the cell surface or as slime polysaccharides, loosely associated with the cell. These are distinguished by the degree of cell association following centrifugation (Royan, et al. 1999). Microbes whose exopolysaccharides have been commercially exploited include *Leuconostoc mesenteroides, Xanthomonas campestris, Pseudomonas sp.*, *Azotobactor* sp. and *Sphingomonas paucimobilis* (Ashtaputre, et al. 1995b). In case of *Rhodococcus rhodochrous* S-2, the addition of EPS promoted the emulsification of aromatic fraction of sea water, the growth of bacteria and degradation of the aromatic fraction. This fact suggests that EPS produced by *Rhodococcus rhodochrous* S-2 could be useful for the biodegradation of spilled oil in marine environments, and especially for the

bioremediation of polyaromatic hydrocarbons that remain in the environment even after a traditional bioremediation treatment (Iwabuchi, et al. 2002). Many bacteria produce EPS under various stresses viz. metals, toxins, nutrient limitation etc., thus providing a mechanism to protect cells from toxic effects. EPS buffers cells quickly against the toxic and environmental changes like pH, salinity or nutrient regimes and thus protect the cells against toxic metals and other toxins. Besides, it creates a microenvironment around the organisms allowing it to operate, metabolize and reproduce more efficiently. It also helps in the transfer of heavy metals and organo-metallic compounds from water column and sediments, and serves as an important energy source for protozoans. As the exopolymer is surface active molecule, it possesses high binding affinities for many dissolved compounds present in sea water. Comparatively few studies have directly examined the binding of organic compounds to exopolymers. It also binds with a wide variety of metals such as Pb, Sr, Zn, Cd, Co, Cu, Mn, Mg, Fe, Ag and Ni. Exopolymer binding processes can be important in the downward transport of metals in ocean environment (Decho, 1990). It has been reported that *Xanthomonas campestris, Sphingomonas* sp. and *Escherichia coli* of gram negative genera secretes exopolysaccharides, acquired resistance to antibiotics bacitracin by stopping synthesis of exopolysaccharides (Pollock et al. 1994). The most of the work is focused on EPS produced by metal binding are those that from capsule or slime layers. The majority of these exopolymers are composed of polysaccharide, glycoproteins and lipopolysaccharides, which may be associated with proteins. Generally, a correlation exists between high anionic charges of EPS and their metal complexing capacity (Gadd and White 1993). Microbial cells can attach to solid surface forming biofilms with the help of EPS where it sequesters and localizes nutrients, hence increases biofouling of pipeline, boat and ships (Wilkinson, 1984).

1.9.2 Effect of tributyltin on surfactant activity of exopolymer.

Microbially produced exopolymer behaving as surfactants, have been isolated and used for different industrial applications. The distinct advantage of biosurfactants over whole cells or exopolymers, is their small size, generally biosurfactant molecular weights are less than 1500 KDa. A second advantage is that biosurfactants have a wide variety of chemical structure that may show different metal selectivities and thus metal removal efficiencies and pollutants from different ecosystem (Miller, 1995). Marine microorganisms are known to produce exopolymer which are biosurfactant and useful for cleaning the oil spills (Chandramohan, 1997 and Bertrand et al. 1993) and other xenobiotics which are present as contaminants in marine environment. Although much is known about biosurfactant produced by organisms from terrestrial and fresh water environment, the one produced by organisms from marine ecosystem is less investigated.

Because of insufficient knowledge about *in situ* bioavailablity and biodegradation of low solubility hydrocarbon pollutants, it is difficult to predict clean up till the end points. Reported mechanisms for bacterial metabolism of sparingly soluble hydrocarbons are : (1) dissolution and diffusion of dissolved hydrocarbon to cells with uptake via active or passive transmembrane transport, (2) Invagination of hydrocarbon non-aqueous phase liquid (NAPL) into cells with subsequent intracellular metabolism of the hydrocarbon inclusion, (3) Bacterial production of surface active compounds such as surfactant and emulsifiers (bioemulsifiers) that increases the local pseudosolubility of hydrocarbons and thus improves the mass transfer to biodegrading bacteria. Bacteria are known to produced surface active compounds, such as rhamnolipid biosurfactant and the Plasminogen Activator bioemulsifying protein produced by *Pseudomonas aeruginosa,* can improve bioavailability and biodegradation in liquid culture. Biosurfactant reduces the interfacial tension in two phase NAPL-water mixtures, while being non-toxic and fully biodegradable. Biosurfactant produced by *Pseudomonas aeruginosa* improves biodegradation of various hydrocarbons (Holden, et al. 2002). It has been observed that

specific surfactants are known to be necessary for the efficient uptake of long chain alkanes by various *Pseudomonas* and *Candida* species (Schmid, et al. 1998).

1.9.3 Effect of tributyltin on pigment synthesis

Pigmented bacteria are predominant in areas subjected to stress conditions such as intense sunlight, nutrient limitation, high concentration of organic pollutants, heavy metals, drugs and high salt concentration (Seligman, et al. 1986). These bacteria produce different types of pigment for example *Vibrio* sp. produces zeaxanthin and pheomelanin while *Pseudomonas* sp. also produces zeaxanthin besides phenazines (Ceiglar 1964,Weiner 1997).The yellow compound pyoverdin isolated from the bacteria *Pseudomonas chlororaphis* has significant role in triphenyltin (TPT) degradation. The degradation of TPT by pyoverdin decreased with increase of phosphorus at 0-35 mg/l and Fe-EDTA at 0-2 mg/l. The degradation was unaffected by manganese and zinc (Yamaoka et al. 2002). It is interesting to mention that fractions F–I and F-Ia of pyoverdin which were produced by *Pseudomonas chlororaphis* can degrade triphenyltin to monophenyltin via diphenyltin (Inoue et al. 2003). It has also been reported that the process of pigment formation converts potentially toxic metabolite into innocuous end products or pigments (Williams, 1973). There are reports that pigmented bacteria show heavy metal resistance possibly encoded on the same or co-transmissible plasmids along with antibiotic resistance therefore they show a higher survival rate in harsh environments and are capable of degrading a wide range of pollutants (Higham et al. 1984).

1.9.4 Protein profile for tributyltin resistant bacteria

In general, the toxicity of triorganotin compounds is believed to be due to their ability to bind certain proteins, and the results obtained so far permit to propose that cysteine and histidine residues are involved which bind with organotin compounds, indicating that a highly specific binding site is required to bind with the compound (Santroni et al. 1997). Among the TBTC resistant bacteria, *Vibrio* M1 isolated from

natural marine environment showed interesting properties when grown with TBTC. It was found that in the middle phase of growth, synthesis of two polypeptides was induced (Suzuki et al. 1994). Protein profiles of *Vibrio* sp. Strain M1 clearly showed that biosynthesis of 30 KDa and 12 KDa polypeptides increased dramatically when the strain was cultured in the medium supplemented with 125μM TBTC, although the function of the polypeptides are not known definitely. Preliminarily studies have shown that both polypeptides were detected in the 0.1% CHAPS (3-[(3-cholamidopropyl) dimethylammonio]-1-propane sulfonate) (detergent) treated fraction, but not in the cytosol indicating that they are transmembrane bound proteins. These polypeptides might have similar function in organotin resistance as do 56 KDa and 16 KDa polypeptides of Hg-resistant bacteria (Fukagawa et al. 1992). It was also known that *Pseudomonas aeruginosa* produces a copper binding protein called azurin (Rosato et al. 1991) and also a metallo-enzyme (protein) which initiates the metabolism of herbicides atrazine (Seffernick et al. 2002). Kitamura and Suzuki (2003) have reported that SecA protein (metallo-protein) of 116KDa may be involved in TBT resistance in *Pseudoalteromonas* sp. strain M1 (unpublished data).

1.10 Genetic basis of organotin resistance in bacteria

Genetic studies on TBT resistant and degrading bacterial strains from terrestrial and aquatic environment are extremely limited with very few reports demonstrating presence of plasmids but no correlation with TBT resistance (Singh and Singh, 1984; Singh, 1987; Fukagawa and Suzuki, 1993; Suzuki et al 1994; Miller et al. 1995). In most of the cases, it has been demonstrated that the resistance conferring genes are located on chromosomal genome (Suzuki et al. 1994; Suzuki and Fukagawa, 1995). Fukagawa and Suzuki (1993) have reported for the first time that the presence of genes conferring TBT resistance in *Alteromonas* sp. strain M1. They have successfully isolated, cloned and sequenced the gene, which seems to be involved in efflux of TBT employing a membrane bound TBTC induced transport protein, possessing 108 amino acid residues

encoded by an ORF of 324 nucleotides. This membrane protein has 48.5% of hydrophobic residues and shows more homology with transglycosylases of *E. coli* and other bacterial strains (Fukagawa and Suzuki, 1993). Therefore, this membrane protein has been predicted to be the most prominent resistance mechanism in this marine bacterial strain. Suzuki et al (1994) have further confirmed the taxonomic position of this strain by 16SrRNA sequencing and genomic sizing by Pulse field gel electrophoresis (PFGE) using contour clamped homogeneous electric field (CHEF) technique. These studies have revealed that *Alteromonas sp.* - M1 possesses a genome of 2,240 Kb. It is interesting to note that this strain is devoid of any plasmid suggesting the exclusive presence of TBT resistance genes on chromosomal genome (Fukagawa and Suzuki, 1993).

The short-term test that have been used extensively to identify mutagen and potential carcinogens are increasingly being used to identify antimutagens and potential carcinogens. Three model mutagens, N-methyl-N'nitro- N- nitrosoguanidine (MNNG) , aflatoxin B1 and benzo(a)pyrene were selected from data surveyed in the published literature (Waters, et al. 1990).Simple alkylating agent are recognized as a class of potent mutagens and carcinogens which act through covalent modification of cellular DNA resulting in various types of primary lesions. *E. coli* strains deficient in *ogt* gene, responsible for methyltransferase (MT), are very sensitive to mutagenesis by alkylating agent, such as propylmethanesulfonate, ethylnitrosourea . *Salmonella typhimurium,* a closely related species of *E. coli,* also has two kind of MTs for removing alkylated lesions from DNA in a self protecting process (Yamada et al. 1997). Alkylating agents are involved not only in induced mutagenesis but also in spontaneous mutagenesis and it is possible that some of the endogenous mutagens that contribute to spontaneous mutation are methylating agent (Yamada et al. 1997). N-nitroso compounds are environmental mutagens that are present in the air, water, soil etc., or can be formed by nitrosation of various nitrostable compounds (Gichner et al. 1988). Alkylating agent,

such as MNNG, cause mutations and cell death in both human and bacterial cell. These effects are related to formation of various alkylated bases in DNA (Gichner et al. 1988).

Plasminogen activator (PA) is a specific serine protease closely associated with cellular transformation, neoplasia, tumor promotion and metastasis. It has been reported that MNNG induces the production of PA in Mer^- cell, but not the Mer^+ cells. This observation suggests that PA induction in alkylation repair deficient cell is caused by unrepaired DNA damage and that it may represent eukaryotic SOS-like repair (Lonarek, et al. 1998). DNA damaging agents produce a variety of DNA adducts that have a variable potency with respect to cytotoxicity and mutagenicity. Accordingly, O^6 –Meg is usually considered to be the most efficient cyto-toxic lesion produced by MNNG, even though it represents less than 10% of the total DNA adduct (Lonarek et al. 1998).

Most chemical mutagens and carcinogens produce multiple DNA adducts and lesions. It is difficult to identify the potency level of the chemicals responsible for the specific damage caused by mutagenicity and carcinogenicity. Techniques for site directed mutagenesis have made it possible to place certain specific nucleotide bases at predetermined sites on the DNA and to measure the frequency and type of mutation produced by various agents at these sites (Ogawa et al. 1997). The mechanism by which a number of diverse types of DNA damages repaired are closely similar in *E. coli* and mammalian cells. This implies that the biological consequences (mutation or cell death) of particular unrepaired lesion will be similar in both cell type (Lonarek et al. 1998).The NTG mutagenicity study on *Sphingomonas sp.* which can degrade dibenzofuran or dibenzo-p-dioxin had shown stable mutants which can change their pathway of degradation (Bunz et al. 1999).

Table 1.2 Marked Tributyltin affected areas in the world

Sr. No.	Location	Yr. of detection	Amt. of TBT	Reference
1	Ontario lakes & rivers, Canada	1982	N.D.	Maguire, 1982
2	Vancouver Harbour, Canada	1982-85	11,000 ng/g dry wt.	Maguire et al.1986
3	Arcachan Bay, France	1982-85	N.D.	Alzieu, 1986
4	South west England,	1986	N.D.	Cleary et al. 1987
5	San Diego Bay, USA	1986	N.D.	Seligman et al. 1986
6	San Diego Bay, USA	1986	0.005 mg/lt	Valkirs et al. 1986
7	Poole Harbour, USA	1985-87	520 ng/g dry wt.	Langston et al. 1987
8	Atlantic Coastal water	1986-87	N.D.	Alzieu et al. 1989
9	Boston Harbour, USA	1988	518 ng/gm dry wt.	Makker et al. 1989
10	Mediterranian Sea (French, Italy, Turkey, Egypt coast)	1988	N.D.	Gabriellides et al.1990
11	East Gulf & Pacific coast of USA	1986-91	770 ng/gm dry wt.	Krone et al. 1996
12	Mariana, HongKong	1988-89	1160 ng/gm dry wt.	Lau, 1991
13	Auckland, New Zealand	1990	N.D.	de Mora et al. 1995
14	Boston Harbour, USA	1990	N.D.	Wuertz et al. 1991
15	Funck Bay, Hakkaido, Japan	1990	N.D.	Fukagawa et al. 1992
16	Hakodate Bay, Hakkaido, Japan	1991	N.D.	Fukagawa et al. 1994
17	Bohemia River, Chesapeak Bay USA	1991	590 ng/g dry wt.	McGee et al. 1995
18	Sewage and sludge in five cities in Canada	1990-91	N.D.	Chau et al. 1992
19	Cadiz in SW-Spain	1992	N.D.	Gomez Ariza et al. 1999
20	Portland and Booth Bay Harbour, USA	1990-92	12400 ng/gm dry wt.	Page et al. 1996
21	Mariana, HongKong	1994	3200ng/gm dry wt.	Ko et al. 1995
22	Kanpur- Unnao Ind. Region, India	1995	32.6 ng Sn/lt	Ansari et al. 1998
23	Coast of Thailand	1995	4500 ng/gm dry wt.	Atirekalp et al. 1997
24	Suva Peninsula, Fiji	1996	N.D.	Davis et al. 1999
25	Strait of Malacca & Tokyo Bay	1993-96	N.D.	Hashimoto et al. 1998
26	Killybegs Harbour, Ireland	1997	N.D.	Minchin et al. 1997
27	Harbours of Western Mediteranian Sea	2000	244 ng/gm dry wt.	Diez et al. 2000
28	Coastal environment of Chiana	2001	N.D.	Gui-bin et al. 2001
29	Alang Ship Building, India	2001	N.D.	Kanthak et al. 2001
30	Shipping Strait between Denmark and Sweden	2003	19 ng/gm dry wt.	Strand et al. 2003

N.D = Not Detected

Table 1.5 Tributyltin resistant bacteria

Bacterial strains	Tolerance limit	Reference
Aeromonas sp.	2 μg/ ml	Roy et al 1988.
Staphylococcus aureus	2 μg/ ml	Singh 1987
Bacillus subtilis	2 μg/ ml	Singh 1987
Streptococcus lactis	5 μg/ ml	Singh 1987
Escherichia coli	500 μg/ ml	Singh 1987
Pseudomonas fluorescens	100 μg/ ml	Singh 1987
Proteus mirabilis	>1000 μg/ ml	Singh 1987
Pseudomonas aeruginosa	2.5 ppm	Suzuki et al. 1992
Enterobacter sp.	37.1μM	Wuertz et al. 1991
Citrobacter sp.	> 84μM	Wuertz et al. 1991
Vibrio sp.	125 μM	Fukagawa et al 1992
Bacillus sp.	4.2 μM	Wuertz et al 1991
Alkaligenes sp.	0.6 μM	Wuertz et al 1991
Flavobacterium sp.	6.3 μM	Wuertz et al 1991
Serratia sp.	> 84μM	Wuertz et al 1991
Alteromonas sp.	100 μM	Suzuki et al. 1995
Leginonella pneumophila	500 – 1000 μg/ lit	Jonas et al. 1984
Pseudomonas fluorescens	> 500 mg/lit	Barug. 1981
P. putida	> 500 mg/lit	Barug. 1981
P. aeruginosa	> 500 mg/lit	Barug. 1981
Alcaligenes faecalis	2 mg/lit	Barug. 1981
Escherichia coli	> 500 mg/lit	Barug. 1981
Bacillus subtilis	5 mg/lit	Barug. 1981
Nocardia paraffinae	2 mg/lit	Barug. 1981
N. rubropertincta	5 mg/lit	Barug. 1981
Arthrobacter globiformis	2 mg/lit	Barug. 1981
Micobacterium phlei	0.5 mg/lit	Barug. 1981

Table 1.6 Biodegradation study of Tributyltin

Environment	TBT concentration	Rate (half life $t^{1/2}$)	Reference
Harbour water	0.5 μg/L	50-75 days	Seligman et al.1986
Fresh water	20 μg/L	25 days	Clark et al.1988
Marine sediments	21 ng TBT-Sn g-1	1.3-4.4 years	de Mora et al 1995
Sea water and esturine	2.5 mg/ml	34 weeks	Clark et al.1988
Sea water and esturine	1 μg/L	1 week	Clark et al.1988
sea water and esturine	2.5 mg/ml	16 weeks	Clark et al.1988

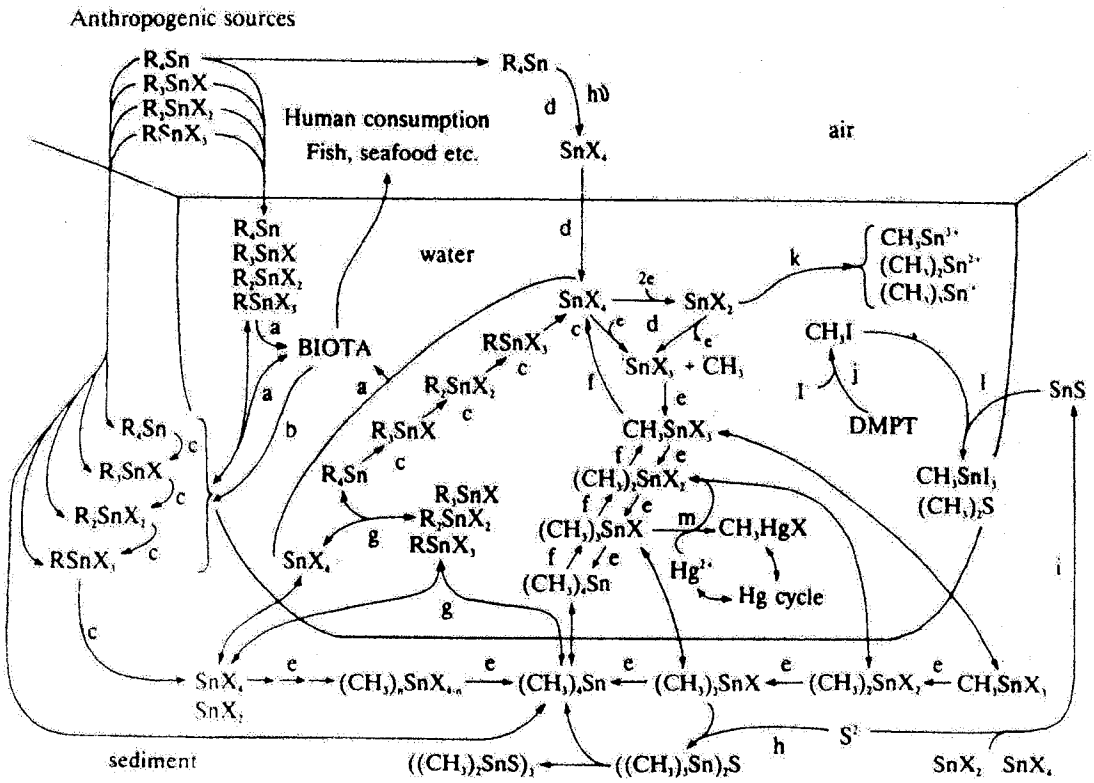


Fig. 1.1 A model for the biogeochemical cycling of organotins. Tributyltin compounds are included in the nomenclature R3SnX. Little is known of the influence of the anionic radical (X) on breakdown. The main reactions detailed are : (a) bioaccumulation; (b)deposition or release from biota on death or other process; (c) biotic and abiotic degradation; (d) photolytic degradation and resultant free radical production; (e) biomethylation; (f) demethylation; (g) disproportionation reactions; (h) sulphide-mediated disproportionation reactions; (i) SnS formation; (j) formation of methyl iodide by reactionof dimethyl -propiothetin (DMPT) with aqueous iodide; (k) Ch3I methylation of SnX2; and (m) transmethylation reactions between organotins and mercury.

Source :Gadd, 2000

AIMS AND OBJECTIVES OF PRESENT WORK

Many marine bacterial strains have an inherent capability to degrade the organotins (TBTs) that enter into the marine environment in the form of insecticides, fungicides and antifouling paints. Natural degradation of these biocides in ambient environment may take several years, therefore, it is important to consider ways or strategies which can accelerate the organotin degradation process (Gadd, 1993; Alzieu, 2000). There are very few reports on biological organotin degradation, which are exclusively restricted to only laboratory scale experiments (Barug, 1981; Gadd, 1993). In spite of serious environmental threat of organotin biocides to non- target organisms, still no report is available on *in situ* bioremediation of these biocides involving living organisms (Cooney, 1988).

Little is known about the incidence of microbial organotin resistance, and the resistance mechanism with which microorganisms tolerate high levels of organotins (Wuertz et al., 1991; Gadd.1993, 2000). Earlier investigations have revealed that the outer cellular membrane is the primary target site for TBT binding due to its lipophilic characteristics. Ultimate cellular ATP depletion could be induced in living organisms or delipidation of anionic phospholipids or by formation of tributyl, stannyl and peroxy radicals, resulting in lipid peroxidation (Gray et al. 1987). There are several TBTC resistant bacterial strains such as *Escherichia coli, Pseudomonas fluorescens, P. aeruginosa, Proteus mirabilis, Serratia marcescens* as Gram negative and S*taphylococcus aureus, S. epidermidis, Bacillus subtilis* and *Mycobacterium phlei* as Gram positive (Singh, 1987), but the biochemical and genetic basis of resistance in these bacterial isolates is not yet understood.

Organotin degradation involves sequential removal of organic groups attached to tin atom, which generally results in a toxicity reduction (Cooney, 1988). Although the degradation of organotins has been shown to be mediated by several microorganisms including bacteria, fungi, algae and yeast, information is still severely limited in relation to

biochemical and genetic mechanism of degradation (Belliveau et al 1987; Cooney 1988; Gadd, 1993). It is interesting to note that biotic processes have been demonstrated to be the most significant mechanisms for tributyltin degradation in fresh water, marine and estuarine environment (Dowson et al. 1993). Genetic studies on TBT resistant and degrading bacterial strains from aquatic environments are extremely limited with very few reports demonstrating the presence of plasmids but no correlation with TBT resistance (Singh, 1987; Fukagawa and Suzuki, 1993; Fukagawa et al. 1994; Suzuki and Fukagawa, 1995).

The paucity of information on environmental fate of this extensively used biocide and antifoulant present in marine environment prompted us to explore the role of TBT tolerant microorganisms (especially bacteria) in biodegradation process as they are naturally enriched in organotin contaminated marine environment (Fukagawa et al. 1994). These studies on TBTC tolerant bacterial strains of west coast of India would enable us to understand mechanisms of degradation and employ natural as well as genetically modified bacterial strains in the task of organotin bioremediation of marine contaminated sites of India.

The main objectives of the study:

1. Screening and identification of TBTC tolerant and degrading marine bacterial strains in addition to morphological and physiological characterization of bacterial isolates with reference to viable count, colony characteristics, growth behaviour, environmental optimas (temperature, pH, salinity), TBTC tolerance, antibiotic resistance and cross resistance to selected heavy metals viz. Cd, Hg, Zn.
2. Biochemical characterization of TBTC resistant and degrading bacterial isolates with respect to TBTC biosorption , toxicity regulation by thiol and chelating agent , TBTC degradation profile (TLC analysis of degradation product with reference to growth phase and various growth media), role of selected carbon and nitrogen sources viz. succinate , glycerol, glucose, NH_4NO_3 , KNO_3 and NH_4Cl on growth.
3. Physicochemical characterization and identification of TBTC degradation product using IR, NMR spectroscopy and Gas Chromatography.
4. Role of TBTC in exopolymer and pigment production and its biological characterization with reference to biosurfactant activity of EPS.
5. Protein profile (SDS-PAGE) of TBTC tolerant *Pseudomonas aeruginosa* strain USS25 to reveal TBTC induced specific polypeptides.
6. Molecular biological and genetic characterization of TBTC tolerant bacterium, *Pseudomonas aeruginosa* strain USS25, with reference to plasmid profile, confirmation of location of TBTC resistance and degradation genes on plasmid or chromosomal genome, NTG mutagenesis to screen TBTC-hyper tolerant mutants and their characterization with reference to growth behaviour, TBTC tolerance limit and TBTC degradation capability.

Chapter II

MATERIALS AND METHODS

2.1 Collection of environmental samples

Marine surface water samples were collected from different sites of Bombay High Oil field area, 150 km of west from west coast of India during a cruise organized by Oil and Natural Gas Commission, Goa, India in October 1999. Water samples from surface level were also collected from Goa Shipyard, Goa, India, in some particular location like painting yard, vicinity of ships and other ship building areas around the Goa Shipyard.

Water samples, from both the sampling sites were collected by Niskin sampler (Tamburini et al. 2002; Sardessai et al. 2001) in sterile polycarbonate bottles and kept at 4°C after collection. All the samples were used within seven days of collection for physicochemical and bacteriological analysis. The water sample bottles were mechanically shaken prior to use and kept for 10 min to allow the heavy particles to settle down. The approximate volume of upper layer of water was taken for physicochemical and bacteriological analysis.

2.2 Physicochemical analysis of samples

The physicochemical parameters of different water samples such as pH, temperature, salinity, nitrites, nitrates and phosphates were determined as per the Standard protocols.

2.2.1 Salinity

This method is based on titration of halide ions present in seawater to a standard silver nitrate solution using potassium chromate as an indicator (Grasshoff, 1983).

Exactly 10 ml of seawater sample was pipetted into a 250 ml conical flask and about 20 ml of distilled water was added to it followed by six drops of potassium chromate indicator solution (10% stock solution) (Annexure-B4). The content was mixed well and titrated against silver nitrate solution. When silver nitrate solution is run down

from a burette a white precipitate begins to appear and the solution turns yellow. The first colour change starts when solution becomes dull red due to excess addition of silver nitrate which immediately disappears on shaking. From this point onwards, silver nitrate solution was added drop wise every time stirring well and observing the colour of the solution. This procedure was repeated until the end point is reached i.e. when the entire solution becomes dull red / dirty orange in colour, which persists for at least 30 Sec, by the addition of one drop of silver nitrate solution. The burette reading i.e. the volume of silver nitrate solution used is determined and salinity was calculated using Harvey's table.

2.2.2 Nitrite content (NO_2)

Determination of nitrite in sea water is based on the reaction of NO_2 with an aromatic amine leading to the formation of diazoamine compound which couples with a second aromatic amine to form an azo dye (Koroleff, 1983).

10ml of water sample was taken into 50 ml graduated tubes and 10 ml of distilled water was added followed by 1ml of Sulphanilamine and 1ml of N-naphthyl ethylene diamine-di-hydrochloride (Annexure- B.1). The volume of the solution was made up to 50ml with distilled water and the absorbance was measured after 20min using Spectrophotometer (SHIMADZU, UV-1601) at the wavelength of 543 nm. The concentration of nitrite is determined using standard curve of sodium nitrite which was prepared by plotting concentration of NO_2 vs O.D. at 543nm (Annexure- H.b).

2.2.3 Nitrate content (NO_3)

It is based on reduction of nitrate to nitrite which is then determined via the formation of an azo dye. The reduction of nitrate is performed in the heterogeneous system using cadmium granules (Koroleff, 1983).

10ml of sample was taken in a 125ml polythene bottle and the volume was made up to 100ml with distilled water. 2ml of concentrated ammonium chloride solution

(Annexure- B.2) was added to this solution, before passing it through the cadmium column. About 40ml of the solution was eluted through the column and concentration of nitrate was determined indirectly from nitrite.

2.2.4 Phosphate content (Koroleff, 1983)

Phosphate dissolved in water when treated with acidic ammonium molybdate gives phospho-molybdic complex, which can be reduced with ascorbic acid to give phospho-molybdenum blue. The absorbance of the solution was measured at 880nm.
10ml of sample was taken in a 50 ml graduated tube and distilled water was added to make the final volume upto 50ml. This solution was treated with 1ml of mixed reagent (Annexure-B.3) and mixed thoroughly. 1ml of ascorbic acid was added to this and shaken well. After 15 min the absorbance of the solution was measured at 880nm using spectrophotometer. Standard curve of phophate was used to estimate the concentration of phosphate in water samples (Annexure –H.a).

2.3 Determination of viable count and screening of bacterial isolates

Water samples were shaken for 10 min before use and 0.1 ml of each sample was surface spread on Nutrient Agar (Appendix- A.2), Nutrient Agar + 0.1mM TBTC and Mineral Salts Medium agar plates (MSM) (Subert, 1960; Mahtani, and Mavinkurve, 1979) (Appendix- A.1) with 0.1mM, 0.2mM, 0.3mM and 0.4mM TBTC respectively. The respective plates were incubated at 28°C for 24hrs, 48 hrs and for one week and total viable count was determined as colony forming unit (cfu)/ ml. The bacterial isolates which had grown initially in MSM agar with 0.4mM TBTC were sub-cultured continuously on MSM agar plate at the same concentration. The bacterial isolates, which showed consistent growth on that medium, were screened for further study.

2.4 Maintenance of TBTC resistant bacterial isolate

The bacterial isolates, which were growing on Mineral Salt Medium (MSM) with 0.4mM of TBTC, were sub-cultured in MSM agar with increasing concentration of TBTC. All the bacterial isolates were checked for their growth MSM agar up to 6mM TBTC. Though the viable cells were observed only up to 2mM TBTC, the resistant isolates, which were growing well in MSM spiked with 2mM TBTC after repeated subculture, were selected for further studies. These TBTC resistant isolates were then maintained in MSM + 2mM TBTC at 28°C. The strains were designated as 25W, 25B, 3(4sub), 9(3A), 5Y2 for experimental convenience.

2.5 Identification of TBTC resistant bacterial isolate

Biochemical tests for all five strains i.e. 25W, 25B, 3(4sub), 9(3A), 5Y2 were done according to Cruickshank et al. (1972) and identified as per *Bergey's Manual of Systemic Bacteriology* (Krieg, and Holt 1984). Two identified strain i.e. *Pseudomonas mendocina* P_2d and *Sphingomonas paucimobilis* were kept as standard with present five strains. Then the strain was tentatively identified on the basis of percentage similarity by plotting them in phenogram (Goodfellow.,1977). As a confirmatory test for *Pseudomonas aeruginosa*, five strains were spot inoculated in Nutrient Agar with 1% dettol and growth was observed after 24 hrs (Ferrao and Mavinkurve, 1977, Mavinkurve and Ferrao, 1979). Strain 25W was ~~further~~ exactly identified as Pseudomonas aeruginosa sequencing by 16srRNA generated by PCR amplification following primers. using fD1 5'AGAGTTTGATCCTGGCTCAG-3' & Rp2fD1 5'ACGGCTAC-CTTGTTACGACTT-3' and homology search done by Prof. Satoru Suzuki and Dr Kitamura from C.M.E.S, Ehime University, Matsuyama, Japan in 2003.

2.6 Determination of environmental optimas for the growth of TBTC resistant bacterial isolate

2.6.1 pH

In order to determine pH optima of the bacterial strains, overnight grown cultures were inoculated to a final concentration of 5% in MSM containing 2mM TBTC. These flasks were separately adjusted to three different pH values i.e. 5.0, 7.0 and 9.0 before inoculation and were incubated on an incubator-shaker for 48 hrs at 28°C. After incubation period, the culture broth was centrifuged at 8000 rpm. The cell pellet was collected and re-suspended in 1ml of saline. Growth in terms of total protein/ml was determined using Lowry's method (Lowry, et al.1951) and result was recorded as a graph of growth (total protein/ml) v/s pH values.

2.6.2 Temperature

For determination of optimum temperature for growth for potential isolates, overnight grown cultures were inoculated to a final concentration of 5% in MSM containing 2mM TBTC and incubated on an incubator-shaker for 48 hrs at three different temperatures i.e. 28°C, 37°C and 42°C. After incubation period, the culture broth was centrifuged at 8000 rpm. The cell pellet was collected and re-suspended in 1ml of saline. Growth was determined in terms of total protein content/ml using Lowry's method (Lowry et al. 1951).

2.6.3 Salinity

All the five overnight grown isolates were inoculated in MSM+2mM of TBTC with increasing concentration of NaCl (0.5%, 1.0%, 1.5%, 2.0%, 2.5% and 3.0%) and incubated in an incubator-shaker for 48 hrs at 28°C. After incubation period, the culture broth was centrifuged at 8000 rpm. The cell pellet was collected and re-suspended in 1ml of saline. Growth was determined in terms of total protein content /ml vs NaCl concentration.

2.7 Determination of heavy metal tolerance limit of bacterial isolate

Three heavy metals i.e. Hg, Cd and Zn were chosen for determining the metal tolerance limit. Metal tolerance of the five strains were determined by growing the five isolates with increasing concentration of metal salts (Appendix- C.3) in Luria Bertani medium (Appendix- A.3). After incubation at 28°C for 24 hrs, the growth was measured as Optical Density of the culture at 600nm. The survival graph was plotted based on percent growth of the strains at different concentrations of metal salts. LD_{50} values of different metal salts viz. $CdCl_2$, $HgCl_2$ and $ZnSO_4$ for five TBTC tolerant isolates were determined from percent survival curve.

2.8 Determination of antibiotic resistance of bacterial isolates

Antibiotic sensitivity of natural isolates was determined using different antibiotics such as chloramphenicol, ampicillin, streptomycin, neomycin, rifampicin, tetracycline, kanamycin, nalidixic acid, spectinomycin, penicillin, antimycin, amikacin (Annexure-C.4). Different antibiotic plates were prepared using Luria Bertani agar and five bacterial isolates were spot inoculated on the plates and incubated at 28°C for 24 hrs. After incubation the sensitivity of the isolates was detected based on their inhibition of their growth on the respective antibiotic plates.

To determine the LD_{50} value of different antibiotics for isolate 25W (*Pseudomonas aeruginosa* strain USS25), it was grown in Luria Bertani broth with different concentration of selected antibiotics at 28°C for 24 hrs and O.D of the culture was taken at 600nm. The graph was plotted based on percentage survival of bacterial isolate at different concentration of antibiotics and the concentration at which the isolate showed the 50% survival, is considered as LD_{50} value of the isolate.

2.9 Selection of potential strain for TBTC degradation study

overnight grown cultures were inoculated to a final concentration of 5% in MSM with various concentrations of TBTC viz. 0.1mM, 0.5mM, 1mM, 1.5mM, 2mM,

2.5mM and 3mM and then incubated on incubator-shaker at 180rpm for 48 hrs at 28°C. Final growth yield was determined in terms of O.D at 600 nm for respective concentration of TBTC. Highest concentrations of TBTC which permits growth of the isolate serves as a tolerance limit.

2.10 Biochemical characterization of *Pseudomonas aeruginosa* strain USS25

2.10.1 Regulation of TBTC toxicity by thiol (Monothiol: β-mercaptoethanol) and chelating agent (EDTA-Na_2)

In order to find out non inhibitory level of β-mercaptoethanol (thiol) and chelating agent (EDTA), 5 overnight grown cultures were inoculated to a final concentration of 5% in MSM containing 2mM TBTC with different concentrations of monothiol (Appendix-C.6) and EDTA-Na_2 (Appendix-C.5) separately and incubated for 48 hrs at 28°C in incubator shaker at 180rpm. Absorption was recorded at 600nm after incubation. From the graphical representation of growth vs. concentration of monothiol and EDTA-Na_2 separately, the optimum concentration of thiol and EDTA-Na_2 for the growth was determined and the same concentration was used for further experiment.

Overnight grown culture (5%) was inoculated in MSM with different concentrations of TBTC supplemented with optimum concentration of monothiol and EDTA-Na_2 separately. The tubes were kept at 28°C in incubator shaker at 180rpm for 48hrs. After incubation, the growth was measured in terms of O.D. at 600nm. The graph was plotted as growth vs. concentration of TBTC.

2.11 Utilization of carbon and nitrogen source

2.11.1 Carbon source: succinate, glycerol and glucose

overnight grown cultures were inoculated to a final concentration of 5% in MSM containing 2mM TBTC, supplemented with different concentrations of and glycerol (Appendix- C.7), succinate (Appendix - C.8) (0.1%, 0.5%, 1.5, 2%, 2.5%, 3% and 3.5%) and glucose (1%, 2%, 3%, 4%, 5%, 6%) (Appendix - C.9) separately. The culture was

incubated on incubator shaker at 180rpm for 24hrs at 28°C. Growth was determined as protein/ml by Lowry's method and optimum concentration of succinate, glycerol and glucose for growth was determined. The growth of the isolate in terms of protein/ml with different carbon sources was represented in graphical forms. The optimum concentration obtained by succinate and glycerol was used for further TBTC degradation studies.

2.11.2 Nitrogen source: nitrates (NH_4NO_3, KNO_3) and ammonium chloride (NH_4Cl)

Overnight grown cultures were inoculated to a final concentration of 5% in MSM containing 2mM TBTC with various concentrations of NH_4NO_3, KNO_3, NH_4Cl (0.1%, 0.5%, 1%, 2%, 3%, 4%, 5%, 6%) (Appendix-C.10) and incubated on incubator-shaker at 180rpm for 48 hrs at 28°C. After incubation, growth was determined as protein/ml by Lowry's method and optimum concentration of nitrogen salts for growth were determined by plotting graph with concentration of nitrogen salts vs. proteins/ml.

2.12 Study the growth behaviour of TBTC resistant isolate in different media and selection of suitable media for TBTC degradation

Growth behavior of *Pseudomonas aeruginosa* strain USS25 was observed in Mineral Salt Medium (MSM) with 2mM of TBTC, MSM+TBTC (2mM)+Glycerol (3.5%), MSM+TBTC (2mM)+Succinate (3%) and MSM+TBTC (2mM)+Glycerol (3.5%)+Succinate (3%), MSM+2mM of crude TBTC, Luria Bertani Broth + TBTC (2mM) and ZMB + TBTC (2mM) respectively. Overnight grown cultures were inoculated to a final concentration of 5% and incubated for 48 hrs at 28°C. Growth was observed in terms of increase in O.D at 600nm. Growth characteristics were expressed in terms of graphical representation of O.D vs. time.

Degradation study was carried out after growing the cells in above-mentioned media for 24 hrs at 28°C. The cell pellet and supernatant was extracted separately by chloroform and degradation profile was observed through Thin Layer Chromatography (TLC). Degradation studies were also done using Zobell Marine Broth (ZMB) (ZoBell et

al. 1940).

2.12.1 Study of TBTC degradation profile (TLC analysis)

These selected bacterial strains were tested for their growth with different concentration of TBTC as sole source of carbon in MSM broth. Cultures, which grew up to 2mM of TBTC as a final concentration in media, were allowed to grow for 72 hrs and cell pellet was harvested by spinning at 8000 rpm (4 x 100, REMI, C-24, CUCT-5578) and supernatant was separated. The degradation product was extracted from cell pellets and supernatant separately with double volume of distilled chloroform using separating funnel. The organic layer was collected in a tube. The emulsion in chloroform was treated with Hyflo Super Cell. Chloroform extract was concentrated with nitrogen gas and the concentrated samples were analyzed by thin layer chromatography (TLC) (Hamilton and Hamilton, 1987) using the solvent system petroleum ether (40-60^{0}C) and acetic acid (9.5:0.5) and subsequently TLC plate was exposed to iodine vapour to develop the spot.

2.12.2 Time course study of TBTC degradation

The bacterial strain 25W was grown in MSM broth with 2mM TBTC under above-mentioned condition to monitor the time course of production of degradation product. 10 ml of culture broth was taken out each time at intervals of 6hrs for a period of 48 hrs and then at the interval of 24 hrs for 75days. The equivalent amount of fresh medium was replaced. For each sample, the degradation product was extracted from cell pellet, TLC was performed as above, and TLC plate was exposed to iodine vapour to check the disappearance of TBTC spot and appearance of the spot of degradation product.

The GC (6890 series plus, Alliance Biotech) profile of culture broth was studied consecutively at different time of incubation viz., one week, one month, three months etc., keeping the standard of TBTC and DBT. The 0 hr culture broth was taken as

negative control.

2.12.3 Effect of selected carbon sources (Glycerol and Succinate) on TBTC degradation

This experiment was carried out to study the utilization of 2mM TBTC in the presence of succinate and glycerol. The isolate was grown in MSM+ TBTC (2mM) + Glycerol (3.5%), MSM+ TBTC (2mM) + Succinate (3%) and MSM+ TBTC (2mM) + Glycerol (3.5%) + Succinate (3%) respectively. The MSM+ TBTC (2mM) medium grown culture was taken as control. At required time interval, samples were collected and extracted as mentioned earlier and TLC profile was checked to observe the yield of degradation product qualitatively.

2.12.4 Purification, estimation and characterization of TBTC degradation product

The pure degraded compound was analyzed using IR and NMR spectrophotometer. To confirm the molecular structure of the degraded compound sodium fusion test of the compound was done for the detection of halogen group present in the molecule.

Sodium fusion test (Shriner et al., 1964)

A small piece of dried Na metal (0.05gm) was taken in fusion tube. The tube was heated very gently over a low flame for about 20-30 seconds until the Na melts and turned black. Equal quantity of degradation compound was added carefully to the fusion tube containing fused Na metal. The resulting mixture, in the fusion tube, was first heated slowly and then vigorously until red-hot. This was then plunged in an evaporating dish containing distilled water (1-2ml). The fusion tube was crushed completely and the resulting solution was boiled for few more minutes and filtered. The filtrate known as Sodium Fusion Extract (SFE) was used further for detecting the presence of Nitrogen, Sulphur and Halogen in the degradation compound.

Detection of Halogen when nitrogen and sulphur are absent

0.2ml of SFE was acidified with dilute HNO_3 and few drops of $AgNO_3$ solution was added to it. A white precipitate obtained indicated the presence of chlorine molecule(s). Double distilled water was taken as control.

2.13 Study of TBTC biosorption

2.13.1 Standard curve of TBTC

Different concentration of TBTC (0.02mM to 10mM) was taken from stock solution (Appendix- C.1) and inoculated in MSM and kept in shaker at 180 rpm at 28°C for 24 hrs. After incubation, the MSM broth was filtered to remove un-dissolved TBTC flakes. The MSM filtrate was collected and extracted with chloroform. The chloroform extract was used for spectrophotometric study. Scanning of each extract was done in the range of 190-800nm to obtain the peak of absorption spectrum. O.D of the filtrate was taken at that wavelength and the standard curve was plotted with increasing concentration of TBTC and the respective O.D at 241nm (Appendix -J). The standard curve was used for TBTC estimation for the uptake study of TBTC.

2.13.2 Uptake study by growing cells

Uptake study of TBTC was performed by growing the *Pseudomonas aeruginosa* strain USS25 in different medium viz. MSM+ TBTC (2mM) + Glycerol (3.5%), MSM+ TBTC (2mM) + Succinate (3%) and MSM+ TBTC (2mM) + Glycerol (3.5%) + Succinate (3%) respectively. The MSM+ TBTC (2mM) medium grown culture was taken as control. The culture was incubated at 28°C at 180 rpm for 24hrs. In the beginning, the 5 ml sample was withdrawn as 0 hr reading. Subsequently same amount of broth was withdrawn from respective medium at equal time interval and kept for quantization of cell protein and TBTC content in cell pellet. Each sample was spinned at 8000 rpm (4 x 100, REMI, C-24, CUCT-5578) and supernatant was discarded. The TBTC was extracted from cell pellets by chloroform. The O.D of the chloroform extract was taken at 241nm

and the observed O.D was compared with standard concentration curve of TBTC to get the final TBTC concentration in cell pellet. 100µl of cell sample was taken to estimate the amount of protein / ml of cell pellet by Folin Lowry's method. Same protocol was followed for each sample, which was taken every two hours. The graph was plotted as TBTC (µM TBTC /protein) Vs time (hrs).

2.14 Characterization of exopolymers

2.14.1 Study of EPS production under TBTC stress

A modified method of Fishman et al. 1997, was followed to extract exopolysaccharides from cells. Overnight grown culture was inoculated in MSM and ZMB (Appendix-A.4) separately, each containing TBTC (2mM) along with control flask of ZMB and MSM without TBTC. Subsequently these flasks were incubated on incubator-shaker at 180 rpm for 48 hrs at 28°C. Slime EPS was extracted by centrifugation at 10,000 rpm for 20 min at 4°C. The supernatant containing the slime EPS was transferred into a clean test tube. For capsular EPS, 2ml of 10mM EDTA was added to the cell pellet, vortexed for 10min and centrifuged at 10,000 rpm for 20min, at 4°C, 1ml of the supernatant was added to the slime EPS, mixed, and 1ml of this mixture was then dialyzed against double distilled water at 4°C for 48hrs. The dialyzed sample was then used to estimate EPS. EPS was estimated by Phenol – Sulphuric acid method (Dubois et al, 1956).

2.14.2 Physicochemical characterization of exopolymer

Detection and isolation of polymer (Mengistu et al. 1994, Moreno et al. 1999).

The isolate 25W was grown in Mineral Salt Medium (MSM) with 2mM of TBTC and MSM with glucose (5%) separately for 48 hrs at 28°C. The cells were harvested by centrifugation at 8000 rpm for 10 min and the cell pellet was suspended in MSM for a week at 4°C The cell suspension was centrifuged and part of the supernatant was extracted with double volume of chloroform to check the biosurfactant activity. The cell pellet was washed thrice with MSM, the supernatants pulled together and the polymer

was precipitated using chilled absolute ethanol in the ratio 1:2. The mixture was kept at 4°C overnight and the precipitate was eluted after centrifugation at 8000 rpm for 10 min. The precipitate was then dialyzed against double distilled water at 4°C for 48hrs. and dried by lyophilization using Labconco lyophilizer (Kansas City, MO, USA) to get a white powder.

Determination of hydrophobicity of the cell in presence of TBTC (Sweet et al. 1987)

Bacterial Adherence To Hydrocarbon test (BATH test) of the cells was performed to check the hydrophobicity of the cells grown in the presence of TBTC and glucose separately.

Bacterial Adherence To Hydrocarbon test (BATH test) (Sweet et al. 1987; Rosenberg et al. 1999)

For each bacterium to be tested, 3ml volume of suspension was added to each of two 15mm diameter glass test tube, representing one test and one control. In addition, a test and a control were prepared on the suspending medium alone as spectrophotometer blanks. To each test suspension was added 0.6ml of benzene/xylene. The test and control were kept for while for equilibrium, then taken in turn and vortexed on a cyclo-mixer (REMI) for 30s and kept for 30 min to allow the immiscible organic and aqueous phases to separate. The lower aqueous layer was carefully removed using Pasteur pipettes and transferred to a clean test tube. Any contaminating organic molecule that may have been carried on the pipette or bound to the bacteria was removed by bubbling air through the 3ml of suspension for 1min. Absorbance (A_{450}) were measured as before, followed by vortex mixing for 5sec to disrupt and resuspend any aggregate that may have formed. Finally the absorbance of the aqueous phase of test suspension (with organic layer) (At) compared with control cell suspension (without organic layer) (Ao) were calculated as Fraction of Adherence by the formula (At – Ao)/Ao.

Biochemical characterization of exopolymer

Solubility of the lyophilized powder was checked in water, 1N NaOH, 1N and 6 N HCl, and solvents like chloroform, methanol and benzene. Proteins were estimation by Lowry's method (Lowry et al. 1951) and sugars were estimated by phenol sulfuric acid method (Dubois et al. 1956) in original and hydrolyzed sample (Read and Costerton 1987).

2.14.3 Biosurfactant activity of exopolymer

The biosurfactant activity was also determined by adding different amount of exopolymer, taken in the ground glass stopper tubes containing equal volume of distilled water and benzene or chloroform. Tubes were vortexed for 5 min and kept in stationary condition for phase separation, the height of organic layer, interphase and aqueous layer were measured respectively. The comparative study of surfactant activity of exoplymer produced in presence of glucose and TBTC was expressed through graphical representation with height of interphase vs. amount of exopolymer.

Effect of TBTC on biosurfactant activity

To observe the effect of TBTC on biosurfactant activity, 100 µl of TBTC was added to above mentioned benzene-water or chloroform-water system and change in respective height of three phases was observed and plotted in graphical form height of interphase vs. amount of polymer.

100µl of crude exopolymer powder was mixed with 10 µl of TBTC in 3ml of distilled water and centrifuged at 8000 rpm for 10 min. The pellet and supernatant were separated and extracted with chloroform. The chloroform extract was run on a silica gel-G TLC (Hamilton and Hamilton, 1987), with solvent system, petroleum ether (40-60°C): acetic acid (9.5:0.5) and kept in iodine chamber to visualize the spots.

The amount of exopolymer required to give maximum turbidity was determined by taking different concentrations of exoploymer (0.5mg, 1mg, 1.5mg, 2mg, 2.5mg) in

3ml of distilled water with 10 µl of TBTC. The tubes were vortexed and O.D. of aqueous layer was determined at 450 nm. Graph was plotted with amount of polymer vs. optical density at 450nm and Critical Micelle Concentration (CMC) (Ron and Rosenberg, 2002) was calculated from the graph.

2.15 Study of TBTC induced pigment: production and characterization

Study of pigment production under TBTC stress

Overnight grown culture (5%) of 25W isolate was inoculated in MSM + glucose (0.5%) as well as in MSM + glucose (0.5%) + TBTC (2mM). These flasks were then incubated on an incubator-shaker of 180 rpm for 48 hrs at 28°C. The culture was harvested by centrifugation at 8,000 rpm for 10 min and the clear supernatant was scanned in the range of 190-500nm using a spectrophotometer (SHIMADZU), to study the absorption spectrum of the pigment. Sterile LB broth was taken as control (auto zero) to avoid the interference of media component.

To observe the effect of TBTC on pigment production of bacteria, the culture was grown in MSM + glucose (5%) with increasing concentration of TBTC i.e. 0.5mM, 1mM, 1.5mM, 2mM and 2.5mM etc. in same condition and after incubation the cell supernatant was scanned to check the changes in absorption spectra.

Identification of pigment

In order to identify the pigments, the Luria Bertani grown pigmented culture supernatant of 25W culture broth was observed under UV-transilluminator. The same pigments were analyzed fluorimetrically by using RF-5301 spectrofluorophotometer (Shimadzu). 200 µl of each of the pigment sample was diluted to 3000 µl with Luria Bertani broth and this used for excitation study. Pigment sample were excited at 350, 390, 400, 420, 450nm respectively. Emission range (400 - 600 nm) was also recorded.

To ~~confirm the structure of~~ identify the pigment, concentrated hydrochloric acid was added to the culture supernatant and darkening of culture broth was observed (Gaber, 1973).

2.16 Protein profile (SDS-PAGE) under TBTC stress.

Cells were grown in different concentration of TBTC i.e. 0.1mM, 0.5mM, 1mM, and 2mM, in MSM broth for a week. The culture was grown in only Luria Bertani broth and Luria Bertani broth with 2mM of TBTC as a positive control for same period. The culture broth was centrifuged to separate the cells and supernatant. Protease inhibitor cocktail (SIGMA) was added to both cell and supernatant (1mg/ml final concentration) to inhibit the protease activity. Cell pellet suspended in saline, was sonicated to rupture the cells (65W, 30 Sec, repeated 3 times). Sonicated samples were again centrifuged and cell supernatant was separated from cell debris. Protein estimation of both samples i.e. sonicated cell supernatant and cell debris, was performed to estimate the protein in samples in order to load the same amount of protein. Required amount of sample was boiled with 30 µl of sample buffer for 10min. Then 2µl of loading dye was added and the sample was loaded. The SDS-PAGE was performed as per Laemmli (1970) method at 30V for 2hrs and gel was subsequently stained with Coomassie Blue and destained to observe the protein bands. Result was documented in gel documentation system.

Polyacrylamide gel electrophoresis (Laemmli, 1970)

Sample preparation for whole cell protein profile: Cell pellet obtained from 25 ml of culture broth was re- suspended to an O.D of 4.0 at 540 nm. 100µl of cells were treated with 50µl of sample buffer and 100µl of cells were used for protein estimation by Folin-Lowry's method. Cells with sample buffer were boiled for 10 min in a boiling water-bath. 2µl of loading dye solution was added to the treated sample prior to electrophoresis.

Preparation of Gels: A modified method of Laemmli was followed. The glass plates after washing and drying were wiped with acetone and clamped together with spacers in place. The assembly was sealed from inside using molten 1% agar and allowed to solidify. All the reagents (Appendix-D.1) used for separating gel were taken in a clean

beaker and mixed well. APS and TEMED were added, together, just before pouring the gel. The mixture was added in between the 2 plates using a 10 ml pipette, upto 3/4th of the space. Distilled water was added above the separating gel to get a uniform surface. Water was drained off after the gel had solidified. Similarly, stacking gel was prepared and added over the separating gel. A comb was introduced into the stacking gel and it was allowed to set. For denatured protein separations, 0.2% of sodium-dodecyl-sulphate (SDS) was added to separating and stacking gels during preparation. The lower spacer and comb were removed and the assembly was placed in electrophoresis chamber. Samples were loaded in the wells with the help of a syringe. Tank buffer was added to upper and lower tanks. Electrodes were connected to power pack and the gel was run at 30 V until the tracking dye reached the bottom of gel. Plates were separated carefully after removing the spacers and the gel was put in staining solution.

Staining of gels: Protein staining was carried out using Coomassie- Brilliant Blue R-250 stain. Gel was placed in this staining solution for 6-8 h. It was then destained in Destaining solution I for 1 h and in solution II until clear bands appeared. Gel was washed with deionised water and stored in the same.

2.17 Molecular biological and genetic characterization of *Pseudomonas aeruginosa* USS25

2.17.1 Plasmid purification and agarose gel electrophoresis

Plasmid DNA of 25W isolates was purified using Alkaline Lysis method (Birnboim and Doly, 1979), because plasmid yield was better than other methods such as Boil prep method (Holmes & Quigley, 1981). The isolated and purified plasmid was kept at -20°C for further use.

i. ***Alkaline Lysis Method*** (Birnboim and Doly, 1979): A single bacterial colony was transferred into 10 ml of Luria Bertani broth and incubated overnight at 28°C at

180 rpm. 1.5 ml of culture was taken in a microfuge tube and was centrifuged at 8,000 rpm for 5 min at 4°C. The supernatant was discarded leaving the bacterial pellet as dry as possible. The pellet was suspended in 100µl of ice-cold glucose EDTA tris buffer (solution I) (Appendix-E.1.i) which was vortexed and kept in ice for 10min. 200µl of freshly prepared SDS (solution II) (Appendix-E.1.ii) was added and the contents were mixed by inverting the tube rapidly 4 -5 times, making sure, the entire surface of the tube came in contact with solution II (Appendix-E.1.ii). The tube was stored in ice for 10 min. Then 150µl of ice-cold Potassium acetate (solution III) (Appendix- F.1.iii) was added and the tubes were gently mixed by inverting, to disperse solution III through the viscous bacterial lysate. The tubes were stored on ice for 3-5 min, after which it was centrifuged at 12,000 rpm for 5 min at 4°C. The supernatant was then transferred to a fresh microfuge tube. Double stranded DNA (ds DNA) was precipitated with double volume of the cold ethanol. The contents were mixed gently and allowed to stand for 2 hrs in ice. The tube was centrifuged at 12,000 rpm for 5 min at 4°C. The supernatant was removed and the tube was allowed to stand in inverted position on a paper towel, to drain all the fluid. The pellet of DNA was rinsed with 70% (v/v) chilled ethanol and centrifuged at 12,000 rpm at 4°C. The supernatant was discarded and the pellet was allowed to dry in air for 10 min and then dissolved in 50µl of TE buffer containing DNase free RNase (20µg/ml). The tubes were gently mixed by tapping and DNA stored at -20°C.

ii. ***Boil Prep method*** (Holmes and Quigley, 1979): A single bacterial colony was transferred into 10 ml of Luria Bertani broth and incubated overnight at 180 rpm. 1.5 ml of culture was taken in a microfuge tube and was centrifuged at 8,000 rpm for 5 min at 4°C. The supernatant was removed leaving the bacterial pellet as dry as possible. The pellet was suspended in 350µl of STET buffer (Appendix- F.2.i). 25µl of freshly prepared solution of lysozyme (Appendix- F.2.ii) was added to the

tube and mixed by inversion. The tube was placed in dry bath at 95°C for 2 min and the bacterial lysate was centrifuged at 12,000 rpm for 10 min at room temperature. The pellet of bacterial debris was removed from the tube and supernatant was collected separately. To the supernatant 40µl of 2.5M sodium acetate and 420µl of chilled isopropanol was added. The contents were mixed gently and the tubes were stored for 5 min in ice. The pellet of nucleic acids was recovered by centrifuging at 12,000 rpm for 5 min at 4°C. The supernatant was removed and the tubes were allowed to stand in an inverted position on a paper towel to drain all the fluid. The pellet was rinsed once in 1ml of 70% (v/v) ethanol and centrifuged at 12,000 rpm for 5 min at 4°C. Again all the fluid was removed and the pellet of nucleic acids was dissolved in 50µl of TE buffer containing DNase free RNase (20 µg/ml). The tube was gently mixed by tapping and DNA stored at -20°C.

iii. ***Gel Electrophoresis of plasmid DNA*** (Meyers et al. 1976; Kado and Liue, 1981,)

a. ***Preparation of gel slabs:*** Agarose gel 0.8% (w/v) was prepared in 1X-TAE buffer (pH 8.0) (Appendix-E.4.ii) by heating in a microwave oven for 2 min. The platform for electrophoresis was sealed on open sides with leucoplast. The comb was adjusted to 1mm above the gel slab and 1.5 cm from one sealed side. To the molten agarose (50 ml), 5µl of ethydium bromide (10mg/ml) (Appendix-E.4.iii) was added to get final concentration of approximately 4µg/ml in molten agarose, poured into the platform to a thickness of 0.5 cm and allowed to set at room temperature. After setting, the comb and leucoplast were carefully removed. The gel slab was placed in the electrophoretic chamber and the 1X TAE buffer was poured to the chamber till the gel was just below the buffer.

b. ***Loading of DNA sample*:** DNA sample (10µl) was mixed with 2µl of tracking dye and added in the sample slots of agarose gel using micropipette.

c. ***Running of the gel*:** The lid of the electrophoresis chamber was closed. The electrodes were connected to the power supply by means of connecting wires. The

voltage was adjusted to 72V and the electrophoresis was carried out at constant voltage for 2 h.

d. ***Visualisation of DNA*:** After electrophoresis the gel was observed on a UV photodyne transilluminator.

2.17.2 Restriction mapping and agarose gel electrophoresis

Plasmid DNA of *Pseudomonas aeruginosa* strain USS25 was digested using different restriction enzymes like BamHI and NcoI with different concentrations as per the Genei catalogue, Bangalore, India and Sambrook et al., 1989. The final volume of reaction mixture was 10µl. As soon as the reaction got over the restriction digestion of DNA was observed through agarose gel electrophoresis and visualized using UV photodyne transilluminator to calculate the size of fragmented DNA. Result was documented in gel documentation system (Vilber Lourmat, ~~USA~~ France).

Restriction mapping of plasmid DNA:

Required amount of DNA sample was taken in separate microfuge tubes. Buffer for the respective enzymes were added to a final concentration of 1X. Then BSA for specific enzyme was also added to a final concentration of 1X. Sterilized deionized water was added to make up volume of reaction mixture. Finally, the respective enzymes were added in a final concentration of 1U/ µl. The total volume was made upto 10µl. After adding the enzymes, the reaction mixture was immediately kept in dry bath at 37°C for one and half hour. After incubation, the reaction mixture was again kept at 65°C for 10min to inactivate the enzymes. The reaction mixture was immediately loaded to 0.8% agarose gel to perform gel electrophoresis. When the electrophoresis was done, the gel was observed under UV photodyne Tranilluminator. A known molecular marker was loaded for reference to calculate the molecular weight of DNA fragment digested by different restriction enzyme.

2.17.3 Curing of plasmid

Curing the plasmid of *Pseudomonas aeruginosa* strain USS25 (25W) was performed using acridine orange (Trevors. 1986). 500μg/ml stock solution of acridine orange was prepared in deionised water and filter sterilized. The solution was kept in amber coloured bottle in dark and cold condition. Different concentration of acridine orange ranging from 10-300 μg/ml was then added to Luria Bertani broth and overnight grown culture of *Pseudomonas aeruginosa* strain USS25, was inoculated and then incubated in standard optimum condition for 24 hrs. After incubation, O.D of the culture broth was taken and graph was plotted with concentration of acridine orange (μg/ml) vs. percentage survival. LD_{50} value of acridine orange for the culture was determined from the plotted graph.

The plasmid bearing strain was subcultured without TBTC in acridine orange (LD_{50} concentration) containing Luria Bertani medium for 10 times. Appropriate dilution of this culture was plated on Luria Bertani agar to get discrete colonies. After incubation, the discrete colonies were replica plated on Luria Bertani agar, Luria Bertani agar with 2mM TBTC and MSM agar with 2mM TBTC respectively and kept for incubation at 28°C. The colonies, which grew on TBTC containing agar plate were checked for plasmid content in order to correlate the loss of TBTC resistance with loss of a specific plasmid.

2.17.4 Nitrosoguanidine mutagenesis of *Pseudomonas aeruginosa* strain USS25 and selection of NTG induced mutants

Killing Curve

Overnight grown culture (5%) was inoculated in to 5ml of LB broth in a test tube and aerated at 28°C until a density of 3 -5 $x10^8$ cells/ml was obtained. The culture was centrifuged and the pellet was washed twice with citrate buffer (Appendix-F.2) (incase of more sample, the sample was pulled together, centrifuged and washed). 4ml of the sample was measured into each of nine test tubes after resuspending in citrate buffer (pH

5.5). Three sets of test tubes were kept for three different concentrations of NTG i.e. 50µg/ml, 100µg/ml and 200µg/ml. Required amount of NTG (Appendix-F.1) was added in each test tube from stock solution. The tubes were placed in a 28°C environmental shaker 180 rpm for incubation. One set of tubes were withdrawn immediately for zero hrs readings.

At different time intervals, the culture was withdrawn, centrifuged, and washed once with phosphate buffer (pH 7). Each pellet was finally re-suspended in 5ml of phosphate buffer, immediately serial dilution was made, and spread plated on LB agar plates. Samples were withdrawn at the following times intervals 0, 15, 30, 45, 60, 75, 90 and 105 min. 0.1ml of 10^{-3}, 10^{-4}, and 10^{-5} dilution for respective samples were plated. The viable cell count was determined from the titre plates for each time interval. The graphs have been plotted with the survival against the time of exposure to NTG.

NTG mutagenesis

Wild type isolate was subcultured and allowed to grow overnight in LB broth and then again inoculated in LB broth and aerated at 28°C until a density of 3-5 x 10^8 cells/ml is reached.

5ml of culture broth was centrifuged and the pellet was washed twice with in the same volume of citrate buffer. Then the pellet was resuspended in 4ml of citrate buffer. Required amount of NTG was added to get a final concentration of 100µg/ml. The flask was incubated at 28°C for 45min in dark condition. At the end of the incubation period, the pellet was centrifuged and washed in phosphate buffer (pH 7) (Appendix-F.3).The washed pellet was resuspended and directly plated on MSM agar containing 5mM and 10mM of TBTC. As a control the unmutagenised cells were also allowed to grow in MSM broth containing +2mM of TBTC broth and after incubation, the cell pellet was collected by centrifugation and plated on MSM Agar plate with 5mM and 10mM of TBTC. Both the plates were kept for incubation for a week at 28°C and after incubation the colonies were observed and mutated colonies were selected for comparative growth

and degradation study.

2.17.5 Comparative study of mutant and wild type strain with reference to growth and TBTC degradation capability

Growth studies

Over night grown, wild and mutant type cultures were taken as inoculum for degradation study. Five percent of inoculum was used for both wild and mutant type cell in MSM+ 5mM of TBTC and MSM+ 10mM of TBTC. Both type of cells were inoculated in respective medium and incubated at 28°C for 42 hrs. Growth was observed in terms of increasing O.D at 600nm. The graph was plotted with time vs. O.D at 600nm.

TBTC Degradation studies

Both the isolates, wild and mutant type were grown in MSM+5mM TBTC in above-mentioned condition for one week and after incubation the broth was centrifuged down to collect the cell pellet and extracted with double volume of chloroform. The extract was concentrated, TLC was performed for both wild type, and mutant type extracts respectively. The comparative study of degradation was observed on TLC plate when exposed to iodine vapour.

2.18 Analytical techniques

UV- Visible spectrophotometry

UV-Visible scans of the culture supernatants and other samples were done using UV-1601 Shimadzu spectrophotometer.

Thin layer chromatography (TLC) (Hamilton and Hamilton, 1987)

The slurry, prepared by mixing 6 to 8g of Silica Gel G (Acme's synthetic chemicals) in 15 ml of distilled water, was poured on glass plates (20 x 20cm) and drawn into thin layer of 0.2 mm. The plates were air-dried and then activated at 110°C for 30

min. Samples were spotted on the activated plates using thin glass capillaries. After drying the spots, plates were developed in solvent chambers, which were previously saturated overnight. Solvent was allowed to run up to $3/4^{th}$ of the plate, the solvent front was marked and coloured spots, if any, were noted. The plates were then placed in iodine chamber. Rf values were calculated using the formula:

$$Rf = \frac{\text{Distance travelled by solute}}{\text{Distance travelled by solvent}}$$

Preparative TLC method to extract organotin compounds (Kimmel et al. 1977)

The slurry was made as above and poured on clean glass plates to make a 0.5mm layer of slurry. After activation of plate, maximum amount of sample was loaded on TLC plate and developed in solvent chamber. As solvent was allowed to run upto $3/4^{th}$ of the plate, the solvent front was marked. $3/4^{th}$ of the plate was covered by another clean glass plate and exposed to iodine vapour to locate the degradation product. The spot was marked and it was removed from iodine chamber. TLC powder was scraped off from plates leaving the exposed part. Then the degradation product was extracted by repeated washing of TLC powder with chloroform. The chloroform extract was concentrated with nitrogen gas.

Gas chromatography (GC) analysis

The TBTC extraction was carried out as per Matthias et al. (1986b) and 1µl of extracted sample was analysed using FPD-Gas chromatography (6890 series plus, Alliance Biotech).

Infrared spectra (IR)

IR spectra were recorded on Shimadzu (Model 8201PC) FTIR spectrophotometer. The samples for IR spectra were prepared in KBr diluted pellet in the solid state and the IR

signals reference to polystyrene bands.

Nuclear Magnetic Resonance Spectrum Analysis (NMR)

H^1 NMR spectra were recorded on FT NMR Bruker WT (300MHz) in $CDCl_3$ with tetra methyl silane (TMS) as an internal standard. The spectra are presented in the chemical shifts of various protons are documented.

2.18.1 Standard estimation method of protein and sugar estimation

a. Protein estimation by Folin-Lowry's method (Lowry, et al. 1951): The protein concentration in samples was quantitated by Folin Lowry's method. To 1ml of sample; 5ml of alkaline copper sulphate reagent was added. Mixture was thoroughly mixed and incubated at 28°C in dark for 10 minutes. To this solution, 0.5ml of diluted Folin-ciocalteau reagent was added and incubated in dark for 30 minutes at 28°C. Absorbance of this solution was taken in dual beam UV-Visual Spectrophotometer (Shimadzu UV 1610) against protein blank at 660nm.

b. Sugar estimation by phenol sulphuric acid method: Samples were analyzed for their sugar content by the phenol-sulphuric acid method (Dubois, et al. 1956). To 1 ml of aqueous sample containing polysaccharides, 1 ml of 5 % aqueous phenol was added. The tubes were placed in ice and 5 ml of concentrated sulphuric acid was added carefully into the tubes. Tubes were incubated in ice for 10 min and subsequently kept at 25-30°C for 10min. The absorbance was measured at 490 nm. Standard curve was plotted using glucose (0-100 mg/ml) as standard.

Chapter III

SCREENING, ISOLATION, IDENTIFICATION AND PHYSIOLOGICAL CHARACTERIZATION OF TBTC RESISTANT ISOLATES

3.1 Details of sampling sites.

The present sampling sites include west coast of India, an important offshore oil field of Maharashtra, 150 miles away from west coast of India and Goa shipyard, one of the biggest shipyard in west coast of India which was also subjected to have organotin pollution because of unregulated ship repairing and ship building activity. Bombay High Oil field area is subjected to have heavy organotin contamination due to heavy traffic of oil tankers, barges, regular goods carrier vessels and coast guard vessels (Fig-3.1). The detailed description of both sampling sites with their respective longitudes and latitudes are given in (Table-3.2). These kinds of contamination at large polluted sites often share critical properties such as toxicity, high level persistence, often high mobility prone to contamination of ground water and high lipophilicity resulting in bioaccumulation in vital components of food webs (Fent, 2003).

3.2 Physicochemical characteristics of water samples.

The sampling sites chosen for the present study i.e. Goa Shipyard Ltd (GSL) and Bombay High Oil field Area are directly or indirectly involved with estuarine water, which brings large amount of nutrients from land to the sea. The nutrient determines the potential fertility of the water masses (Subramanyam and Sambamurthy, 2000). The distribution of nutrient elements in estuarine water is controlled, by nature of estuarine circulation, mixing and other physical process, together with biological, sedimentological and chemical processes (Aston, 1980). The biodiversity of estuarine microflora largely depends upon above characteristics. Since organotins are present in these estuarine econiches in appreciable amount, interestingly, bacterial strains which

can degrade them get enriched. Therefore several TBTC degrading bacterial strains are abundant in these econiches (habitat) (Wuertz et al. 1991).

In addition to dissolved molecular nitrogen the sea water 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 associated with marine organisms as products of their metabolism and decay (Riley and Chester, 1980). The samples of Goa Ship yard showed nitrate content of 4.8μg/ml whereas the values are 2.21μg/ml and 2.08μg/ml for NL-9 and NL-2 sites of Bombay High oil field area respectively (Table-3.1). The phosphate content of water sample of Goa Shipyard was 5.6 μg/ml where as samples of Bombay High Oil field area (3.4-3.6μg/ml). The temperature, pH and salinity of water sample of specified sampling sites was ranged from 8-8.3 pH, 28-30°C and 33-35‰ respectively (Table-3.1).

As both the 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 ‰ (Subramanyam and Sambamurthy, 2000). In case of Indian Ocean the nitrate, nitrite and phosphate content was found to be 0-7μg/L, 0-280μg/L and 3.1 to 31 μg/L respectively (Lobban et al. 1985), which coincides with present observation (Table-3.1). 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. Atmospheric precipitation has been reported to contain large amounts of ammonia and nitrate (Quasim and Sengupta 1981). The phosphate content of water sample of Goa Shipyard was higher than the Bombay High Oil field area. This data clearly indicated that nutrient content of estuarine water is higher than open sea.

TABLE 3.1- Physicochemical characteristics of water samples

Sr. No	Sampling Sites	Physicochemical characteristics					
		pH	Temperature	Salinity	Phosphate (PO_4) µg/L	Nitrate (NO_3) µg/L	Nitrite (NO_2) µg/L
1	Goa Shipyard	8.1	28°C	33‰	0.07	0.8	4.6
2	Bombay High Oil Field area (NQ-9)	8.3	28°C	35‰	13.60	2.21	5.63
3	Bombay High Oil Field area (NL-2)	8	28°C	35‰	12.96	2.08	5.13

3.3 Viable count of bacteria in water sample

The total viable count of all water samples obtained from Bombay High Oil field were ranging from 23 x 10^2 to 408 x 10^2 cfu/ml when plated on Nutrient Agar. The viable count of the same samples on NA + 0.1mM TBTC and MSM + 0.1mM TBTC was 8 x 10^2 to 85 x 10^2 cfu/ml and 18 to 697 cfu/ml respectively (Table-3.2). It clearly indicates that approximately 16% of natural bacterial population is resistant to 0.1mM of TBTC as it utilizes TBTC as sole source of carbon (Table- 3.2). When the TBTC concentration was increased upto 0.4mM in MSM agar medium, the viable count was reduced to 24.3 %.

Viable count of water sample obtained from Goa Shipyard Ltd (GSL) ranged between 32 x 10^2 to 383 x 10^2 cfu/ml on Nutrient Agar, but the viable count was 198 to 765 cfu/m on MSM agar plates with 0.1mM TBTC. Approximately 11.4 % of natural bacterial population is resistant to 0.1mM TBTC in MSM and 66.8 % of these TBTC resistant isolates could grow on MSM even with 0.4mM TBTC as sole source of carbon (Table-3.2).

The studies have shown that bacterial flora of GSL are more resistant than bacterial flora of Bombay High Oil field area (Table-3.2). The antifoulant in ship paints,

shipyards, harbours is considered to be the prime source of TBT in the marine ecosystem (de Mora and Pelltier, 1997). GSL is also one of the important harbours and shipbuilding yards of west coast of India, therefore organotin level are invariably high in surface water and sediments around the GSL. Similar findings have also reported that coastal water near harbours is appreciably contaminated with TBTC (de Mora et al. 1995; Chau et al. 1997) and considerably high amount of organotin has already been reported in different coastal and harbour areas of the world (Table-1.2).

3.4 Screening, isolation and purification of TBTC resistant marine bacterial strains

The bacterial isolates which appeared on Mineral Salt Medium (MSM) with 0.4mM TBTC were repeatedly sub-cultured separately in Mineral Salt Medium with increasing concentrations of upto 4mM TBTC. The predominant bacterial isolates which were growing well on MSM with 2mM TBTC after subsequent sub-culturing, were selected for further studies. These TBTC utilizing isolates were then maintained on MSM with 2mM TBTC and incubated at 28°C, which is ambient temperature of marine /estuarine samples.

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 (0.4mM to 4mM). Most of the bacterial isolate 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. Predominant isolates were selected, purified and screened on the basis of their growth on MSM agar with increasing concentrations of TBTC. The TBTC concentrations used are 0.1mM, 0.4mM, 1mM, 1.5mM, 2mM, 2.5mM and 3mM respectively. Out of forty-six isolates five bacterial strains namely 3(4Sub), 9(3A), 25B, 25W, $5Y_2$ showed consistent good growth in presence of 2mM TBTC as final concentration in MSM broth after 48hr incubation with the optimum condition for growth i.e. temperature at 28^0C, pH 7.2 and 2.5% (25‰) salinity and 180 rpm.

Although Singh (1987) and White, et al. (1999) have reported the range of microbial resistance from upto 0.07mM of 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 and this provides one route for detoxification of Tributyltins. In addition, microorganisms are capable of accumulating TBT compounds, which is another mechanism of removal of TBT from marine environment (Gadd, 2000). The high lipid solubility of organotins ensures the interaction of TBTC with intracellular sites by penetrating through cell wall and cell membranes (Gray, et al. 1987; Gadd, 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 et al. 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 and Fukagawa, 1995). Further more, not much is known on TBTC degradation rates under ambient environmental conditions in marine coastal waters (Seligman et al. 1986) 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 and Roy, 2003).

3.5 Identification of TBTC resistant bacterial strains

Initially colony characters of these five bacterial isolates [25W, 25B, 3(4sub), 9(3A), $5Y_2$] revealed that 25W, 25B and $5Y_2$ were green pigmented colony which turned to brown after 48hrs. The other two bacterial isolates 3(4sub) and 9(3A) showed yellow pigmentation. The detailed colony characters are cited in Table-3.3. The Gram's characters of all the five isolates showed that these bacterial isolates were Gram's negative short rods (Table-3.4). Biochemical tests for all five Gram's negative strains were initially done according to Cruickshank et al. (1972) (Table-3.4). On the basis of biochemical tests (Table-3.4) strains were identified according to *Bergey's Manual of*

Systemic Bacteriology (Krieg and Holt. 1984) (Table-3.3) and for further confirmation two standard strains i.e. *Pseudomonas mendocina* P_2d and *Sphingomonas paucimobilis* were kept for comparison. As per the biochemical test all five isolates were showed 80% similarity with *Pseudomonas mendocina* P_2d. All the isolates were grouped as *Pseudomonas* spp. as they show more than 70% similarity among themselves in phenogram with standard *Pseudomonas* sp. (Fig-3.2) (Goodfellow, 1977; Baya, et al., 1986). The biochemical characteristics of the bacterial isolates, 9(3A) and 3(4Sub) led to identification as *Pseudomonas* sp. strain 9(3A) and *Pseudomonas* sp. strain 3(4Sub) respectively. The other three isolates (25W, 25B, $5Y_2$) formed a cluster with 90% similarity, are tentatively identified as *Pseudomonas aeruginosa* strain USS25, *Pseudomonas* sp. strain 25B and *Pseudomonas* sp. strain $5Y_2$ (Table-3.3). The growth on Nutrient Agar with 1% dettol after 24 hrs also identified that 25W and 5Y2 were *Pseudomonas aeruginosa* (Ferrao and Mavinkurve, 1977; Mavinkurve and Ferrao, 1979). 25W strain was exactly confirmed as *Pseudomonas aeruginosa* by 16s rRNA sequencing and homology search by Prof. Satoru Suzuki and Dr. Kitamura from C.M.E.S., Ehime University, Matsuyama, Japan. It was designated as *Pseudomonas aeruginosa* strain USS25 (Data not shown).

Table. 3.3 Morphological Characteristics of potential TBTC degrading marine bacterial isolates

Sampling sites	Isolates	Colony characteristics on MSMA+TBTC (2mM)	Identified bacterial strains
Goa Shipyard (Painting yard)	3(4sub)	Yellow, 2mm, irregular opaque, sticky, flat	***Pseudomonas fluorescens*** **strain 3(4Sub)**
Goa Shipyad (Close to ship)	9(3A)	Yellow, pinpointed, Irregular opaque, sticky, flat.	***Pseudomonas stutzeri*** **strain 9(3A)**
Bombay High Oil Field area (PlatformNQ-9)	25B	Brown, 3mm, round, opaque, sticky, elevated	***Pseudomonas aeruginosa*** **strain 25B**
Bombay High Oil field area. (PlatformNQ-9)	25W	Brown, 4mm , irregular, opaque, sticky ,elevated.	***Pseudomonas aeruginosa*** **strain USS25**
Bombay High Oil field area (PlatformNL-2)	$5Y_2$	Green, 2mm, round, opaque, non-sticky, elevated.	***Pseudomonas aeruginosa*** **strain $5Y_2$**

3.6 Characterization of potent TBTC resistant bacterial strains

3.6.1 TBTC tolerance limit

The selected five bacterial isolates were primarily screened on the basis of TBTC utilization as sole carbon source. Most of the isolate which initially grown on MSM agar supplemented with 2mM TBTC, lost their viability on the same medium after repeated subculturing, because organotin compounds inhibits or kills the aquatic microorganisms (Pettibone and Cooney, 1986), but very few of them could survive at higher concentration of TBTC i.e. 2mM. The selected five isolates consistently showed good growth on MSM agar supplemented with 2mM TBTC with zone of clearance (Fig-3.3) as well as in MSM broth with in 48hrs. In subsequent higher concentration i.e. 3mM, 4mM etc isolates did not grow in MSM broth supplemented with 2mM TBTC.

3.6.2 Optimum temperature for growth

In order to determine optimum temperature for growth of TBTC utilizing isolates, the five selected isolates were grown in MSM + TBTC (2mM) at three different temperatures viz. 28°C, 37°C and 42°C. The total protein content of the isolate which was grown at 28°C was high as compared to the protein content of other isolates grown at 37°C and 42°C. It is interesting to note that *Pseudomonas aeruginosa* strain USS25 showed maximum growth at 28°C, whereas the lowest growth was recorded at 42°C for all the isolates after 48hrs of incubation. The protein content of the isolates 3(4Sub), 25B, $5Y_2$, 25W and 9(3A) with 48hrs of incubation showed 430μg/ml, 455 μg/ml, 450μg/ml, 580μg/ml and 410μg/ml respectively at 28^0C (Fig 3.4).

Therefore it was evident from this experiment that all five isolates grew and utilized 2mM TBTC better at ambient temperature of sea water i.e. 28°C whereas at 42°C, growth as well as TBTC utilization declined significantly (Fig-3.5). *Pseudomonas chlorophis* also showed its optimum growth and triphenyltin degradation activity of at 28°C (Inoue et al 2000). On the contrary, Fukagawa et al. (1994) and Callow and Willingham. (1996) have reported that 25°C is the optimum temperature for growth of *Vibrio* sp in presence of TBTC and other bacteria respectively. Lower than 28°C i.e. 25°C, prolonged the incubation period for appearance of colonies on sea agar plates (Callow and Willingham, 1996). Gomez-Ariza et al., (1999) have reported that phenyltin remained unaltered higher than room temperature for 60 days.

3.6.3 Optimum pH for growth

Organotin belongs to the most toxic environmental pollutants known for aquatic life. The bioavailability of organotin depends on the pH and the content of organic matter (Fent, 2003). Considering this fact all five isolates were grown at three different pH values i.e. 5, 7 and 9 in order to check the optimum growth of the isolates. All five isolates showed better growth at pH 7 in terms of protein content in MSM supplemented with (2mM) TBTC as compared to cultures growth at pH 5. The highest protein content

was observed for isolate 25W (550μg/ml) at pH 7 (Fig.3.5), which correlates well with the previous findings of Gomez –Ariza (1999) which revealed butyltin remained stable in un-acidified sea water at 4°C for 7months. So, easy accessible of stable organotin helps the bacterial isolate to grow in the medium. On the contrary, there was marked decrease in growth of 25W isolate, at pH 9. In case of 25B, 3(4Sub) and 9(3A) the amount of total protein in culture broth was 380μg/ml, 360μg/ml and 280μg/ml respectively at pH 7, but at pH 9, it decreased to 190μg/ml, 100μg/ml and 200μg/ml respectively. Hence pH 7 was most favourable for growth with TBTC as sole carbon source. Therefore, slightly alkaline growth conditions were comparatively more favourable than acidic conditions, for all the strains (Fig 3.5). In case of *Rhodospirillum rubrum,* triphenyltin inhibits the hydrolysis of chromophore, membrane bound pyrophosphatase in a pH depedent manner, being maximal at pH 9-10. The basic pH values at which the inhibition produced by this organotin on membrane bound pyrophosphatase is very similar to that produced on the chromatophor H^+ ATPase (Celis et al. 1998). Another factor that affects the growth was solubility of TBT in water; it was observed that the minimal solubility of TBTs in water at 25°C was 1mg Sn /L at pH 6-8 and 15 mg Sn /L at pH 7-8 at the same temperature (Inaba et al. 1995).

Thus, it is evident from this experiment that TBTC is cytotoxic at acidic pH of growth medium (i.e. pH 5). Similar observations have also been reported by Gadd (2000). It is interesting to note that reduction in pH of the medium results in enhanced TBTC toxicity possibly due to increased availability of the tributyltin cation, $[Bu_3Sn(H_2O)_2]^+$ in the medium. Microbial interaction with organotins is influenced by environmental conditions. As in aquatic system, both pH and salinity may determine organotin speciation and therefore bioavailability and reactivity. These environmental factors like pH may also alter selectivity for TBT resistant microorganisms in polluted system as it is evident from selective enrichment of TBTC resistant bacterial strains in TBTC contaminated marine environment (Fukagawa et al. 1994; White et al. 1999).

3.6.4 Optimum salinity for growth

When all the five isolates were grown in MSM supplemented with 2mM TBTC containing different concentrations of NaCl ranging from 0.5 to 2.5% they all showed a similar pattern of growth in both media as growth increased proportionately with increase in salt (Sodium chloride) concentration up to 2.5% (Fig 3.6).

Since, TBTC is very hydrophobic compound, its solubility greatly depends on pH and salinity of the environment (medium) (Alzieu 2000). The aqueous solubility of organotin compound such as TBTC was decreased with increased salinity (Inaba et al. 1995). These results suggest that TBTC toxicity is reduced at higher salinity levels. As the availability of TBTC is high at low concentration of NaCl, it becomes toxic to the cells; the toxicity of TBTC is attributed to Na^+ and Cl^- moieties as well as the possible osmotic response of the organisms that included changes in intracellular compatible solutes and membrane composition (Cooney and Wuertz, 1989). Amelioration of metal toxicity in the presence of NaCl could possibly be due to formation of anionic co-ordination complexes of test compounds with Cl^-, which could be comparatively less toxic to microbes than the free metal cations (Dubey and Rai, 1990a).

3.7 Cross tolerance to heavy metals i.e. Hg, Cd, Zn

Zinc (Zn^+)

The five isolates showed considerably high level of Zn resistance with maximum resistance of 4.9mM for 3(4Sub) and 9(3A) isolates which were found very sensitive as the LD_{50} values obtained from the survival curve was 0.07mM (Fig 3.7, 3.8, 3.9, 3.10, 3.11) (Table-3.5).

The prokaryotes possess a range of Zn^{+2} import/export/binding proteins, some of which utilize either ATP or the chemiosmotic potential to drive the movement of Zn^{+2} ions across the cytosolic membrane, together with proteins that facilitate the diffusion of this ion across either the outer or inner membrane (Leonard et al. 1986; Blencowe and

Morby, 2003). Zinc concentration as low as 4.2 μg/L (0.06μM) has also been reported to affect microbenthos. In presence of 0.4ppm of $ZnSO_4$ algal growth sharply declines with abnormality in cell structure (Jayachandran and George, 1991). Paulsson et al. (2000) has reported that 0.12-0.42 μM was considered as the threshold concentration for bacteria and algae in most of the European river. Zinc interferes with the cellular phosphorus in algae (Bates et al. 1985) and observation of increased activity of alkaline phosphatase after zinc exposure implies existence of a zinc-phosphorus interaction extracellularly, making less available to the cell (Patt et al. 1987). This observation leads us to hypothesis that the impact of Zinc might be due to two different mechanisms, one direct due to zinc toxicity and another on nutrient un-availability (Paulsson et al. 2000).

It is noteworthy that five of the isolates were resistant upto 5mM. These isolates may have some mechanisms to remove this metal from cell or to prevent their entry into the cell.

Cadmium (Cd^+)

When all the five isolates were checked for their survival in presence of $CdCl_2$, the 25W isolate showed highest resistance i.e. LD_{50} 2.7mM, while 9(3A) isolate showed very low level of resistance i.e. LD_{50} 0.25mM. Rest three strains 3(4Sub), 25B and $5Y_2$ showed moderate level of resistance 1.35mM, 1.15mM and 1.45mM of $CdCl_2$ respectively (Fig 3.7, 3.8, 3.9, 3.10, 3.11) (Table 3.5).

In case of Cd resistance, plasmid governed system of membrane proteins that pump toxic ions out of the cells are already known (Neis and Silver, 1989; Silver and Laddaga, 1990; Tisa and Rosen, 1990). Unlike the Hg and As resistance systems that are highly homologous in all bacteria studied, Cd resistance appears to have evolved at least three times, giving rise to

(i) Efflux ATPase enzymes in Gram's positive bacteria (Ji and Silver,1995)

(ii) Chemiosmotic cation-proton antiporter in Gram's negative bacteria (Neis, 1992).

iii) Metallothionines of cyanobacteria (Turner and Robinson, 1995).

Mercury (Hg^{+})

It was very interesting to observe that isolate 9(3A), which was very sensitive to Cd and Zn has shown high resistance to Hg as the LD_{50} value was 8.6mM (Fig 3.9). The other TBTC resistant isolates i.e. 25W, 25B, 3(4Sub) and $5Y_2$ showed values of 4.7mM, 6.5mM, 5.5mM and 5.2mM $LD_{50,}$ respectively (Fig 3.7, 3.8, 3.9, 3.10, 3.11) (Table 3.5). These findings definitely matches with earlier reports on phenol degrading *Pseudomonas* sp. (Yoon, 1998). This observation is very similar to present findings. The mechanism of cadmium resistance was through efflux, operating due to plasmid p1258 *Staphylococcus aereus*, and the mechanism of mercury resistance was volatilization, similar to *S. flexneri* (Yoon, 1998). Vasishta et al (1989) mentioned that *Pseudomonas aeruginosa* is resistant to both mercury and cadmium. We are not aware of any such metal resistance mechanisms operating in these TBTC tolerant bacterial strains.

Bacterial resistance to inorganic and organic mercuric compounds is one of the most widely observed phenotypes in eubacteria. Mercury resistant Gram's positive or Gram negative bacteria typically possess a mercuric reductase enzyme that reduces reactive Hg^{+2} to volatile and relatively inert elemental mercury vapour (Hg^{0}) (Foster, 1987; Barkay et al. 2003). Resistance to mercuric compounds is well studied for both Gram positive and Gram negative bacteria. The genetic determinants are usually located on plasmid or transposons, particularly in Gram negative bacteria (Summers and Silver, 1978). Another detoxification mechanism is the production of mercuric sulfide due to the action of H_2S on Hg. There has been speculations that permeability barriers to Hg^{+2} may also exist, limiting the access of the toxic ion to sensitive intracellular targets (Summers and Silver, 1978; Silver and Kinscherf, 1982; Robinson and Tuovinen, 1984).

In the present study, the high mercury tolerant bacterial strain 9(3A) may possess of one of these mechanisms. Fukagawa et al, (1994) have reported that out of the 55 bacterial strains which are TBT resistant (250nM), only 11 of them showed cross resistance to methyl mercury (20nM). Most of the isolates were identified as *Vibrio* sp.

resistance to methyl mercury (20nM). Most of the isolates were identified as *Vibrio* sp. and it is evident that TBT tolerant bacteria may possess common genetic determinants for mercury, cadmium and methyl mercury on plasmid or chromosomal genome.

Table 3.5 LD_{50} values of different heavy metals for the five TBTC tolerant bacterial isolates

Bacterial isolates	Metal Salt (mM)		
	Zn	Cd	Hg
***Pseudomonas aeruginosa* strain USS25**	2.5	2.7	4.7
***Pseudomonas aeruginosa* strain 25B**	4	1.2	6.5
***Pseudomonas fluorescens* strain 3(4Sub)**	5.4	1.2	5.5
***Pseudomonas aeruginosa* strain $5Y_2$**	1.8	0.9	4.9
***Pseudomonas stutzeri* strain 9(3A)**	0.3	0.25	8.5

3.8 Antibiotic resistance

All the five isolates were found resistant to most of the broad range antibiotics such as Penicillin, Ampicillin, Tetracycline, Chloramphenicol and Streptomycin including some other antibiotics viz. Neomycin, Spectinomycin, Rifampicin, Kanamycin, Nalidixic acid etc. (Table-3.6). *Pseudomonas aeruginosa* strain USS25 showed highest resistance to Ampicillin (LD_{50} 225ug/ml) and high sensitivity to Novomycin (LD_{50} 45ug/ml). It also showed considerably resistance to Penicillin and Streptomycin (LD_{50} - 60μg/ml) (Fig 3.12) (Table 3.7).

Wuertz et al (1991) has reported that TBT resistant (8.2μM) bacteria, which are isolates form Boston harbour were resistant to Cephalothin, Ampicillin, Novobiocin, Carbenicillin, Erythromycin and Penicillin. It has also been reported that most of the bacterial isolates, which can resist high level of heavy metal, can resist high concentration of different antibiotics. Bruins et al. (2003) have reported that a strain of

Pseudomonas pickettii which is resistant to cadmium as well as some broad range antibiotics. *Pseudomonas aeruginosa* has been reported to be multi-drug resistant like Ampicillin, Penicillin, Amoxicillin, Clavulanic acid, Piperacillin, Streptomycin, Gentamycin (Sader et al. 2002). Yomoda et al (2003) also reported that *Pseudomonas putida* has resistance to several antibiotics like Amikacin, Norfloxancin, Piperacillin, Ceftazidime, Tobramycin etc. These facts also satisfy the findings of Esiobu et al. (2002) which reported that *Pseudomonas* sp. has plasmid mediated multiple drug resistance such as Ampicillin, Penicillin, Tetracycline, Streptomycin, Kanamycin, etc. These reports clearly confirm that organotin resistant natural bacterial communities invariably demonstrate resistance to toxic heavy metals as well as commonly used antibiotics. The genetic determinants (gene) for microbial resistance are generally plasmid borne.

Table 3.6 Antibiotic resistance limit of TBTC tolerant marine bacterial isolates:

Serial No.	Antibiotic (Concentration in μg/ml)	25W	25B	3(4Sub)	9(3A)	$5Y_2$
1.	Chloramphenicol (25)	+	+	+	+	+
2.	Rifampicin (100)	+	+	+	+	+
3.	Tetracyclin (15)	+	+	+	+	+
4.	Kanamycin (400)	+	+	+	-	+
5.	Ampicillin (400)	+	+	+	+	+
6.	Nalidixic Acid (50)	+	+	+	+	+
7.	Streptomycin (240)	+	+	+	+	+
8.	Neomycin (100)	+	+	+	+	+
9.	Spectinomycin (100)	+	+	+	-	+
10	Penicillin (500)	+	+	+	+	+
11	Antimycin (300)	+	+	+	+	+
12	Amikacin (3)	+	+	+	+	-

Resistant = (+), **Sensitive** = (-)

Table 3.7 LD_{50} values of Antibiotics for TBTC tolerant *Pseudomonas aeruginosa* strain USS25:

Serial No.	Antibiotics	LD_{50} (μg/ml)
1	Novomycin	45
2	Streptomycin	60
3	Nystatin	120
4	Ampicillin	225
5	Antimycin	250
6	Kanamycin	220
7	Chloramphenicol	90

3.9 Selecion of potent strain for TBTC degradation study

Among the five isolates, isolate 25W showed rapid growth within 48hrs in MSM broth with 2mM TBTC and prominent degradation ability. There this isolate was selected for further characterization.

In order to determine the optimum concentration level of TBTC for growth of 25W it was grown separately in MSM broth with different concentration of TBTC ranging from 0.1mM to 3mM. It was interesting to note although an initial lag phase was restricted to 6-12h depending on the concentration, growth yield was high upto 2mM of TBTC in MSM broth, whereas at 2.5mM and 3mM of TBTC, marked decrease in growth was observed clearly (Fig 3.13). This indicated that they inherently possess inducible/constitutive enzymes to metabolize high levels of TBTC, certainly upto 2mM of TBTC. Above this level cells get killed due to cytotoxic effects on cell metabolism, which involves Ca^{2+} overload, cytoskeletal damage, and mitochondrial failure leading to apoptosis (Stridh et al. 1999). Thus optimum level was considered for growth of isolate 25W was found to be 2mM TBTC in MSM broth. It is interesting to mention that this isolate surprisingly exhibits 20 times higher TBTC tolerance as compared to other TBTC tolerant Gram positive or Gram negative bacterial strains viz. *Bacillus, Alcaligenes, Alteromonas, Vibrio* and *Pseudomonas* as it is known that these bacteria could tolerate up to 100μM TBTC (Yamada, et al. 1978; Boopathy and Daniels, 1991; Suzuki et al. 1992; Fukagawa, et al. 1992; Suzuki et al. 1994; Sinha, 1997).

TABLE 3.2. Total viable count of bacteria in marine water samples collected from various sampling sites

Sampling sites	Geographical positions		(cfu/ml± SE) x 10^2 in NA		cfu/ml ± SE in MSMA with			
Bombay High Oil fields	**Latitude**	**Longitude**	**0mM TBTC**	**0.1mM**	**0.1mM**	**0.2mM**	**0.3mM**	**0.4mM**
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
Goa Shipyard								
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

S.E. - Standard Error, N A -Nutrient Agar, MSMA - Mineral Salt Medium Agar

Table 3.4 Biochemical test for identification of TBTC resistant bacterial isolates

Characteristics	*Pseudomonas mendocina* P_2d	*Sphingomonas macrogaltabidus*	3Sub	9(3A)
Morphology of the organism	Short rods	Coccobacilli	Short rods	Short rods
Gram's stain	-	-	-	-
Motility	+	+	+	+
Catalase activity	+	+	+	+
Oxidase activity	+	+	+	+
HL Media (O/F)	O	O	O	O
V.P test	+	-	-	-
Indole	-	-	-	+
MR	-	-	-	+
Utilization of Gluc., Lac., Galac., Suc., Xylose, Arab., Mannose, Salicin, Raffinose	+	+	+	+
Utilization of Mannitol, Inositol, Sorbitol, Rhamnose	-	-	+	+
Utilization of Fructose, Maltose	-	+	+	+
Utilization- Alanine, Isoleucine	+	N.D	+	+
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	-	-	-	+
YellowPigment on King B Agar	-	+	-	-
Green Pigment on King B Agar	-	-	+	-
Lysine Decarboxylase	-	-	-	-
Argenine Decarboxylase	-	-	-	-
Ornithine Decarboxylase	-	-	-	-
Bioluminescence	-	-	-	-
Growth on Cetrimide agar	+	-	+	-
Pigment on cetrimide agar	Brown	-	-	-
Thiosulphate Citrate Bile Sucrose Agar	-	-	+	-
Eosin Methylene Blue Agar	-	-	-	-
Citrate utilisation	+	-	+	+
H_2S production	-	-	-	-
PHB Production	-	+	-	-
Growth at 4°C	-	-	-	-
37°C	+	+	+	+
43°C	+	-	+	+
Growth at pH 3.6	-	-	-	-
Growth at 1% dettol	-	-	-	-

(+)= Positive, (-)=Negative, O= Oxidative, N.D= Not done

Table 3.4 Biochemical test for identification of TBTC resistant bacterial isolates

Characteristics	25W	25B	5(Y2)
Morphology of the organism	Coccobacilli	Short rods	Short rods
Gram's stain	-	-	-
Motility	+	+	+
Catalase activity	+	+	+
Oxidase activity	+	+	+
HL Media (O/F)	O	O	O
V.P test	-	-	-
Indole	-	-	-
MR	-	-	-
Utilization of Gluc., Lac., Galac., Suc., Xylose, Arab., Mannose, Salicin, Raffinose	+	+	+
Utilization of Mannitol, Inositol, Sorbitol, Rhamnose	+	+	+
Utilization of Fructose, Maltose	+	+	+
Utilization- Alanine, Isoleucine	+	+	+
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	-	-	+
YellowPigment on King B Agar	-	-	-
Green Pigment on King B Agar	+	+	+
Lysine Decarboxylase	-	-	-
Argenine Decarboxylase	-	-	-
Ornithine Decarboxylase	-	-	-
Bioluminescence	-	-	-
Growth on Cetrimide agar	+	+	+
Pigment on cetrimide agar	Green	Dark Green	-
Thiosulphate Citrate Bile Sucrose Agar	+	+	+
Eosin Methylene Blue Agar	-	-	-
Citrate utilisation	+	+	+
H_2S production	-	-	-
PHB Production	-	-	-
Growth at 4°C	-	-	-
37°C	+	+	+
43°C	+	+	+
Growth at pH 3.6	-	-	-
Growth at 1% dettol	+	+	+
(+)= Positive, (-)=Negative, O= Oxidative, N.D= Not done			

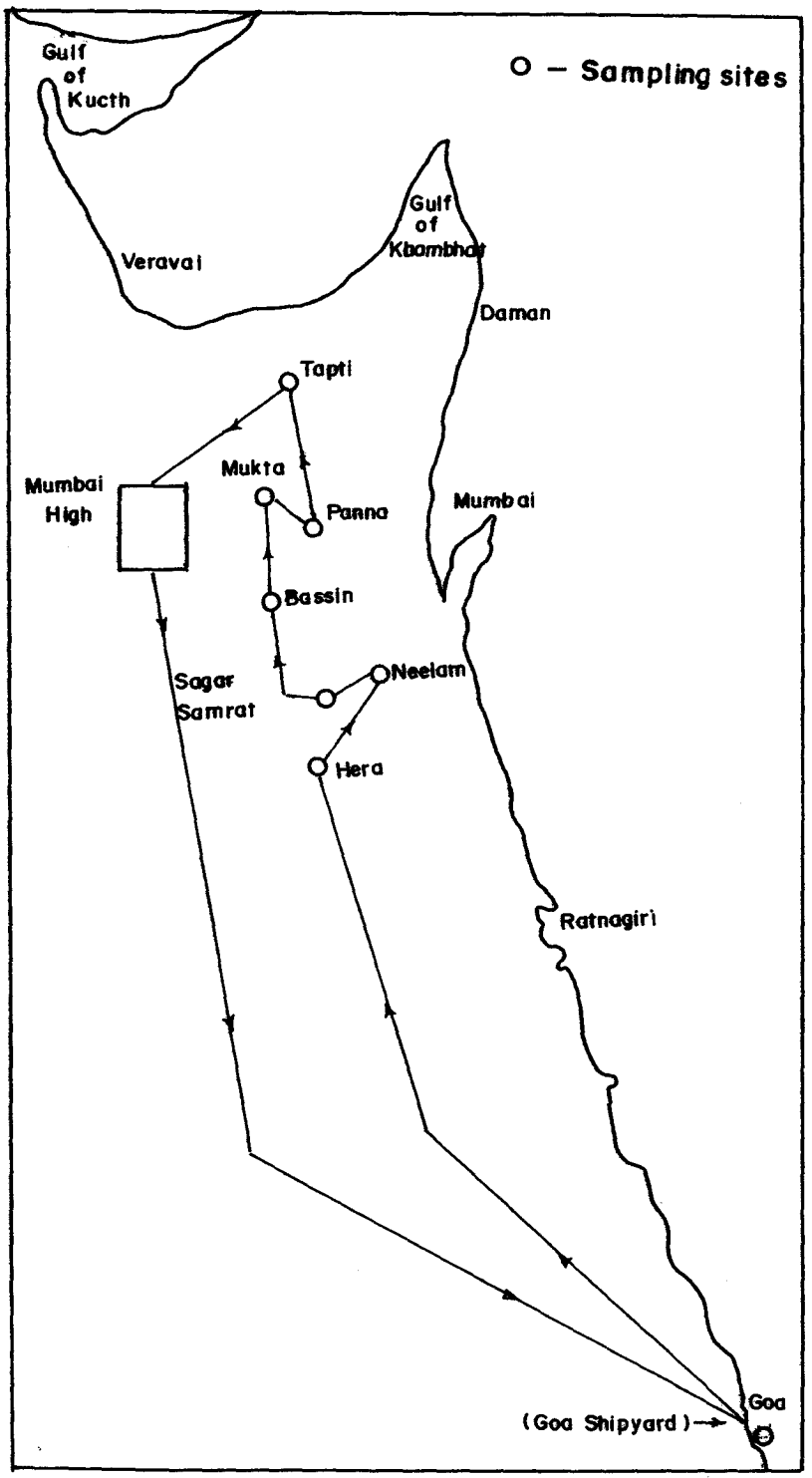


Fig. 3.1 Geographical location of sampling sites.

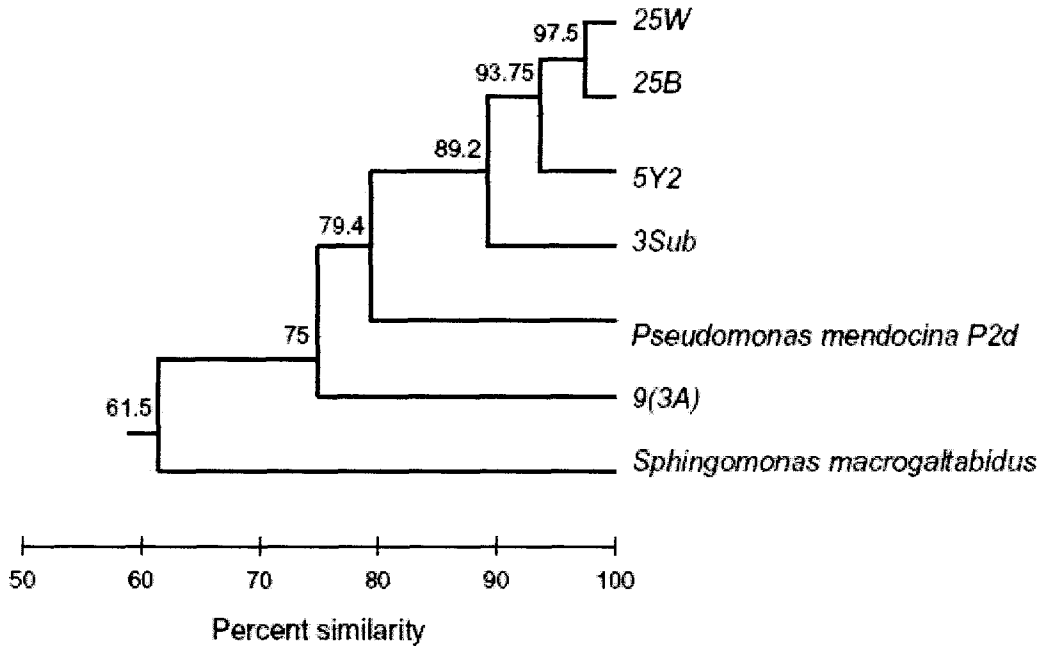


Fig 3.2 Phenogram showing similarity among different isolates

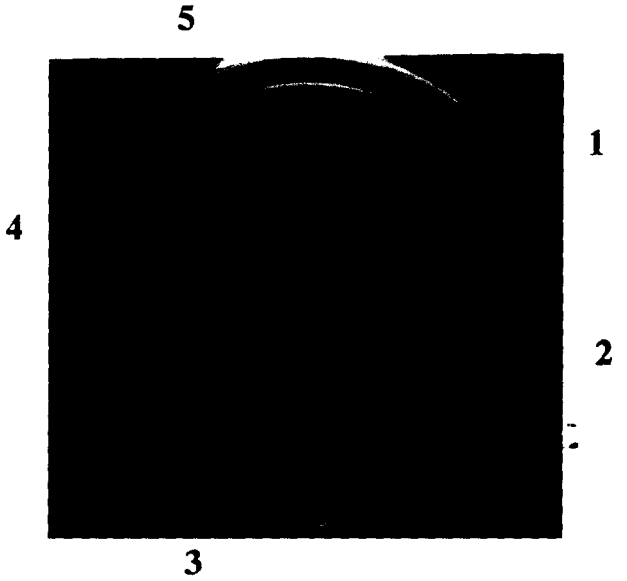


Fig. 3.3 Growth pattern of TBTC resistant isolates on MSM Agar supplemented with 2mM TBTC

1. ***Pseudomonas aeruginosa*** **strain 25B**
2. ***Pseudomonas aeruginosa*** **strain $5Y_2$**
3. ***Pseudomonas fluorescens*** **strain 3(4 sub)**
4. ***Pseudomonas stutzeri*** **strain 9(3A)**
5. ***Pseudomonas aeruginosa*** **strain USS25**

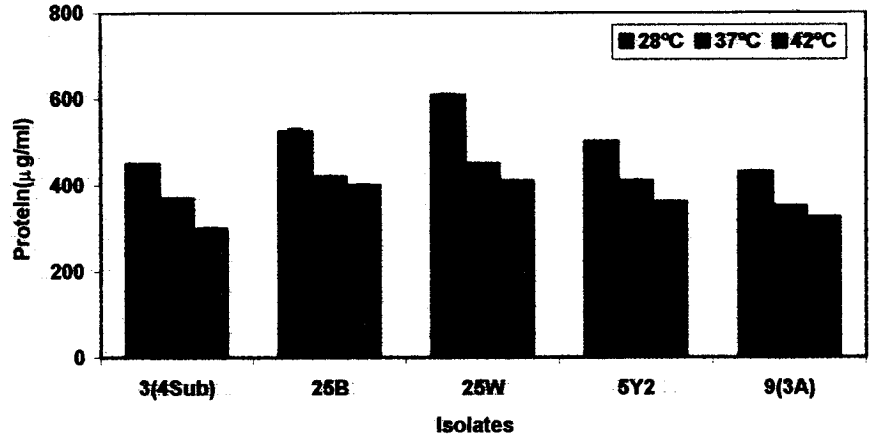


Fig 3.4 Optimum temperature for growth of five isolates grown in MSM+2mM TBTC

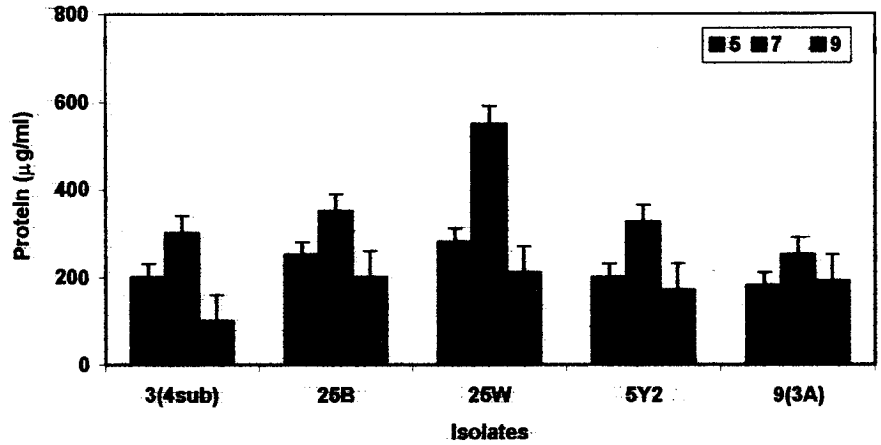


Fig 3.5 Optimum pH for growth of five isolates grown in MSM+2mM TBTC

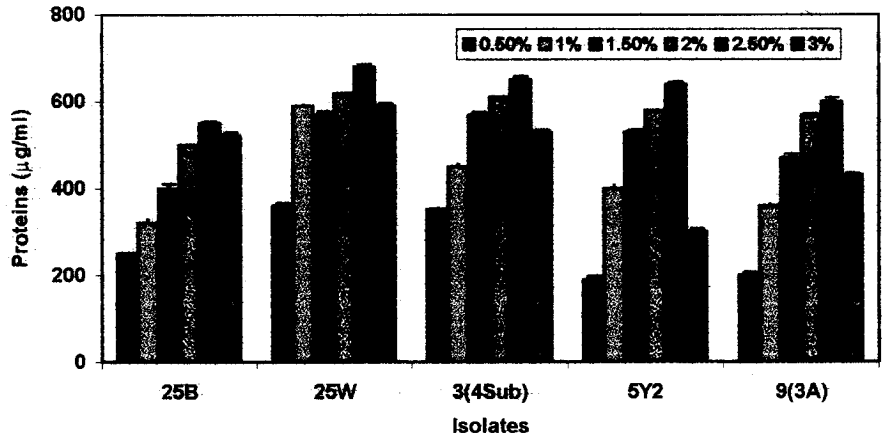


Fig 3.6 Optimum NaCl concentration for growth of five isolates grown in MSM +2mM TBTC

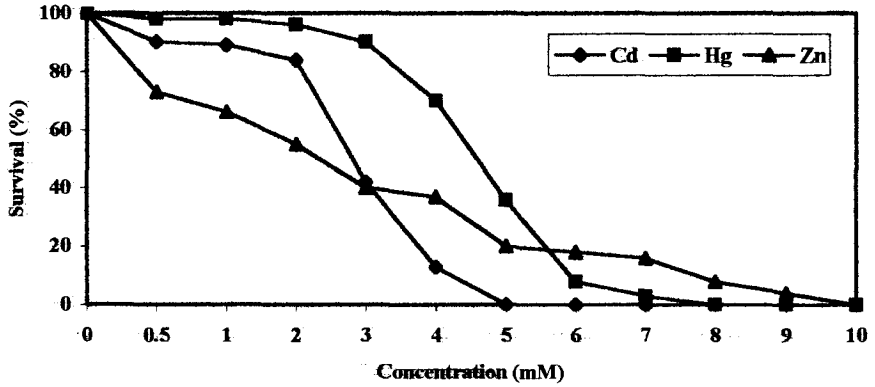


Fig 3.7 Survival of *Pseudomonas aeruginosa* strain USS25 expose to selected heavy metals

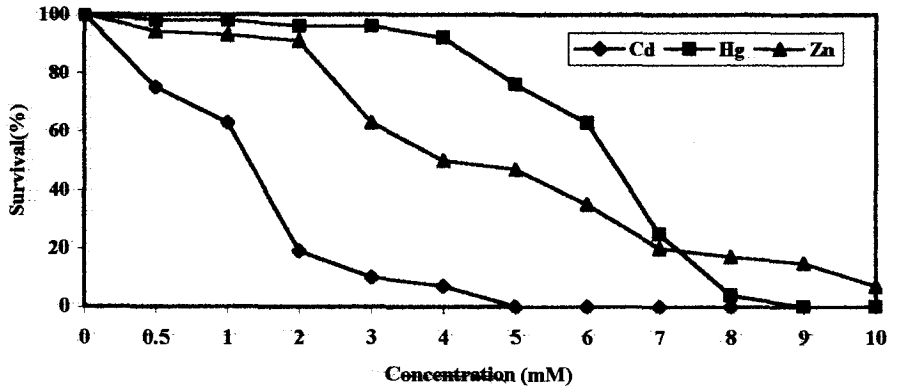


Fig 3.8 Survival of *Pseudomonas aeruginosa* strain 25B exposed to selected heavy metals

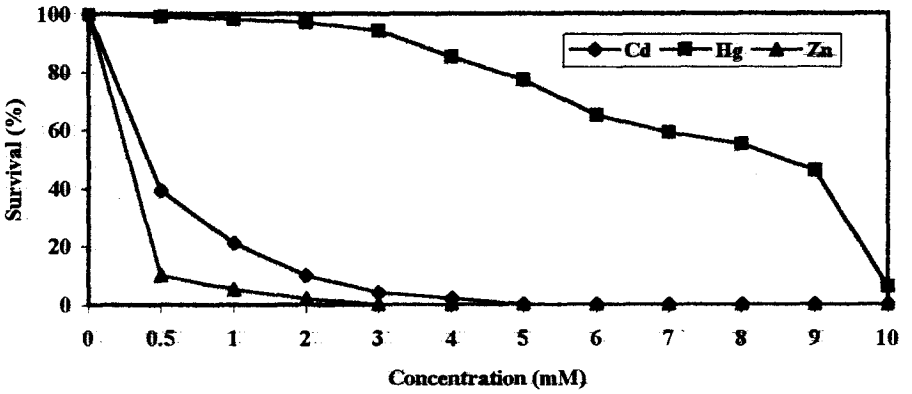


Fig 3.9 Survival of *Pseudomonas stutzeri* strain 9(3A) exposed to selected heavy metals

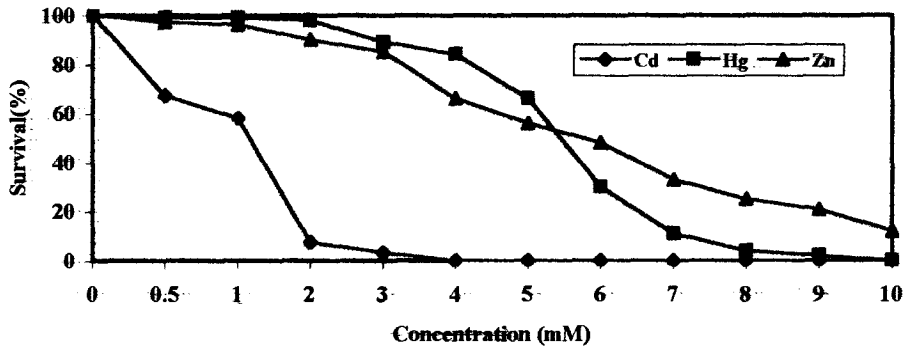


Fig 3.10 Survival of *Pseudomonas fluorescens* strain 3(4sub) exposed to selected heavy metals

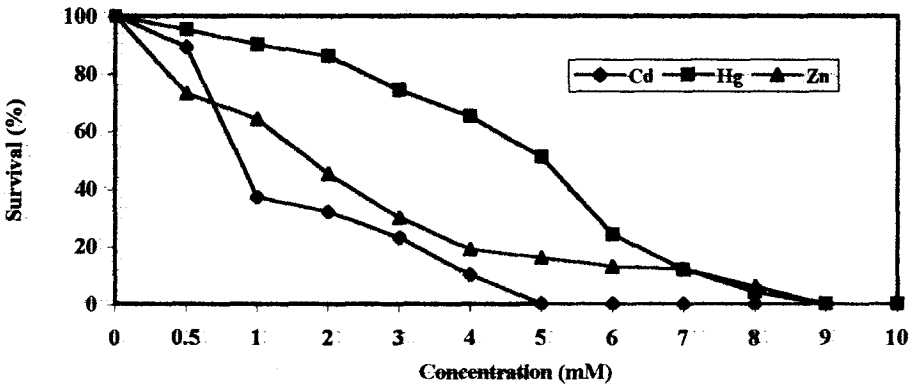
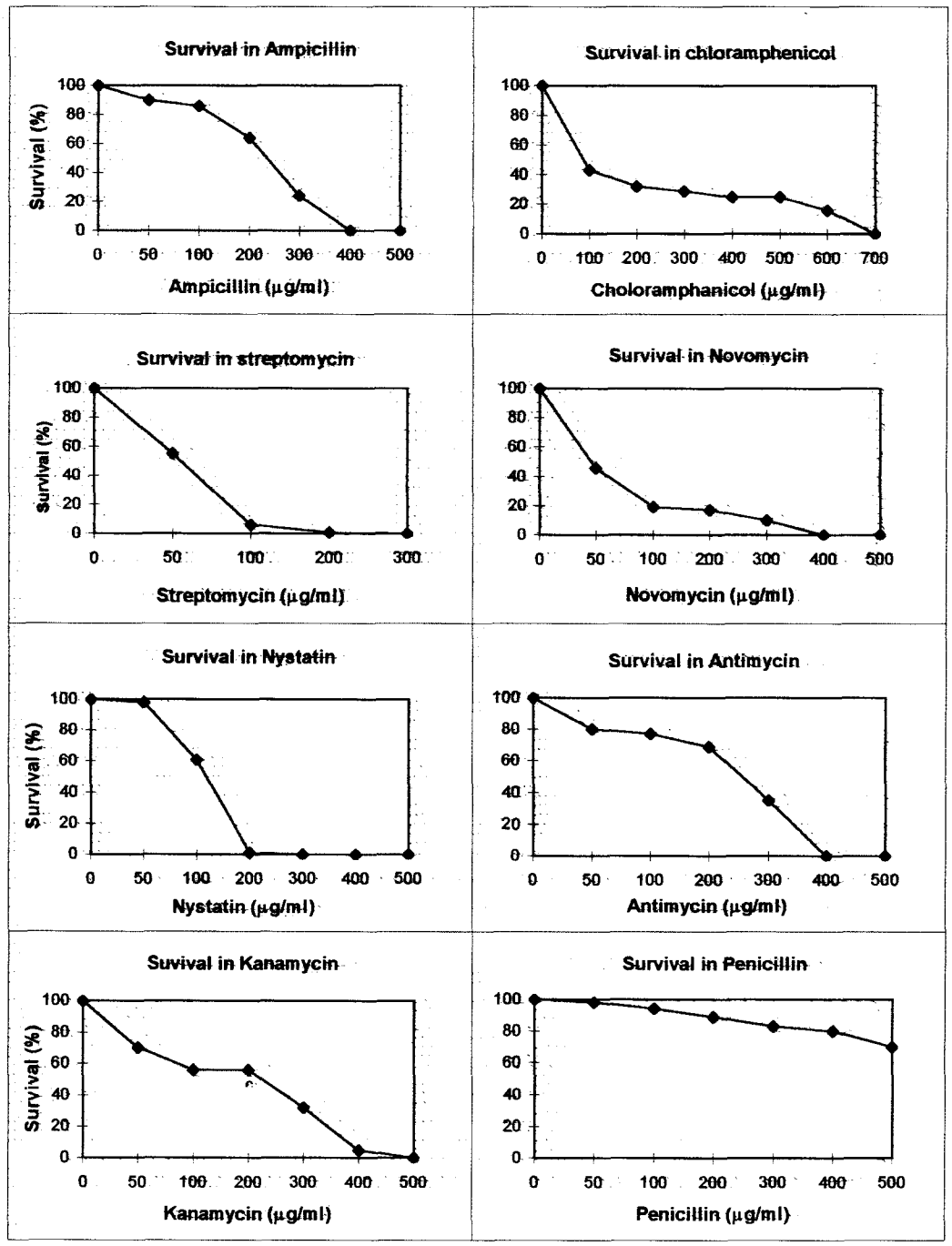


Fig 3.11 Survival of *Pseudomonas aeruginosa* strain 5Y2 exposed to selected heavy metals

Fig 3.12 Survival of *Pseudomoans aeruginosa* strain USS25 in various antibiotics



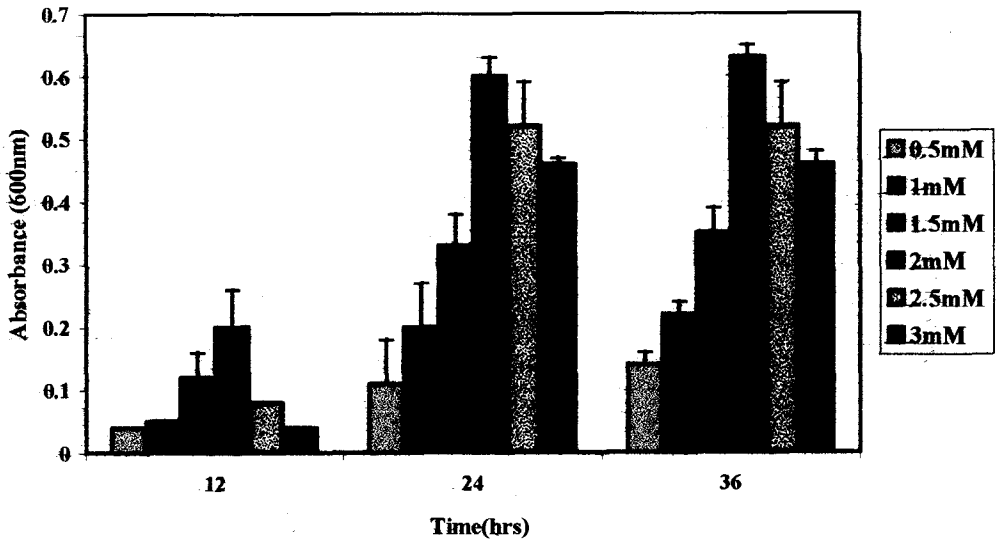


Fig 3.13 TBTC tolerance limit of *Pseudomonas aeruginosa* strain USS25 in MSM

Chapter IV

BIOCHEMICAL CHARACTERIZATION OF TBTC RESISTANT *Pseudomonas aeruginosa* strain USS25

4.1 Selection of suitable growth media for TBT degradation

The primary objective of the present work was to obtain bacterial strains, which can tolerate high concentration of TBTC and utilize it as a sole carbon source. It was only possible when the bacterial isolate was allowed to grow in minimal medium supplemented with TBTC as sole carbon source. It was also important to screen (design) a suitable medium for TBTC degradation studies which depicts faster utilization of organotin biocides by potential isolate, *Pseudomonas aeruginosa* strain USS25 (25W).

In order to find out a suitable growth medium, the selected bacterial isolate was grown in different media such as LB, Zobell Marine Broth (ZMB), MSM with 2mM TBTC. Irrespective of presence or absence of TBTC the isolate showed better growth in nutrient rich media like LB and ZMB compared to MSM containing TBTC (Fig 4.1, 4.2). No lag phase was observed in LB and ZMB with or without TBTC and log phase remained for 18 hrs. Initially the growth was found to be slow in LB and ZMB containing TBTC but after completion of log phase the growth was found slightly higher than the LB and ZMB having no TBTC. The isolate grew better in LB than the ZMB with or without TBTC. In comparison to nutrient rich media a lag phase of 6 hrs was observed in MSM containing TBTC. Lag phase was also extended upto 36hrs. Further the amount of growth in stationary phase was significantly lower.

Singh (1989) have reported that *E. coli* shows a reduction in lag phase when grown in complex medium compared to any minimal medium like MSM. The reduction of lag phase observed in complex medium indicated that reduction of toxicity of TBTC in that medium as compared to minimal medium. This reduction of toxicity could be due to complexation of TBT with organic compounds present in extract added in the complex medium such as tryptone, yeast extract etc. Organic compound like yeast extract, tryptone were present in LB and Peptic digest of animal extracts were present in ZMB. This made it clear that TBTC toxicity to cells could not be studied in any complex

media. This was the confirmation of already reported information that nutrient rich media does not help in degradation (Inoue et al. 2000) in the present case i.e. LB and ZMB.

MSM broth was found to be the suitable medium for TBTC degradation studies in the present investigation. It was found that toxicity of TBTC was more in MSM broth as compared to complex media tested. In case of *E. coli*, toxicity of TBTC was increased atleast three fold in minimal medium than in complex medium (Singh, 1989). As mentioned earlier in complex media TBTC forms complex with organic ligands present in complex media. As TBTC is the only organic source used in MSM, it confirmed that the test organism was utilizing TBTC as carbon source for their growth. The slow and steady growth in specified medium indicated that it was utilizing TBTC with slow biosynthesis. It is definite that the growth of the test organism was due to TBTC utilization. As there was no other organic compound in this medium, the reduction of TBTC bioavailability and toxicity due to complexation was not possible. Similar findings have been reported by Singh (1989) that *E. coli* cells were found to be more sensitive to TBTC in minimal medium than in complex medium. This medium was therefore selected for further characterization of TBTC degrading bacterial isolate, *Pseudomonas aeruginosa* strain USS25 (25W).

4.2 Effect of selected carbon and nitrogen sources on growth

4.2.1 Carbon sources: glucose, succinate and glycerol

The strain 25W responded differently when it was allowed to grow in presence of glucose, succinate, and glycerol in MSM containing TBTC (2mM). In the presence of glycerol, the isolate showed maximum growth (400μg/ml) at 3.5%. In case of succinate, the culture showed best growth (580μg/ml) at 3% and further increase in concentration of succinate or glycerol resulted in decrease of growth. When the isolate was allowed to grow with different concentration of glucose, it was observed that with increase in

concentration there was marked increase in growth, showed maximum growth (730μg/ml) at 4% of glucose (Fig 4.3).

It was observed that all three carbon sources supported the growth of the isolate in the medium having TBTC. As the culture shows best growth in presence of glucose, indicating that glucose was used as easily available carbon source. A similar observation was reported by Singh (1989), which indicated that compared to other carbon sources, glucose enhances the growth of *E. coli* in presence of TBT. The isolate showed the best growth in glucose followed by succinate and glycerol. Inoue et al (2000) has also proposed that the degradation of triphenyltin is possible in presence of glycerol and succinate as the carbon source. The optimum concentrations of these carbon sources were therefore used in study of TBT degradation.

The test organism (bacterium) was grown in MSM containing TBTC along with either glycerol (3.5%), succinate (3%) or both succinate and glycerol together. It was observed that in all three respective media the culture showed good growth without any lag phase while compare to TBTC alone as carbon source (Fig 4.4, 4.5, 4.6).

Inoue et al. (2000) have proposed that the combination of glycerol and succinate as carbon sources enhanced the Triphenyltin (TPT) degradation activity of bacterial isolates by several times compared to their individual use. It might be possible that succinate and glycerol also help in degradation of TBTC due to higher population density of the degrading organism as well as higher levels of enzymes involved in debutylation process. Further, in present investigation it was noted that isolate grew best when glucose was incorporated in medium. However, it was found that when glucose was present, organism was not degrading TBTC.

In order to confirm that the growth of the test isolate was supported by TBTC, the culture was grown in MSM + Ethanol (0.14%), MSM + 2mM TBTC + Ethanol and MSM+ 2mM TBTC (crude) respectively. It was observed that growth of the test organism was best in 2mM TBTC + Ethanol, because of higher availability of TBTC to bacterial cells. In case of crude TBTC supplemented medium the growth was

comparatively less and slow due to low availability of TBTC as a carbon source (Fig 4.7). Therefore, ethanol diluted TBTC has been used as carbon source for further studies.

4.2.2 Nitrogen sources: Potassium nitrate, Ammonium nitrate and Ammonium chloride

The isolate was grown in presence of ammonium nitrate, potassium nitrate and ammonium chloride in MSM+ TBTC (2mM). In presence of ammonium nitrate, the enhancement of growth (300μg/ml) was observed upto 0.5% of the salt concentration (Fig 4.8), with no further increase in growth with increased concentration of salt. When the culture was grown in presence of potassium nitrate, growth (350μg/ml) of the culture increased upto 1% and with further increase in concentration, growth decreased (Fig 4.2). The optimum growth was observed when the medium was supplemented with ammonium chloride. Maximum growth (480μg/ml) was observed at 0.5% of the salt concentration.

Among the three salts, ammonium nitrate and potassium nitrate showed similar pattern in growth response though the optimum concentration differed i.e. 0.5% and 1% respectively. In the present investigation ammonium chloride was found as best among the tested inorganic nitrogen sources. In contrast to this observation Cooney (1994) reported that TBT, DBT and monobutyltin inhibit NH_4^+ uptake in *Bacillus* sp. and *Pseudomonas* sp., because it affects the nitrification events in prokaryotes. They also reported that each organism exhibits its own pattern of response to butyltin compounds vis a vis nitrogen metabolism in identical ways or differently. Gadd (2000) has reported that nitrogenase activity was generally more sensitive to inhibition in presence of tri-, di- and mono-butyltin, which in turn reduce the growth of bacteria.

4.3 TBTC uptake by growing cells

In the MSM broth without additional carbon sources the uptake of TBTC was slow and steady. The uptake of TBTC was increased when cells were growing in

succinate and glycerol. It was observed that at optimum concentration of succinate and glycerol separately, the uptake of TBTC was almost equal irrespective of presence of either succinate and glycerol in medium. But uptake of TBTC was maximum when the culture was grown in the presence of optimum concentration of both succinate and glycerol in the medium (Fig 4.9).

Though the initial amount of inoculum was same, the growth and multiplication of cells in MSM was at a slower rate and the reason might be the low biosorption of TBTC in MSM broth. In presence of both succinate and glycerol, the cell multiplication was faster because of easy availability of carbon source. As the number of cells increases, TBTC also attaches to the cell surface because of high lipophilic property of TBTC. This could be another reason of faster degradation of TBTC in presence of both succinate and glycerol

The strain 25W showed faster biosorption of TBTC than the reported fungal strain *Aureobasidium pullulans,* which takes 30min for biosorption of TBTC (Gadd et al. 1990). The bacterial surface is highly negatively charged due to full deprotonation of surface carboxyl and phosphate sites (Fein and Delea, 1999) and in aquatic environment TBTC is present in cationic form (Hoch, 2001). These negative charges of bacterial cell surface and free cation of TBT (TBT^+) have ionic attraction, which might be a cause of biosorption of TBTC. It has been suggested that the organic moieties of organotins become associated with the surface of biological membranes rather than penetrating them (Avery et al. 1993). It has been suggested that there is release of K^+ from cells, arising from increased cytoplasmic membrane permeability which occurs because of organotin binding or as a consequence of insertion into the membrane (White et al. 1999).

The other possible mechanism of TBTC uptake of the strain *Pseudomonas aeruginosa* strain USS25, is chelation of organotin with EPS or siderophores which have affinity to bind with heavy metal and organic compound. As some strains of *Pseudomonas aeruginosa* are known to produce EPS (Holden et al. 2002) and

siderophores (Inoue et al. 2003), it is quite possible that the present strain also has adapted one of the above mentioned mechanisms. Usually, microorganisms take up normal nutrient cations and anions via specific, energy dependent transport system across epithelia or membrane (Hoch, 2001). Toxic ions are generally thought to enter cells by the same transport system as used for structurally related nutrient ions. This is the mechanism followed by *Pseudomonas fluorescens* for chromium uptake (Ohtake et al. 1987). Furthermore a species of *Pseudomonas* is known to accumulate TBTC up to 2% of dry cell weight and it is not influenced by the metabolic activity of the cell, rather it was attributed to adsorption at the cell surface (Avery et al. 1993). High lipid solubility of organotins ensures cell penetration and association with intracellular sites while cell wall components also play an important role in bioaccumulation of TBTC (Gadd, 2000). It has also been reported that *Pseudomonas aeruginosa* can take up heavy metals like lead, copper, cadmium by cysteine-rich transport protein associated with cell membrane (Chang et al. 1997). The present strain can be used for adsorption and concentration of TBTC present in the environment which will be an important control mechanism concerning distribution and fate of this organotin in the environment. In natural sea water, high salinity affects the biosorption of TBTC, due to less solubility of this organotin compound (Inaba et al. 1995).

4.4 TBTC degradation profile (Studies)

Spectrophotometric analysis of Bacterial Cell extract

In order to confirm the bioaccumulation of TBTC by marine isolate 25W, absorption spectra of chloroform extract of cell pellets were taken in range of 190-500 nm. It was observed that both TBTC and DBT absorb maximally at 241nm (Annexure-H.g). It is interesting to note that chloroform extract of cell pellet of *Pseudomonas aeruginosa* strain USS25 exhibits increase in peak intensity upto 72 hrs starting from zero hr in all the media tested (Fig.4.10). Page, (1989) postulated that higher bioaccumulation can helpful in degradation.

TLC analysis of Bacterial Cell extract

The chloroform extract, which was used for above analysis, were used for TLC analysis. The observation revealed that the pure TBTC compound has different profile (Fig 4.11, Lane-1) than the TBTC mixed in medium (Fig 4.11, Lane-2). When the cell pellet extract was observed on TLC, it was noted that the MSM+TBTC complex had became light and a faint spot was observed on the top of the lane (Fig 4.11, Lane-3). In case of supernatant extract there was no significant spot was observed (Fig 4.11, Lane-4). The Rf. values of TBTC and the transformed compound was 0.8 (Solvent front -15±2, TBTC - 12.5±1.5) and 0.94 (Solvent front - 15±2, Product - 14.1±2) respectively and compared standard values (Orsler and Holland, 1982).

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. The supernatant revealed no significant spot on TLC. This study demonstrate that outer membrane of bacterial cell plays an important role in TBT biodegradation in aquatic system, but limited number of these microorganisms have been identified up till now. It has been reported that two *Chlorella* species can metabolize TBT to the less toxic species DBT. The microalgae species *Skeletonem costatum* is capable of degrading TBT and some bacteria like *Pseudomonas aeruginosa*, *Pseudomonas putida* C and *Alcaligenes faecalis* are able to degrade organotin compounds (Hoch, 2001).

4.5 Time course study of TBTC degradation

The TBTC degradation study was performed with regular time interval. It was observed that within 48hrs, no degradation product could be detected on TLC plate. The TLC profile of the crude TBTC was different than the TBTC+MSM complex (Fig 4.12, Lane-1). The degradation product starts appearing after 7 days of incubation (Fig 4.12, Lane-2). The TBTC+MSM complex starts disappearing with successive incubation, which could be detected through 28 and 45 day extract (Fig 4.12, Lane-3, 4). The 75days

extract showed that there was disappearance of TBTC+MSM complex in a great extent (Fig 4.12, Lane-5).

Time course study revealed that though the culture utilizes TBTC during growth, in the initial phase, due to acclimatization or biosorption of TBTC by cells, the degradation process was very slow, hence no degradation spot could be detected through TLC in one or two days old cell pellet extract. Even the reason of not getting any degradation spot on TLC plates within 7 days, might be the presence of very little amount in the extract. In the extract of subsequent incubation period, culture started utilizing the TBTC+MSM complex and transforming it to some other compound. The disappearance of TBTC+MSM complex in successive extracted sample of 28 and 45 days also indicated the same. The 75days extract clearly indicated that TBTC was almost utilized by isolate 25W.

In laboratory condition, it was found that the half life of TBT found in the freshwater sediment was 360 days (initial concentration of 450ng/g) (Dowson et al., 1996), whereas *in situ* studies on TBT using marine sediments revealed that TBT half-life ranging from 0.91-5.2 years, but there was very little difference in time course study of TBTC degradation profile (Dowson et al. 1996; de Mora et al. 1989). In the present study the culture could degrade TBTC within 75 days which is very promising report.

4.6 Role of selected carbon sources (succinate & glycerol) on TBTC degradation

TLC analysis of chloroform extracts of cell pellets of 7days incubated in MSM + 2mM TBTC culture broth, revealed the presence of a dark brown spot with a fair light spot on the top of TLC plate (Rf- 0.94), was considered as a degradation product (Fig 4.13, Lane-2). The lower dark spot was corresponding to the TBTC + MSM complex of the first lane (Fig 4.13, Lane-2) , which was considered as control i.e. chloroform extract of MSM + 2mM TBTC without culture. TLC profile of chloroform extract of the cells grown in MSM + 2mM TBTC with optimum concentration of succinate and glycerol separately, showed marked decrease in TBTC+MSM complex and formation of

significant amount of degradation product (Fig 4.14) (lane-3 & 5). This observation indicates that succinate and glycerol has stimulatory effect on TBTC degradation. The cell extract of the isolate grown in presence of succinate and glycerol in MSM + 2mM TBTC showed a marked decrease in TBTC+ MSM complex and the formation of new compound (Rf-0.94), which was indicated by the dark spot in lane-4 (Fig 4.14). This observation confirmed that succinate and glycerol together induces faster degradation of TBTC.

Since TBTC is a membrane active lipophilic biocide, it significantly binds to the cell membrane of cultures which were grown in MSM supplemented with (2mM) TBTC. It is evident from the TLC profile that binding of TBTC to the cell membrane is very fast. It is interesting to note that some *Pseudomonas* sp. could accumulate TBTC up to 2% of its dry weight (Gadd, 2000).

The intensity of dark brown spot on TLC plate gradually decreased with time, suggesting that the cell envelop bound with TBTC, was getting utilized by the growing cells gradually. It is interesting to mention that the additional light brown spot of TLC profile may be indicative of TBTC degradation product.

Organotin degradation involves sequential removal of organic group from the tin atom, which generally results in a toxicity reduction (Blair et al. 1982; Cooney, 1988; Cooney, 1995). Several studies have been attempted to identify the mechanism of biodegradation of TBT to determine if it is successively de-alkylated from tri- to di- to monobutyltin (MBT) and finally a form of tin or whether the TBT is converted directly to MBT. Dibutyltin was the initial degradation product in Toronto Harbour sediments (Maguire et al. 1986), whilst MBT was the principal initial product in San Diego Bay (Barug, 1981; Stang and Selgmen, 1986). There are very few reports on biodegradation of TBT mediated by microorganisms viz. bacteria, fungi, cyanobacteria and green algae in aquatic environment (Cooney, 1988; Gadd, 1993). It is interesting to note that *Pseudomonas aeruginosa* could degrade only upto 2.5 µg/ml TBTC (Barug, 1981), whereas the present strain i.e. *Pseudomonas aeruginosa* strain USS25 could withstand

and degrade even upto (2mM) 650.9µg/ml TBTC in laboratory conditions. Debutylation of TBTC by microorganisms such as bacteria is one of the possible routes of detoxification as well as degradation.

4.7 Identification of TBTC degradation product using IR, NMR spectroscopy and Gas chromatography

The degradation product was purified by column chromatography and preparative TLC method. The yield of the compound was found to be 280 mg/gm of TBTC. Further a positive sodium fusion test of the compound produced white precipitate in presence of silver nitrate solution, which revealed the presence of chlorine atom in the molecule. The TLC analysis of TBTC, DBT and degradation product showed Rf values of 0.8, 0.6 and 0.94 respectively which was similar to standard values of TBT, DBT and derivatives of monobutyltin (Orsler and Holland, 1982). Gas Chromatography profile of culture broth also revealed the presence of some amount of DBT and major amount of TBTC, which was also comparable to standard GC profile of TBTC and DBT (4.14a, b). The IR spectra (Fig 4.15a, b) of standard TBTC and degradation compound exhibit several signals in the mid IR region, indicating the presence of organic moiety namely butyl group. This assignment gains credence based on NMR data (Fig 4.16a, b). Based on these spectra, formation of pure tin halide can be ruled out. Both the spectra give characteristic bands at 2850.3, 2927.7 and 2960.5 cm^{-1} which are assignable to C-H stretching vibration. A comparison of both IR spectra (Fig-4.15a, b) of standard TBTC and degradation compounds clearly indicates that degradation product is quite different from that of pure TBTC which is the reference compound. Although several signals are observed in both spectra in finger print region, certain difference can be observed which indicates that the degradation of all the organic groups attached to TBTC can be ruled out. The IR spectrum of degradation product exhibit a doublet at around 1580 cm^{-1} whereas reference compound (TBTC) does not show any signals at that region. The band at 1460 cm^{-1} in the reference compound is observed as triplet for the degradation product.

Similarly a strong band is seen at around 670 cm^{-1} for degradation product which is seen as a doublet for TBTC. These differences can be attributed to the presence of at least one butyl group in degradation product. The ^{1}HNMR spectra are presented and the chemical shifts of various protons are summarised in the following Table 4.1 and the labelling scheme for the NMR spectral assignment is shown in Fig 4.17. The spectrum of purified degradation product showed slight difference in chemical shifts (Fig 4.16a, b) from the standard TBTC and DBT (Atkinson et al. 1999) as shown in the following table. This can be attributed to solvent effect as earlier reported spectra were recorded in DMSO-d_6 (Atkinson et al. 1999) whereas in present study the spectra were recorded in $CDCl_3$. All the spectra exhibit characteristics resonance of the butyl group. The minor difference in the chemical shifts can be due to difference in number of alkyl groups attached to tin. The diamagnetic nature of tin can also be responsible for the observation of resonance with similar chemical shifts. The methyl (CH_3) protons (δ) resonate at around δ 0.9ppm. The methylene (CH_2) proton (α) absorbs at around δ 1.2ppm. β protons are observed down field compared to α and γ protons. The signals of γ protons were observed around δ 1.4ppm. The degradation product exhibit a sharp singlet at δ 2.404ppm and this signal was not observed in other two spectra. This singlet can be assign to a protons bound to tin. Based on this spectrum the degradation product can be assigned as monobutyltin complex with a hydrogen molecule attached directly to tin. In addition a positive chlorine test was observed for degradation product. The combined IR, NMR and halide analysis indicates the degradation product is monobutyltin compound.

Table 4.1 H^{1}NMR analysis: chemical shift of TBTC, DBT and degradation product in $CDCl_3$ (δ 7.26)

Chemical shifts	TBTC (Bu_3SnCl)	DBT (Bu_2SnCl_2)	TBTC Degradation Product
H	-	-	2.040
α	1.282	1.216	1.211
β	1.632	1.781	1.588
γ	1.309	1.427	1.315
δ	0.908	0.959	0.916

Dowson et al (1996) has reported that though DBT was the primary degradation product in most of the studies, it is not apparent on all the cases. The rate of debutylation from TBT to DBT is nearly identical to the rate of DBT to Monobuyltin (MBT). In the present study it has been observed that the culture was utilizing the MSM+TBTC complex and debutylating the TBTC molecule. The IR and ^{1}HNMR spectrum of TBTC and DBT was found to be similar with the literature survey of (Atkinson et al. 1999). The ^{1}H NMR spectrum of the degradation product showed signals which is similar to TBTC signals, but in addition to this, spectrum also showed singlet at δ 2.040ppm. On the basis of integration values of NMR data, it was observed that the molecule consist of one butyl and one hydrogen group. Thus the molecular structure of the compound should be $BuSnHCl_2$ (Monobutyltin dichloro hydride). Several cultures, which are reported to be resistant to TBTC, are known to detoxify this compound by conversion of DBT. DBT is comparatively less toxic compound and easily biodegradable (Seligman et al. 1986). The culture 25W also grows on DBT as sole source of carbon reflecting the detoxification mechanism being initiated with conversion of TBT to DBT and then DBT is then further utilised to support growth of the organism and transform to MBT.

4.8 Role of thiol (β- mercaptoethanol) and Chelating agent (EDTA-Na_2) on TBTC toxicity

When the culture was grown in MSM+TBTC (2mM) with varying concentrations of β-mercaptoethanol (0.01%- 2%) and EDTA-Na_2, it was observed that with increasing concentration of thiol or EDTA-Na_2 The growth of isolate was also increased upto 1% of thiol or EDTA-Na_2 separately (Fig 4.18). At higher concentration growth of the culture was inhibited. Considering 1% of thiol or EDTA-Na_2 as an optimum concentration, the culture was grown with varying concentration of TBTC (1mM – 9mM) in MSM+ 1% thiol or EDTA-Na_2. It was observed that in presence of thiol or EDTA the culture could tolerate upto 9mM TBTC, showing maximum growth at 4mM and 5mM for thiol and EDTA respectively (Fig 4.19).

It was interesting to note that the culture which was growing in presence of 2mM of TBTC could grow up to 7mM and 5mM of TBTC in presence of thiol and EDTA-Na_2. The successive reduction of growth at higher concentrations of thiol and EDTA-Na_2 could be due to decreasing enzyme activities (Lo et al. 2003). This observation indicated that thiol and EDTA-Na_2 has a similar effect of reducing the TBTC toxicity. Generally it was observed that heavy metal resistant microorganisms such as yeast, cyanobacteria (*Synechococcus* sp.) and bacteria (*Pseudomonas putida, Vibrio alginolyticus*) induce metallothionine like proteins, which are capable of binding heavy metal ions via thiolate co-ordination. This plays a very important role in sequestering metal ions intracellularly by rapidly binding to them as they enter the cell, thus effectively reducing the toxicity (Higham et al. 1984; Robinson and Tuovinen, 1984; Gadd, 1986; Khare et al. 1997). The present investigation showed that thiols and EDTA-Na_2 reduce the toxicity of TBTC to cell. A similar kind of observation was also made by Dubey and Rai (1989a), which revealed that mercaptoethanol and dithiothireitol reduced the toxicity of chromium and tin, dithiol being more protective than mono-thiols. The better protective efficiency of dithiothreitol and mercaptoethanol could be due to restoration of proton transfer across the biomembrane. The toxicity of the heavy metal or organic compounds gets reduced by formation of complexes with S^- and SO_4 ions, which are unable to cross the interior of biomembranes because they are too large (Dubey and Rai 1989b). In case of *E.coli,* the inhibitory effect of TBTC on the growth was reduced by 10-25% in presence of dithiothreitol (Singh, 1989). It has been reported that some of the organotin compounds, including TBTC, act like organomercurials and inhibit the activity of many enzymes by interacting with thiol groups of protein (Hallas and Cooney, 1981a). This inhibitory effect of TBTC on above process was completely abolished in presence of various mono- and di-thiols (Singh, 1989).

In a case study, it was observed that aqueous EDTA could strongly compete with the bacterial surface for aqueous heavy metals like cadmium and the presence of aqueous EDTA significantly diminishes Cd adsorption onto *B. subtilis*. The most

possible reason was the formation of Cd-EDTA complex (Fein and Delea, 1999). In *Pseudomonas aeruginosa,* β-mercaptoethanol and EDTA act as best desorption agents of heavy metal ions like Cu^{+2}, Hg^{+2}, Pb^{+2} (Chang, et al. 1997).

4.9 TBTC induced exopolymer production.

When the strain 25W was grown in ZMB, produced 47 μg/ml of EPS after 48hrs incubated at 28^0C. It is interesting to note that culture grown in TBTC (2mM) supplemented ZMB produced 90.3μg/ml EPS. Interestingly, the isolate 25W showed significant production of EPS (i.e. 20μg/ml), when cells were grown in MSM with (2mm) TBTC. Hence we can hypothesize that *Pseudomonas* sp. may be producing EPS for sequestering the toxic effects of TBTC and rendering the cells more tolerant to TBTC (Fig-4.20).

EPS production is more obvious under nutrient rich conditions probably due to increased biomass, as observed in the case of strain 25W in ZMB. As lipid solubility nature of the organotin compound ensures the association of TBT molecule to cell wall component (Gadd, 2000), it is quite possible that attachment of TBT molecule to the cell surface also enhances the EPS production. It has been reported that cell attachment increases the EPS production in *Pseudomonas aeruginosa, Pseudomonas putida* and *Pseudomonas fluorescens* (Read and Costerton, 1987; Danese, et al. 2000). Exopolymers itself also possess high binding affinities for many suspended compounds contained in sea-water. It forms a micro-environment around the microbial cell, which allows it to operate, metabolise and reproduce efficiently under controlled conditions and even in presence of heavy metals and toxin (Decho, 1990, Ashtapurte and Shah, 1995a). *Sphingomonas galtibidus* and *Vibrio* sp. are reported to produce gellan like polymer (Sarradin et al. 1995) and *E.coli* also produces exopolysaccharides (Pollock et al. 1994). There are reports that Gram's negative bacteria that produce exopolysaccharides are resistant to antibiotics (Pollock et al. 1994). EPS from *Ochrobactrum anthropi* has been used for removal of chromium, cadmium and copper from aquatic environment

(Ozdemir et al. 2003). The aromatic hydrocarbon degrading bacterium *Pseudomonas putida* G7 produces exoploymer, which has high metal binding activity (Kachlany et al. 2001). EPS production by *Pseudomonas aeruginosa* has been well characterized (Ashtaputre and Shah, 1995b).The EPS described in the present study increases our understanding of the mechanisms of TBTC resistance and degradation by this well known protobacterium.

4.10 TBTC induced surfactant activity of exopolymer

Chloroform extraction of supernatant yielded a thick emulsion, which was depicted to be water in oil type. Such water in oil type surfactants are known to reduce the interfacial tension behaviour between the liquids, thereby exhibiting the phase separation. The use of water in oil (W/O) micro emulsions as a means for facilitating enzyme dispersal in organic solvents has been widely reported (Luisi and Majid 1986). The micro-emulsions have an advantage of being reproducible, stable and facilitating dispersion of enzymes at molecular level, thereby giving a very efficient enzyme activity. The yield of EPS obtained by alcohol precipitation of cell supernatant, after lyophilisation was found to be 4.9mg/gm of cell mass. The release of polymer into the supernatant also confirmed hydrophobicity of the cell surface, which was determined by the BATH test (Neu, 1996). The EPS obtained from the TBTC grown culture broth showed 59% emulsifying activity (Ao = 0.99, At = 1.58) whereas the EPS obtained from glucose grown culture broth showed only 2% emulsifying activity (Ao = 0.99, At = 1.01). The emulsification activity of the polymer was observed in benzene–water system. It was observed that EPS obtained from MSM+2mM TBTC culture broth has more emulsifying activity than the EPS obtained form MSM+4% glucose culture broth (Fig 4.21), because the height of interphase increased sharply in presence of EPS obtained from MSM+ TBTC broth.

The polymer was found to be partially soluble in methanol and benzene. Further characterization of the compound showed the presence of protein and sugar in concentration of 340 μg/mg and 480 μg/mg respectively.

The EPS gave a turbid solution in distilled water, when 10 μl of TBTC was added. TLC profile of pellet and supernatant of turbid solution extracted in chloroform showed that TBTC was associated with pellet. However the turbid aqueous chloroform layer showed no spot of TBTC on TLC analysis. Biosurfactants are known to have several lipophilic and hydrophobic moieties, which holds the organic compounds in water as emulsion (Bognolo, 1999). Separation of such complex compounds is difficult by simple extraction procedures. In the present study TBTC seemed to be present in aqueous phase in such a complex association, which could not be extracted with chloroform (Cooper and Zagic, 1980).

As the EPS showed surfactant activity during extraction of cell pellet, the surfactant activity of the isolated compound was checked with equal volume of benzene and distilled water. Three layers were formed after vortexing the mixture. It was interesting to note that with increasing concentration of surfactant the height of benzene layer got rapidly reduced, however the height of interphase layer increased very sharply (Fig 4.22) (Lane-3). Though the experiment was also performed with EPS obtained from culture grown in MSM+ 4%Glucose, it did not show the sharp increase in height of interphase (Fig 4.22) (Lane-2).

To monitor the effect of TBTC on surfactant in chloroform-water and Benzene-water mixture (equal volume), 100 μl of TBTC was added. It was significant to note that there was formation of interface in chloroform-water with increasing concentration of surfactant while in control system i.e. only chloroform-water system, very little change was observed with increasing concentration of surfactant (Fig 4.23). In case of benzene-water system with 100 μl of TBTC also showed increase in interphase with increasing concentration of surfactant, but the control showed very slight increase in height of interphase with increase in concentration of surfactant (Fig 4.24).

The effect of surfactant on TBTC in distilled water showed turbidity with increasing concentration of surfactant. Critical Micelle Concentration (CMC) (Ron and Rosenberg, 2002; Holden et al. 2002) showing maximum increase in absorbance at 450 nm, was found to be 1.5 mg for 3ml of reaction mixture with 10 µl of TBTC (3.7M) with further increase resulting in decrease in optical density (Fig-4.25). It was observed that low molecular weight biosurfactants, which have low CMC values, increase the apparent solubility of hydrocarbons by incorporating them into the hydrophobic cavities of micelles (Ron and Rosenberg, 2002). The CMC value of the test surfactant i.e. 0.5mg/ml (1.5mg/3ml reaction mixture), is also quite low which indicates that this surfactant had low molecular weight. Compared with synthetic surfactants, which can be either stimulatory below the CMC or inhibitory above the CMC, biosurfactant reduces the interfacial tension in two phase hydrocarbon non-aqueous phase liquid and water mixture, while being nontoxic and fully biodegradable (Holden et al. 2002).

The above characteristics of emulsifying activity of EPS of *Pseudomonas aeruginosa* USS25, gave a clear idea that biosurfactant has immense importance in solubilizing the hydrophobic pollutant to make it available to living cells. The emulsion formation not only assists in cell substrate interaction (Joseph et al. 1990) but also concentrate the minute amount of the substrate in water oil emulsion thus scavenges and cleans the environment from pollutants. The purified surfactant, which was obtained from alcohol precipitation and lyophilization, was insoluble in water and partially soluble in alcohol and benzene showed the hydrophobic nature of the surfactant.

The biochemical characterization of the surfactant produced by *Pseudomonas aeruginosa* strain USS25 showed that it partially consists of sugars and protein. The surfactant produced by *Pseudomoans oleovorans* has been reported to consist mainly of lipopolysaccharides, but also contains a significant degree of proteins and lipids (Schmid et al 1998). Though the lipid profile was not performed in the present study, it is known from earlier studies that several species of *Pseudomonas aeruginosa* produce

rahmnolipid which is responsible for cell surface hydrophobicity (Ron and Rosenberg, 2002; Miller, 1995). Rhamnolipid containing biosurfactant produced by *Pseudomonas aeruginosa* influences various processes related to hydrocarbon degradation (Maier and Soberon, 2000; Noordman, et al. 2002; Holden et al. 2002). As a viscosifier and stabilizer, alginic acid from *Pseudomonas aeruginosa* was also well characterized (Ashtaputre and Shah, 1995b).

It is noteworthy that the EPS obtained from MSM+2mM TBTC culture broth, showed more surfactant activity than the EPS obtained from MSM+ 4% glucose. When the effect of TBTC on surfactant activity was studied, it was noted that in presence of TBTC, surfactant activity increased. The possible reason could be in presence of TBTC, cell produces more bioemulsifier, which can act on TBTC molecule to bring it to water phase, so that the cells can utilize the compound as a carbon source. This might be the sole mechanism of the strain 25W, which could tolerate and grow in such a high concentration of (2mM) TBTC. Ron and Rosenberg (2002) have reported that emulsifier is advantageous when bacterial growth is slow or in presence of high concentration of pollutant or when the pollutant consist of the compounds that are difficult to degrade, such as polyaromatic hydrocarbons. The BATH test of the EPS obtained form TBTC grown culture showed 50% more emulsifying activity than the EPS obtained from glucose grown culture, which clearly indicates that in presence of TBTC the characteristics of EPS changes which in turns gave more emulsifying activity to dissolve the TBTC in the medium. The cell surface hydrophobicity of *Pseudomonas aeruginosa* has already established by BATH test with reference to cell adhesion on stainless steel surface (Vanhaecke and Pijck, 1988).

Biosurfactant from *Pseudomonas aeruginosa* has been studied mostly in liquid cultures (Holden et al. 2002). The test surfactant, isolated and purified from *Pseudomonas aeruginosa* USS-25 showed significant role in removing TBTC from MSM broth. It has been suggested that biosurfactant may prove useful in a broad

spectrum of potential applications, which is mostly accomplished by synthetic surfactants (Parkinson, 1985). The present biosurfactant would be more effective than synthetic surfactants in increasing bioavailability and degradation of the emulsifying hydrophobic compounds.

4.11 TBTC induced fluorescent pigment synthesis and its characterization

Effect of TBTC on pigment production

Pseudomonas aeruginosa strain USS25 gave a dark green pigmentation on MSM + glucose (0.5%) + 2mM TBTC Agar plate (Fig-4.26). When the culture was grown in MSM + glucose (0.5%) (Fig 4.27, Lane-2) and MSM + glucose (0.5%) + 2mM TBTC respectively, it was observed that pigmentation got enhanced in presence of 2mM of TBTC (Fig 4.27, Lane-3). This extracellular pigment turned brown on further incubation (after 48 hrs) like the other *Pseudomonas* sp. (Decho, 1990; Deley, 1964).

In order to observe the effect of increasing concentration of TBTC on pigment production, the culture was grown in MSM+glucose (0.5%) with increasing concentration of TBTC from 0.1mM to 2.5 mM. When the culture was grown in MSM + (0.5%) glucose + (2mM) TBTC, enhanced extracellular yellow-green pigmentation was observed compared to the control without TBTC. It was very interesting observation that absorption spectra of culture supernatants in the wavelength range of 190–500nm revealed a predominantly intense peak at 250 nm along with a weaker peak at 330 nm (Fig 4.28). The spectrophotometric scans of the pigment showed increase in intensity of pigment with increasing concentration of TBTC from 0.5mM to 2mM TBTC (Fig 4.28).The peaks of pigments of MSM + 2mM TBTC grown cells were significantly higher as compared to the TBTC deficient control cultures broth (Fig 4.28). These results indicated that TBTC significantly stimulates production of extracellular pigments, which may be involved in TBTC sequestration and degradation.

As pigments are also secondary metabolites like EPS, the objective was to observe the role of TBTC on pigment production. The above observations indicated that with the increasing concentration of TBTC, pigment production of the isolate also increased. It is quite possible that production of pigment acts as a defensive mechanism for cells against TBTC. Inoue (2000) reported the involvement of pyoverdin in co-metabolism of triphenyltin (TPT). In present study also pigment has a role in cometabolising the TBTC or inhibit TBTC to enter in to the cell. It has been reported that pyoverdin from *Pseudomonas chlororaphis* CNR15 has a major role in triphenyltin degradation. Among F-I and F-II fractions of pyoverdin, F-I fraction seems to degrade TPT to monophenyltin without releasing an intermediate. It should be noted that pyoverdins are generally produced in response to iron starvation. This suggests that organotin degradation by pyoverdin may be considered a co-metabolism. Therefore, the degradation reaction of organotin compounds is likely to occur by chelation of certain types of siderophores (Inoue et al. 2003). The bacterium *Bacillus megaterium* ATCC 19213 is known to produce two hydroxamate siderophores, which chelates with aluminum, iron and organic substrate and removes them from environment (Hu and Boyer, 1996). Further characterization of this pigment and its role in TBTC resistance or degradation will help us to understand the role of pigment in removal of toxic substance from aquatic environment.

Identification of fluorescent pigment.

This extracellular yellow-green fluorescent nature of the pigment of the culture was indicating that the pigment was either pyoverdin or pyocyanin (Fig 4.29). The fluorescent nature of the pigment of both the culture was confirmed by growing both the isolate in King B medium (King and Adler, 1964). It was interesting to note that the culture, which was producing green pigment, got converted to brown on further incubation. The culture was producing pigment even when it was kept in static state in ordinary laboratory condition. This pigment from culture broth got easily extracted with

chloroform and the extract gave blue colour, which is one of the characteristic features of the phenazine pigment (Gerber, 1973). The spectrophotometric analysis of the chloroform extract showed highest absorption at 250 nm and weak peak in the range of 409nm which was exactly correlating with the spectral analysis of standard phenazine methosulfate (N-Methyldibenzo pyrazine methyl sulfate salt) dissolved in chloroform.

The pigmented culture broth of 25W was used for spectrofluorimetric study separately, in which culture broth was excited at 423nm and sharp emission was observed at 485nm (Fig 4.30). The pigment was confirmed as phenazine pigments as the colour got intensified on addition of few drops of concentrated HCl to pigmented culture broth. This is a distinctive character of phenazine compounds (Gerber, 1973).

The observation clearly indicated that *Pseudomonas* sp. produces phenazine pigments during stationary phase of growth. The production of pigments like pyocyanine, phenazine is very common in different strains of *Pseudomonas aeruginosa* (Krieg and Holt, 1984). As it was observed that the cultures produced pigment even in ordinary laboratory conditions, it confirmed that the pigment is phenazine, because phenazine pigment appears if the culture has been kept for long time in ordinary laboratory condition (Krieg and Holt, 1984). The darkening of the culture broth with Concentrated HCl also proves that *Pseudomonas aeruginosa* strain USS25 produces phenazine pigment. Phenazine produced by cells of the selected isolate is a distinctive and novel feature of TBTC resistant *Pseudomonas.* sp.

4.12 SDS-PAGE analysis of TBTC induced protein.

Heavy metal induced specific polypeptides play a very important role in metal ion homeostasis in cyanobacteria (Olafson et al. 1979). Similarly some bacterial strains are also known to synthesize cystine rich low molecular weight polypeptides which play an important role in biosorption of these metals and ultimately resulting in immobilization of toxic metals and protection of vital metabolic process which may get adversely affected due to toxic effect on enzymes (Higham, et al. 1984; Gadd, 2000).

SDS-PAGE analysis of Pseudomonas *aeruginosa* strain USS25 clearly revealed that cells expose to TBTC at 0.1 mM and 0.5 mM levels inhibit synthesis of certain proteins (fig 4.31). The protein profile of the extracted extract obtained from cells grown in MSM + glucose (0.5 %) and MSM + glucose (0.5 %) +2mM TBTC showed almost similar pattern (lane-4 & 5). The protein profile of the cell extract obtained from cells grown in MSM +1mM TBTC also revealed a faint protein band (Fig 4.31, lane-6). It is interesting to note that protein of MSM+2mM TBTC grown cells clearly revealed a 45KDa protein, which was induced by TBTC(2mM) (Fig 4.31, lane-7). In addition to this novel protein of 45KDa we have noticed intensification of 27KDa protein, which is also present in control lane but faint.

Similar reports have been mentioned earlier as TBTC resistant *Vibrio* sp. also exhibited enhanced synthesis of two polypeptides of 30 KDa and 12 KDa when cells are grown in presence of 125 µM TBTC (Fukagawa, et al. 1992). The expression of 45 KDa proteins in *Pseudomonas aeruginosa* strain USS25 was different from earlier observations. The other protein band, which is gets intensified in presence of 2mM of TBTC is also note-worthy. The possible reason could be that this level of TBTC which is optimum for growth and induction of the isolate of metallothionine like proteins. So at that concentration only the 45KDa protein gets induced, while the 27KDa protein, which is present in all the conditions, gets intensified. This indicated that 27KDa protein is a constitutive protein. Certain heavy metal tolerant bacteria such as *Pseudomonas putida* and *Vibrio alginolyticus* commonly show induced synthesis of low molecular weight, cysteine rich polypeptides like proteins which bind with specific metals such as cadmium and copper making them unavailable to the bacterial cells (Higham et al. 1984; Gadd, 1992, 1993; Pazirandeh, et al. 1995, 1998). There is evidence that the site of toxic action of organotins may be both at the cytoplasmic membrane and intracellular level. While studies on the effect of TBT on certain microbial enzymes indicated that in some bacteria TBT can interact with cytosolic enzymes (White et al. 1999). TBT acts on mitochondria and chloroplast by causing ion exchange through membranes and inhibiting

phosphorylation and ATPase. Some of the enzymes like glucose dehydrogenase, glucose-6-phosphate dehydrogenase, β-galactoside galactohydrase and alkaline phosphatase are affected by TBT except for ATPase and NADH oxidase. In case of *Bacillus* sp ATPase gets activated in presence of TBTC. The NADH oxidase activity was stimulated as the concentration of TBT was increased in relatively resistant strain of *Pseudomonas putida* TBT-6 and *Pseudomonas* sp BP-4 (Tsing and Cooney, 1995). The above identified proteins might be one of them. These studies have confirmed that even toxic compound could affect protein synthesis it has been reported that a 45 KDa protein produced by *Acinetobacter radioresistens* is highly effective in stabilizing the solubilization of hydrocarbon, including polycyclic aromatic hydrocarbon (Ron and Rosenberg, 2002).

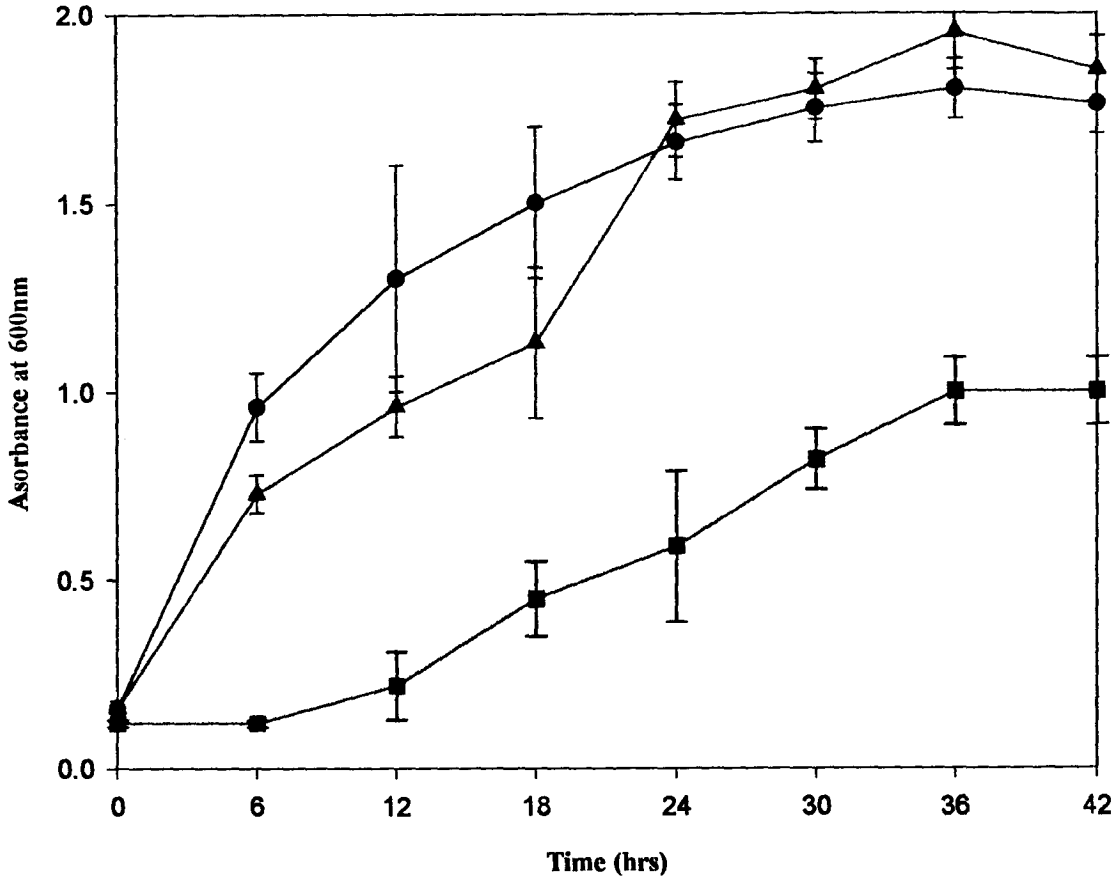


Fig 4.1 Growth behaviour of *Pseudomonas aeruginosa* strain USS25 in Luria Bertani broth and Luria Bertani broth + TBTC

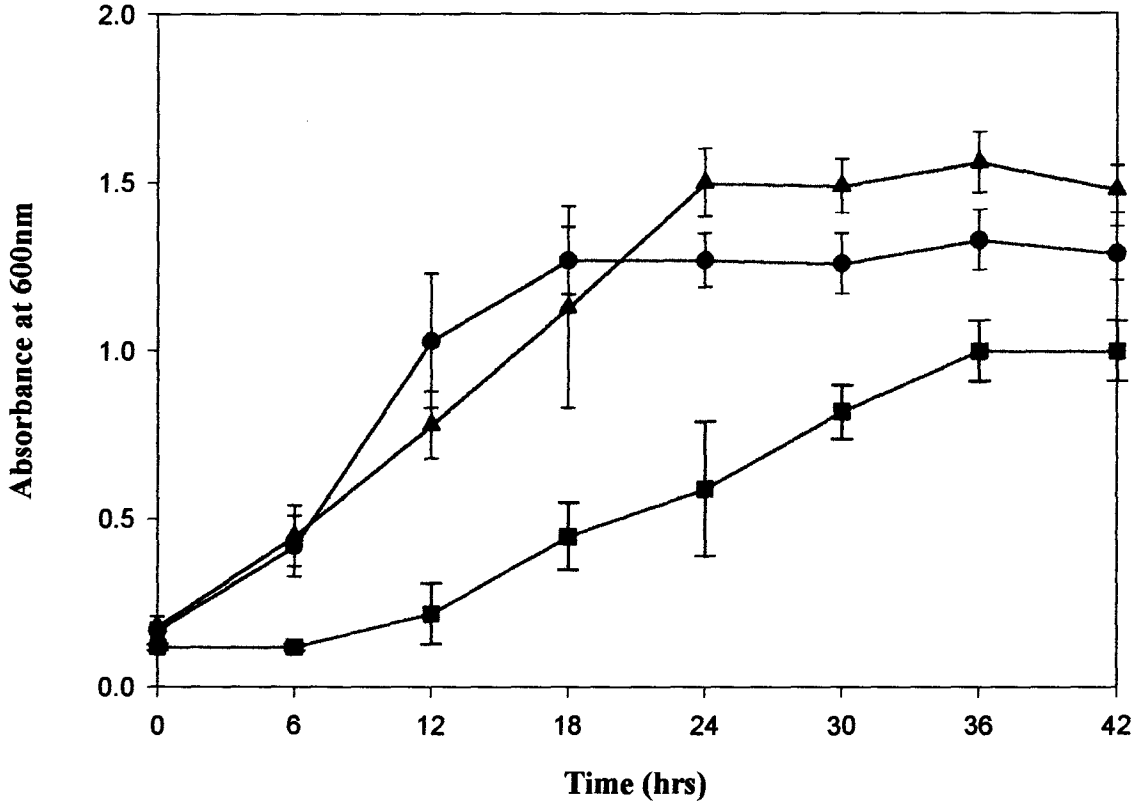


Fig. 4.2 Growth behaviour of *Pseudomonas aeruginosa* strain USS25 in Zobell Marine Broth and Zobell Marine Broth +TBTC

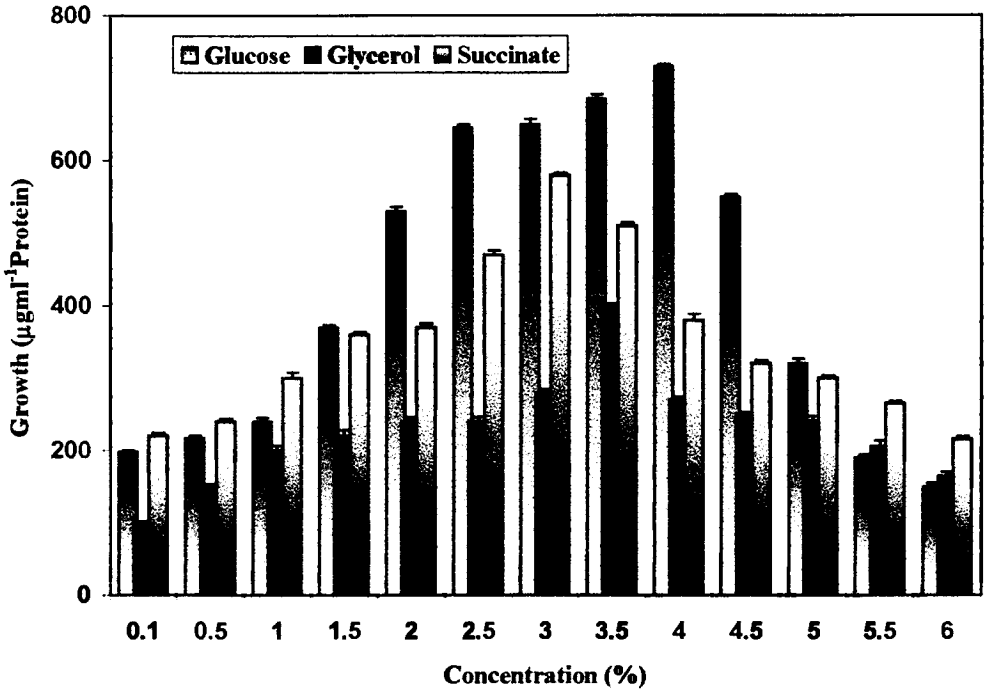


Fig 4.3 Optimum concentration of carbon sources for growth of *Pseudomonas aeruginosa* strain USS25

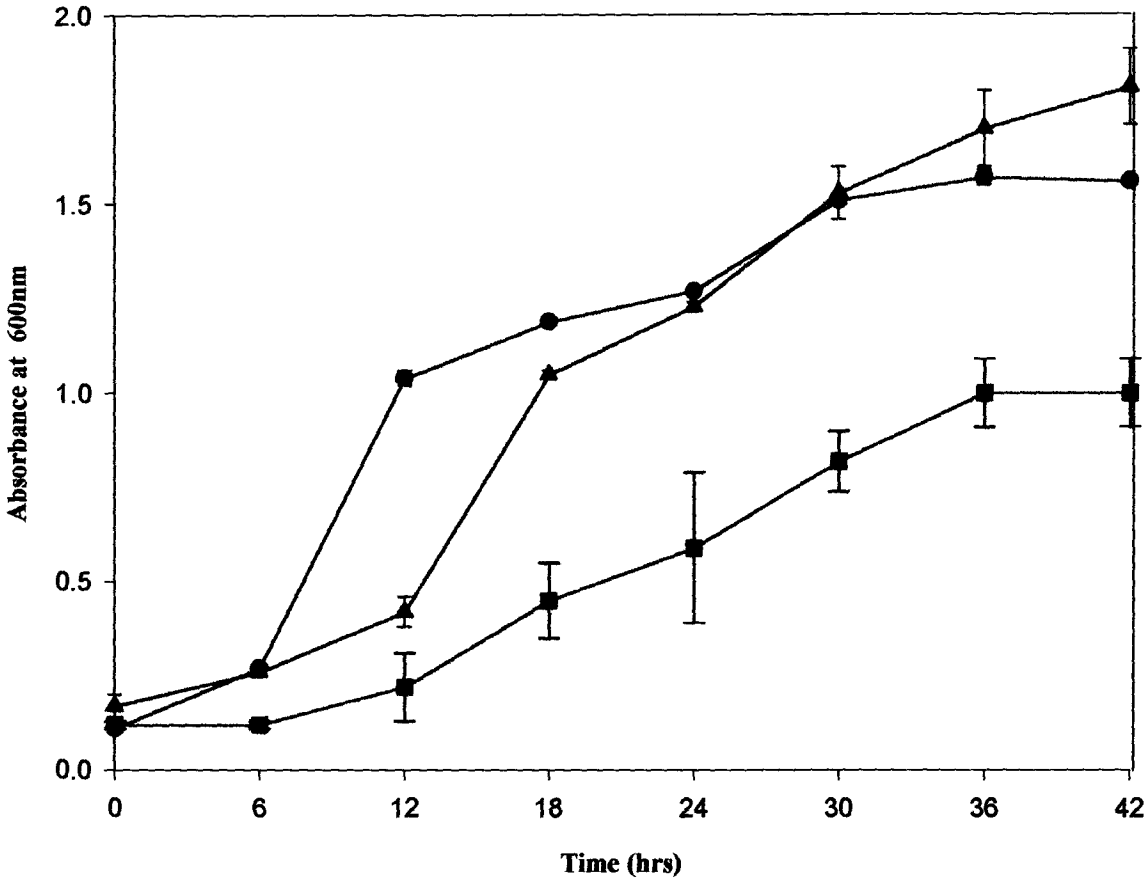


Fig. 4.4 Growth behaviour *Pseudomonas aeruginosa* strain USS25 in MSM+glycerol(3.5%) and MSM+ glycerol(3.5%)+ 2mMTBTC

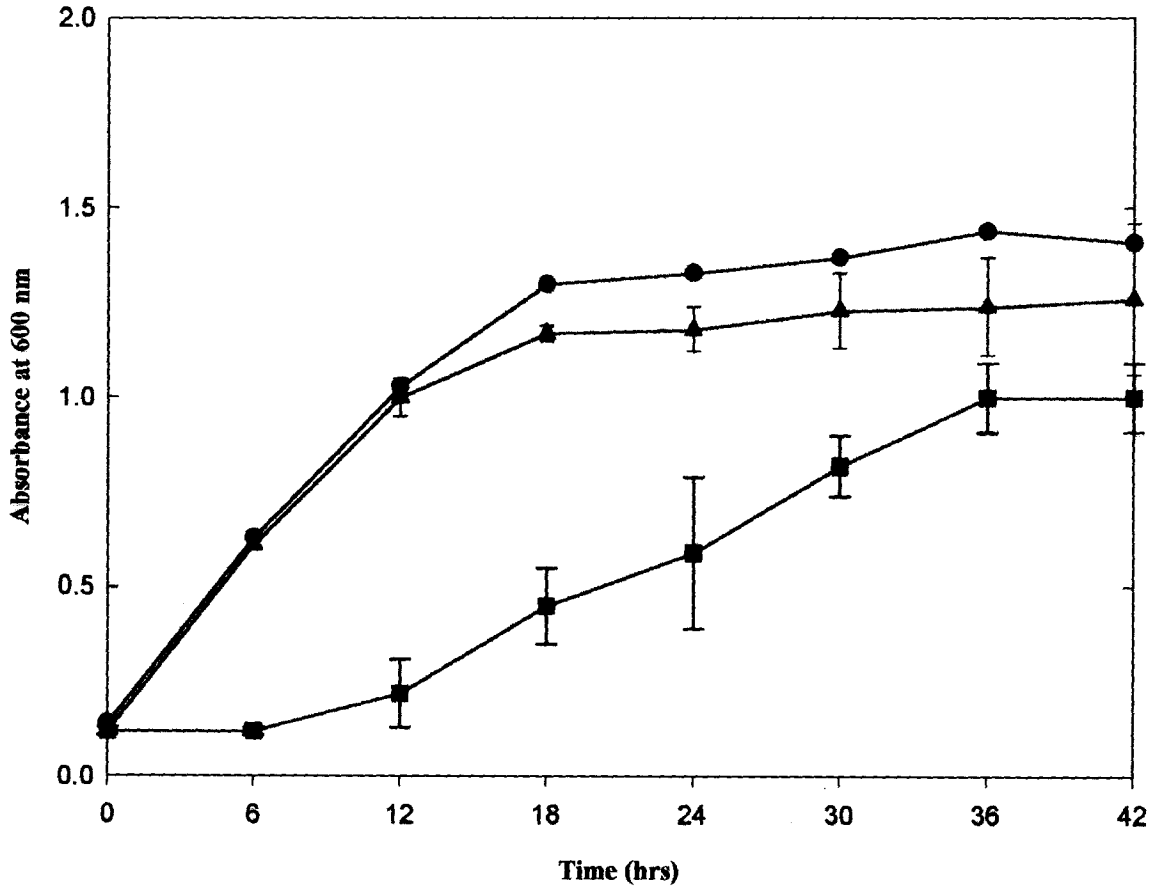


Fig 4.5 Growth behaviour of *Pseudomonas aeruginosa* USS25 in MSM+succinate (3%) and MSM+succinate (3%) + 2mMTBTC

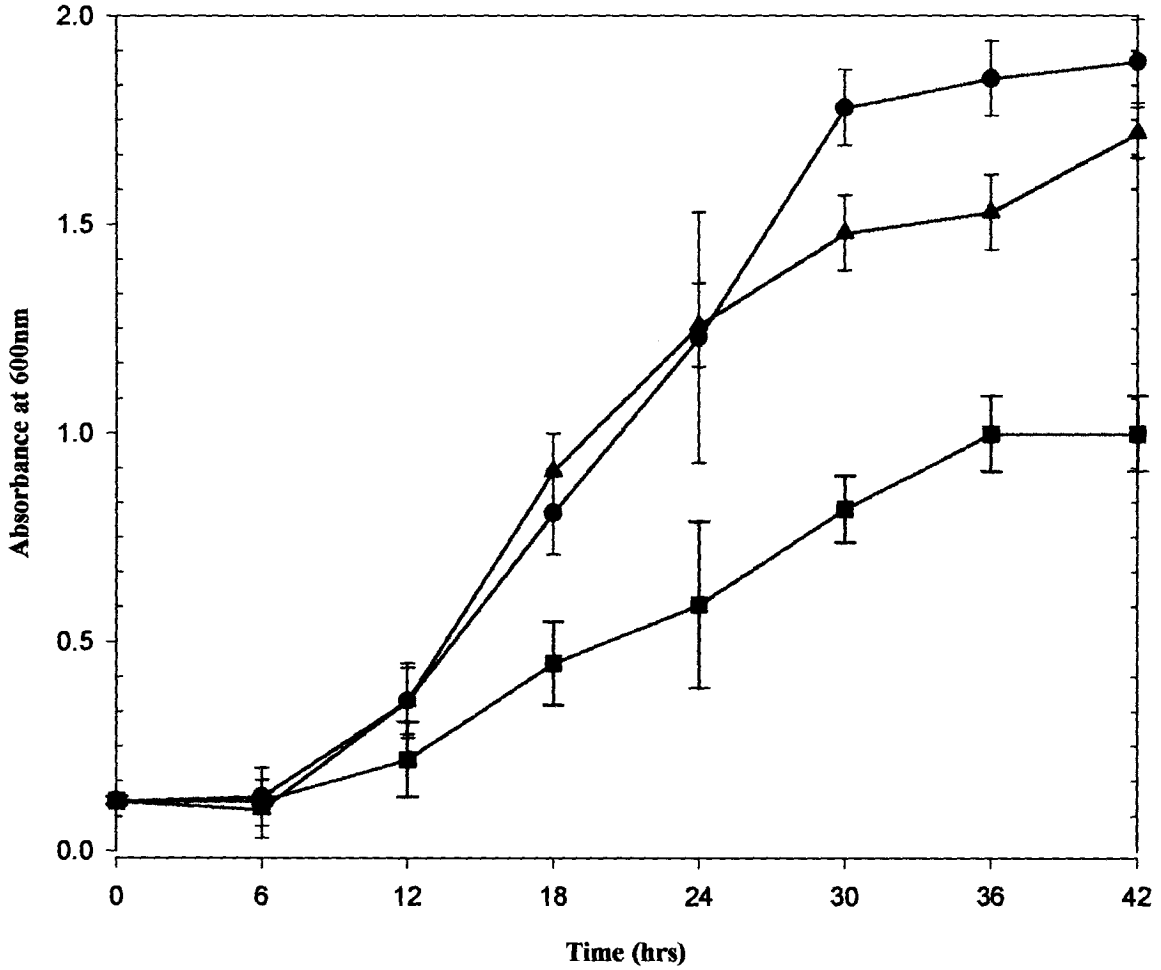


Fig 4.6 Growth behaviour of *Pseudomonas aeruginosa* strain USS25 in MSM with succinate(3%) + glycerol(3.5%) and succinate + glycerol+ 2mM TBTC

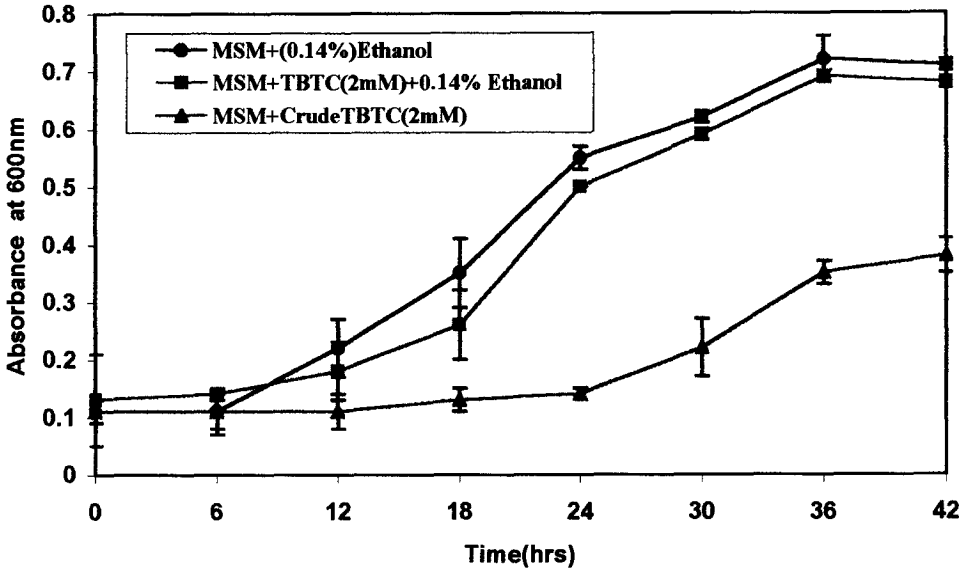


Fig- 4.7 Growth behaviour of *Pseudomonas aeruginosa* strain USS25 in presence of 2mM TBTC with or without ethanol

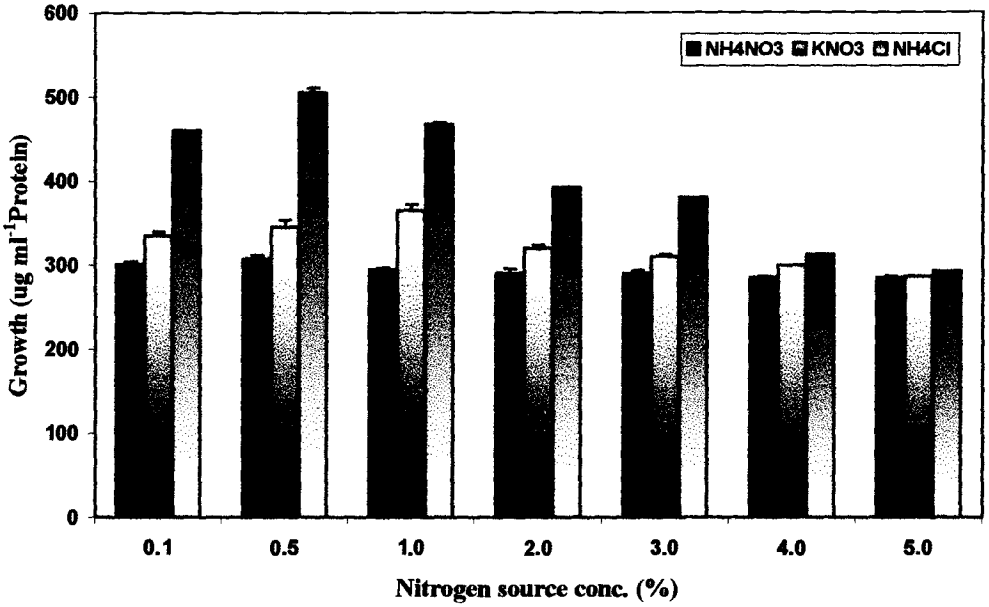


Fig 4.8 Optimum concentration of nitrogen sources for the growth of *Pseudomonas aeruginosa* USS25 grown in presence of 2mM TBTC

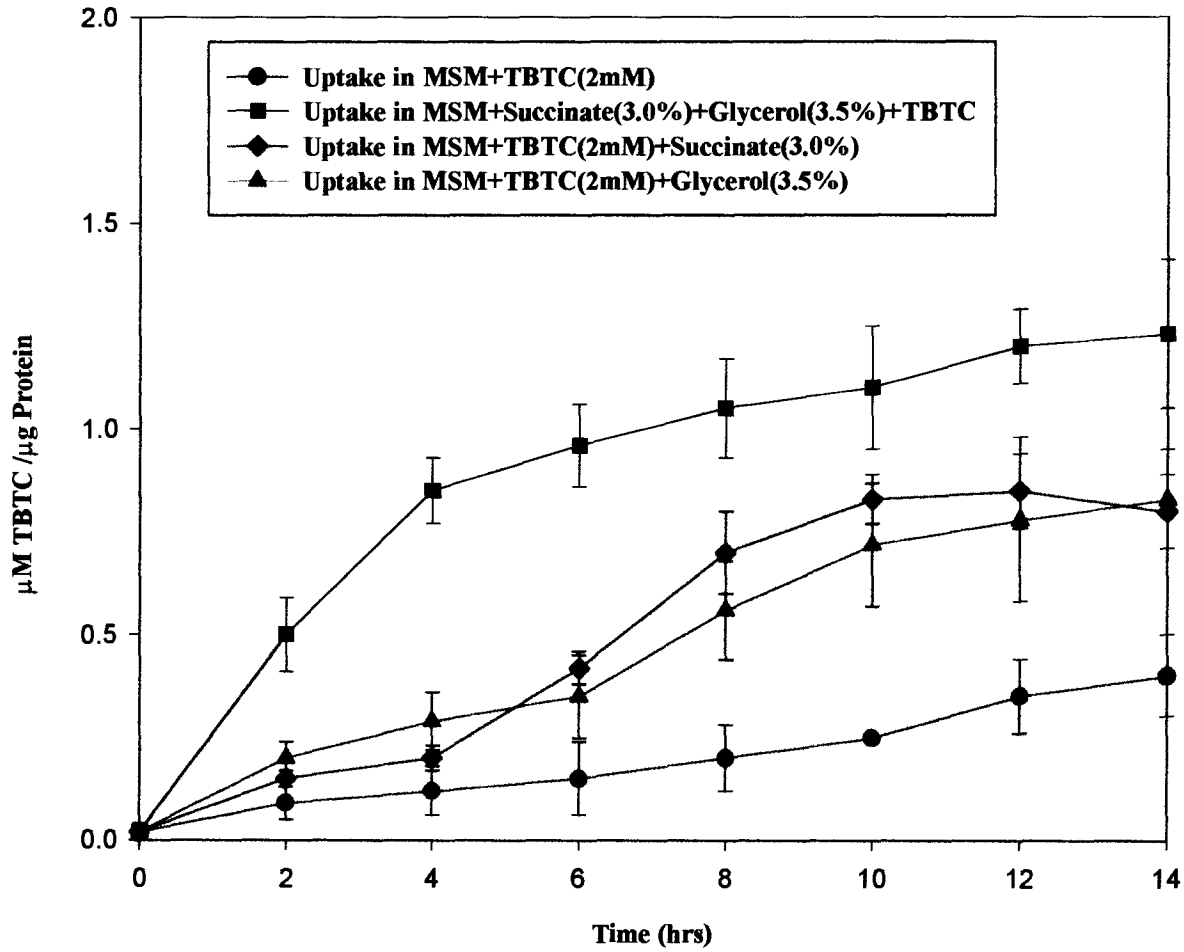


Fig- 4.9 Uptake of TBTC by *Pseudomonas aeruginosa* strain USS25 in different media

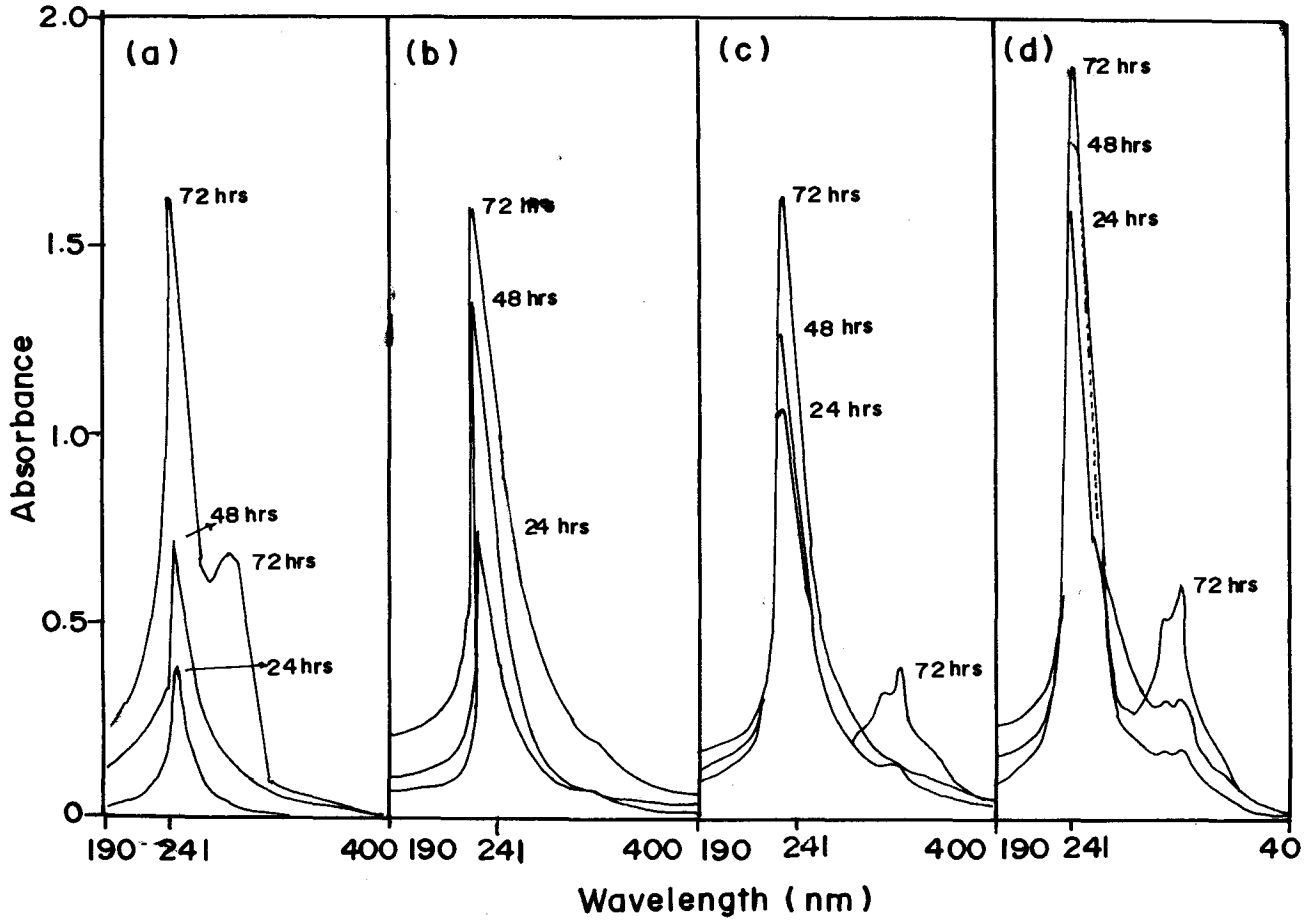


Fig.4.10 Absorbance spectra (190-400nm) of chloroform extract of bacterial cells grown in (a) MSM + 2mM TBTC only (b) MSM + 2mM TBTC + 3% succinate; (c) MSM + 2mM TBTC + 3.5 % glycerol ; (d) MSM + 2mM TBTC + 3% succinate + 3.5 % glycerol.

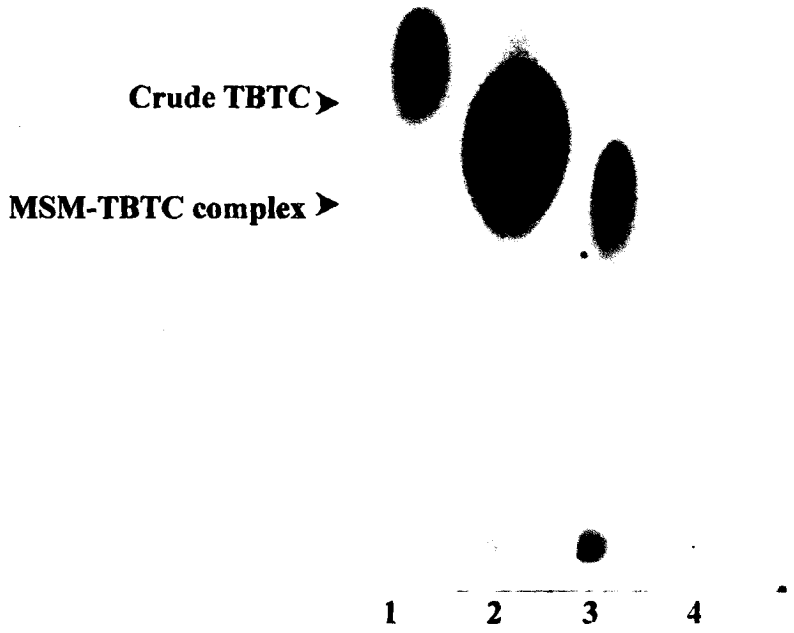


Fig 4.11 TBTC degradation profile of *Pseudomonas aeruginosa* strain USS25

1. TBTC (Control)
2. MSM + 2 mM TBTC
3. Extract of cells grown in MSM + 2mM TBTC
4. Extract of supernatant

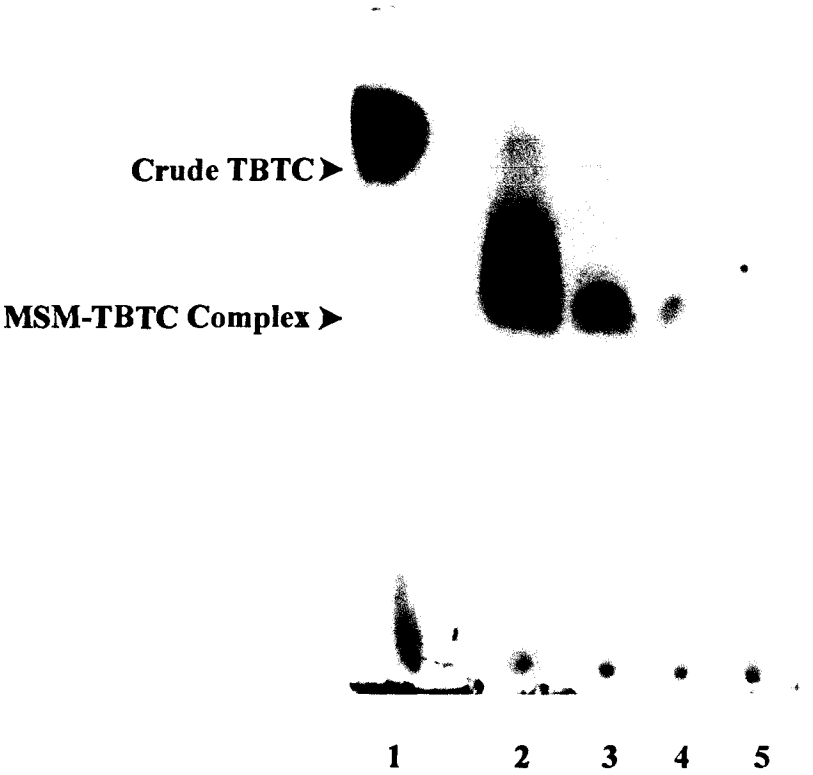


Fig 4.12 TBTC degradation profile of *Pseudomonas aeruginosa* strain USS25 - (Time course study)

1. **TBTC (Control)**
2. **Extract of cells grown in MSM + 2mM TBTC for 7 days.**
3. **Extract of cells grown in MSM + 2mM TBTC for 28 days.**
4. **Extract of cells grown in MSM + 2mM TBTC for 45 days.**
5. **Extract of cells grown in MSM + 2mM TBTC for 75 days.**

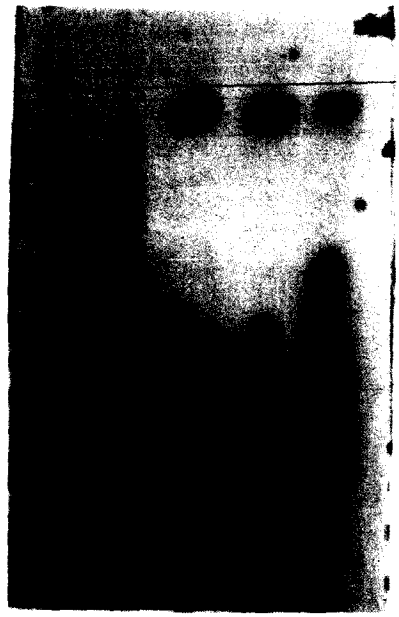


Fig. 4.13 TBTC degradation profile of *Pseudomonas aeruginosa* strain USS25 in presence of succinate (3%) and glycerol (3.5%)

1. **MSM + 2mM TBTC (CONTROL)**
2. **Cell extract from cells grown in MSM + 2mM TBTC.**
3. **Cell extract from cells grown in MSM + 2mM TBTC+ succinate.**
4. **Cell extract from cells grown in MSM + 2mM TBTC + succinate + glycerol.**
5. **Cell extract from cells grown in MSM + 2mM TBTC + glycerol.**

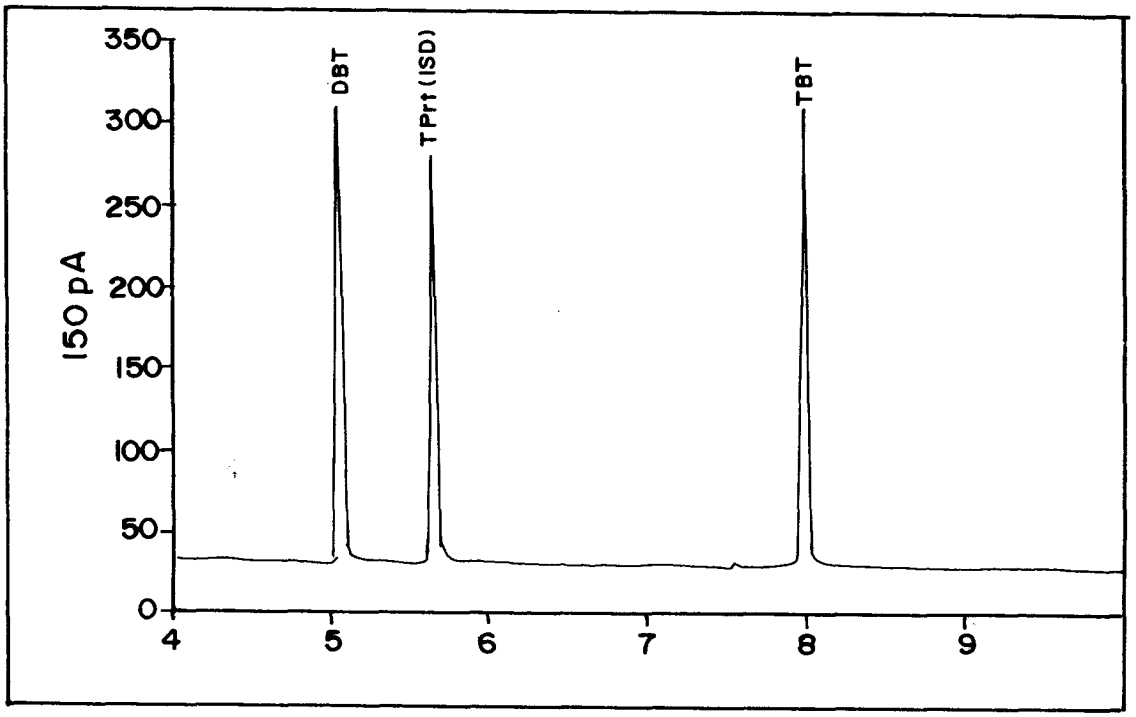


Fig.4.14a Gas Chromatogram showing the separation of standard components such as dibutyltin (DBT), tripropyltin chloride (TPrT) as internal standard (ISD) and tributyltin (TBT)

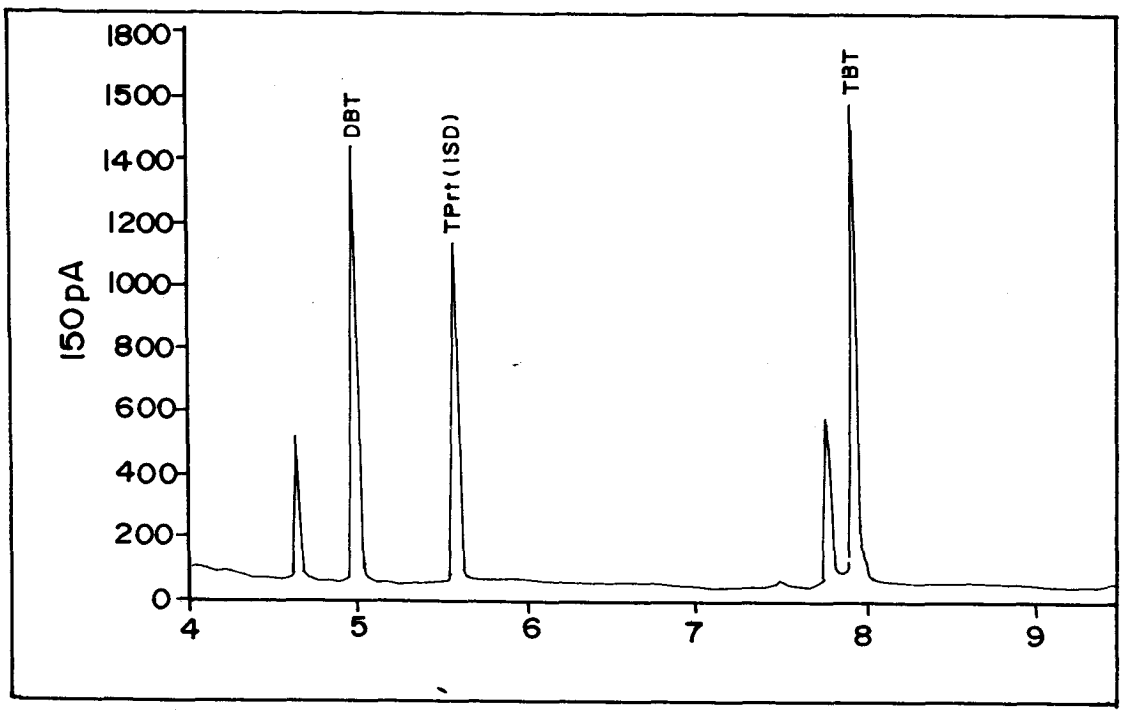


Fig.4.14b Gas Chromatogram of culture suspension showing production of dibutyltin from tributyltin by Pseudomonas acruginasa USS25

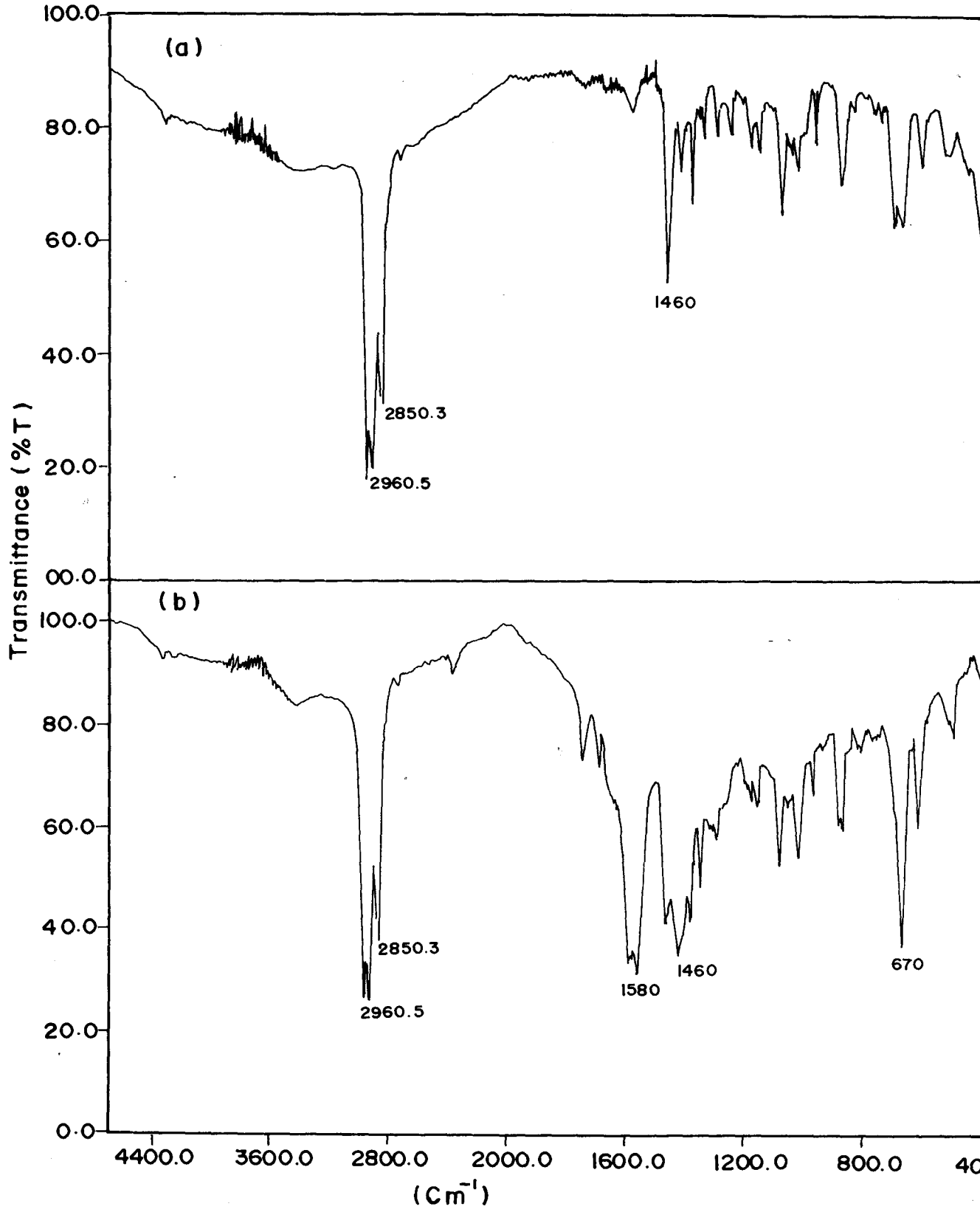


Fig.4.15 IR Spectra of (a) Standard TBTC and (b) Degradation product.

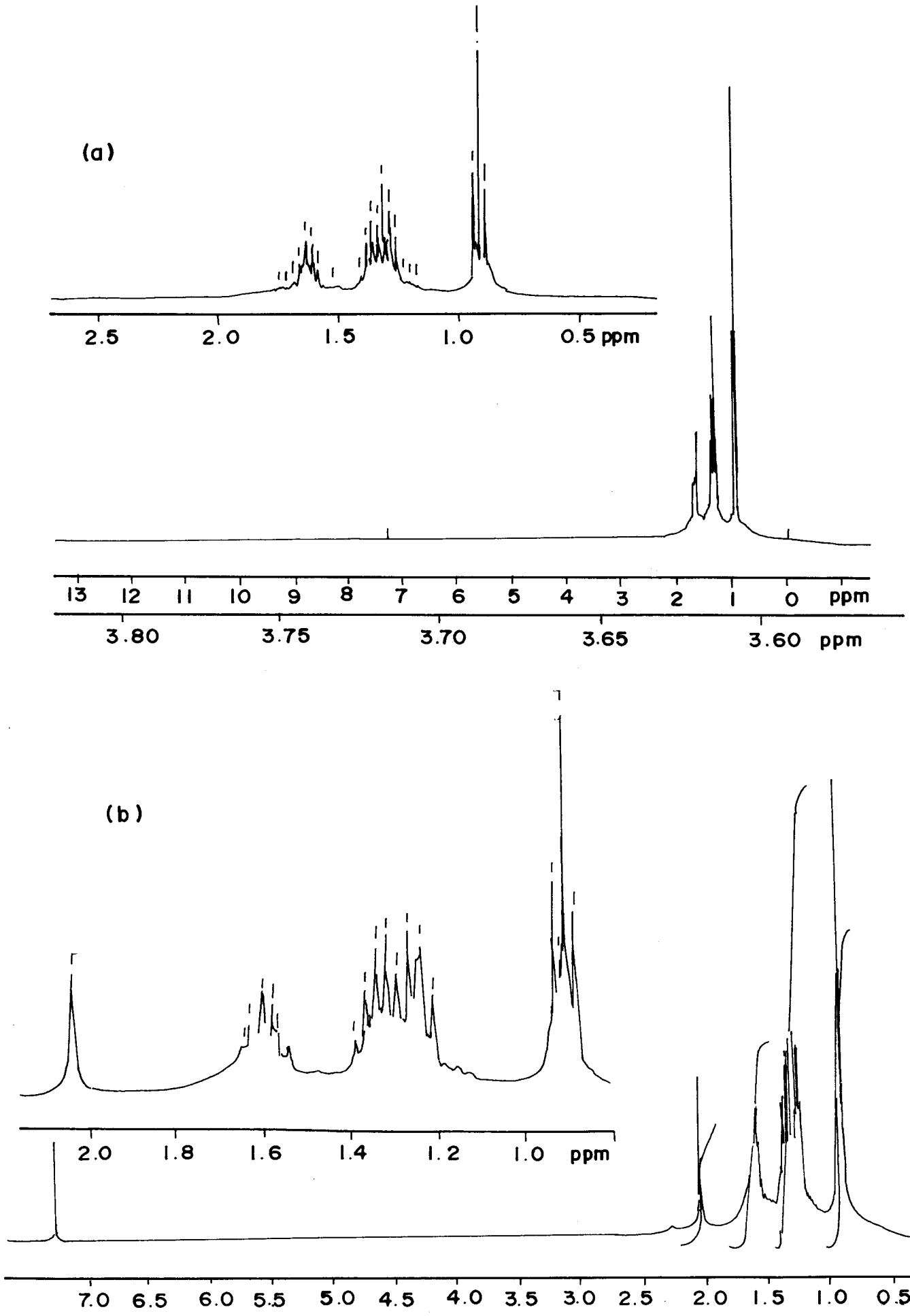
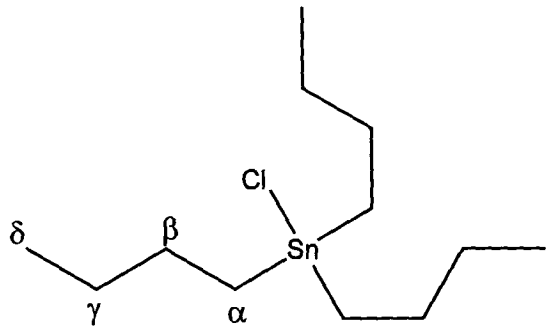
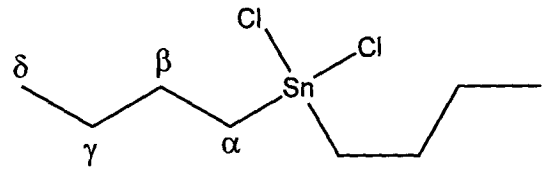


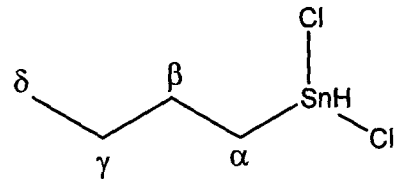
Fig 4.16 ^{1}HNMR Spectra of (a) Standard TBTC and (b) Degradation product.



Tributyltin Chloride



Dibutyltin dichloride



Monbutyltin dichloride

Fig 4.17 Structures of TBTC, DBT & Degradation product (Monobutyltin)

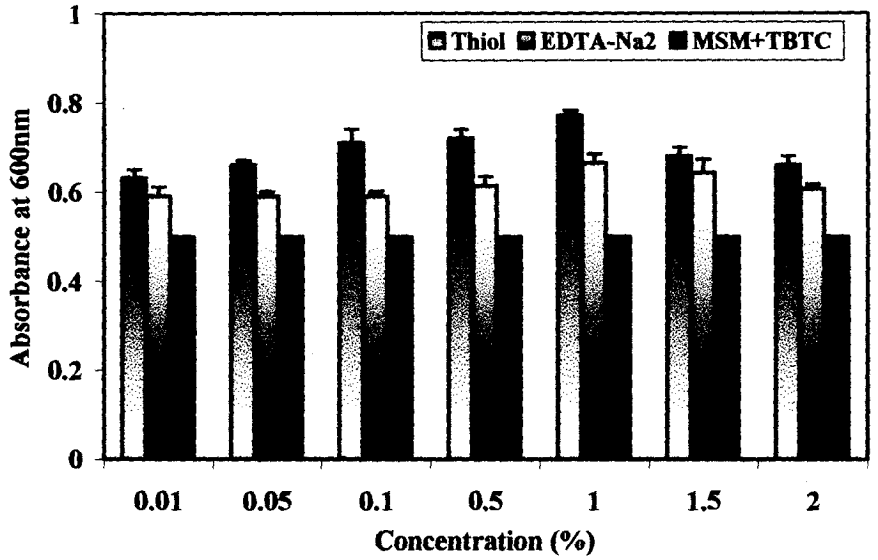


Fig 4.18 Effect of thiol (β–mercaptoethanol) and chelating agent (EDTA-Na_2) on growth of *Pseudomonas aeruginosa* strain USS25 in MSM+2mM TBTC.

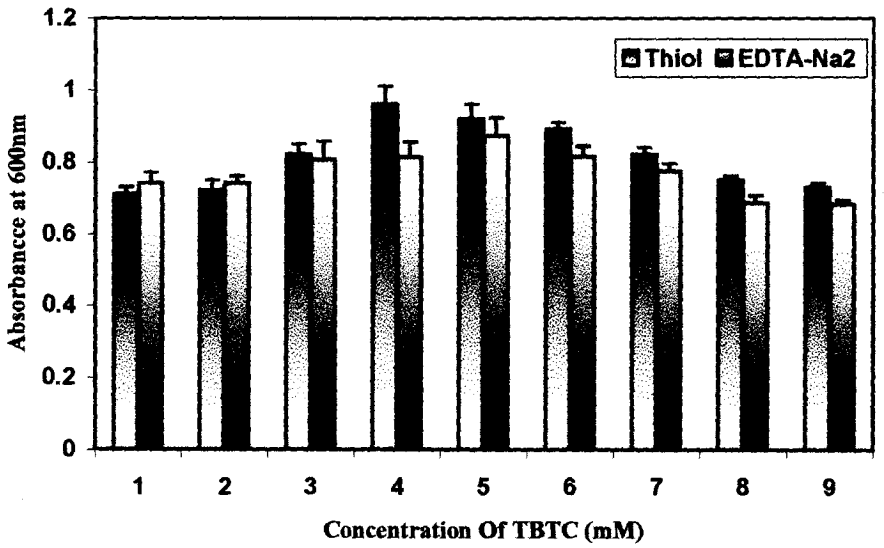


Fig 4.19 Effect of thiol and EDTA-Na_2 on TBTC toxicity of *Pseudomonas aeruginosa* strain USS25 in MSM

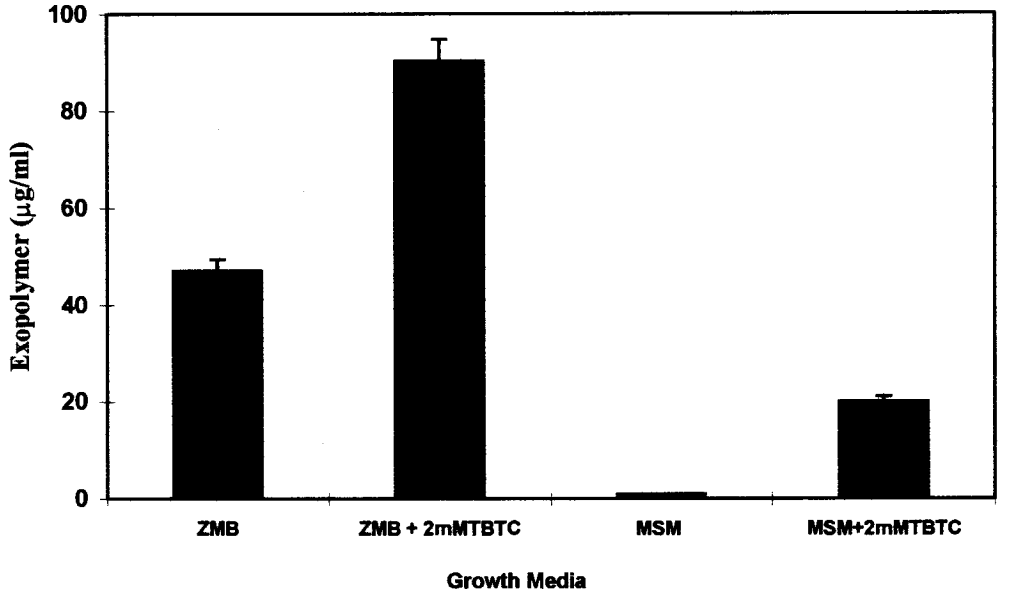


Fig 4.20 Exopolymer production of *Pseudomonas aeruginosa* strain USS25 in different media

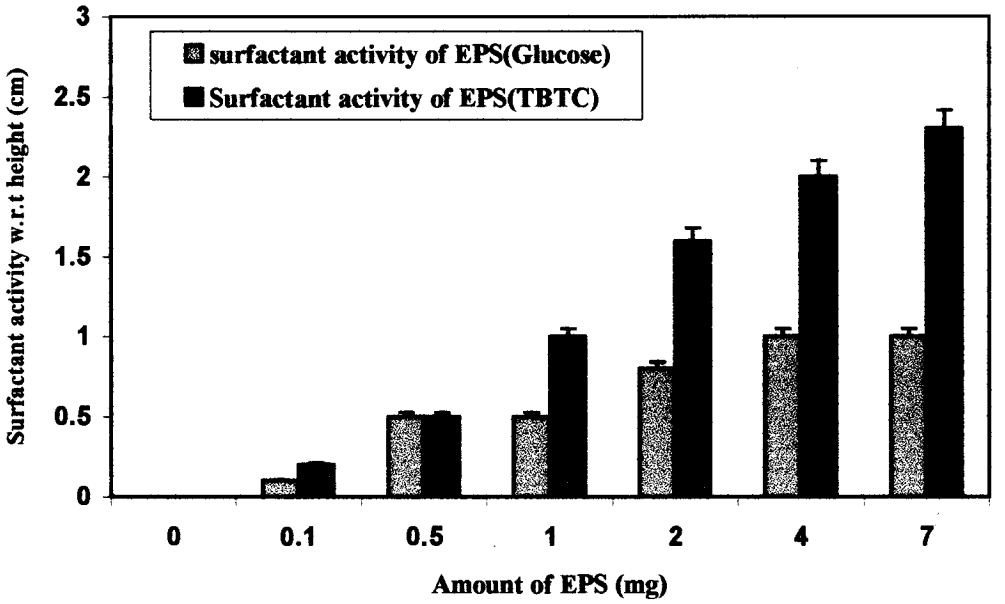


Fig 4.21 Surfactant activity of Exopolymer in Benzene-water system

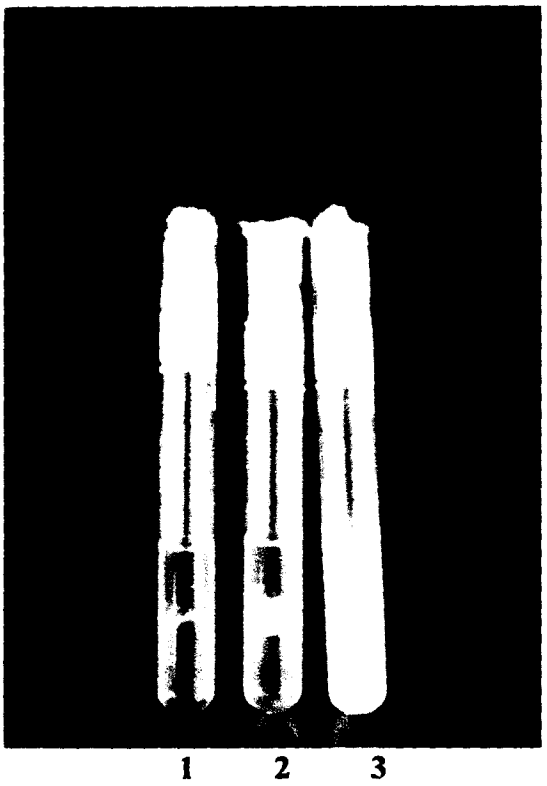


Fig. 4.22 Effect of TBTC on surfactant activity of EPS of *Pseudomonas aeruginosa* strain USS25 (Benzene–Water system)

1. **Benzene-Water System (Control).**

2. **Surfactant activity of EPS of bacterial cells grown in MSM+ 0.5% Glucose.**

3. **Surfactant activity of EPS of bacterial cells grown in MSM + 2mM TBTC.**

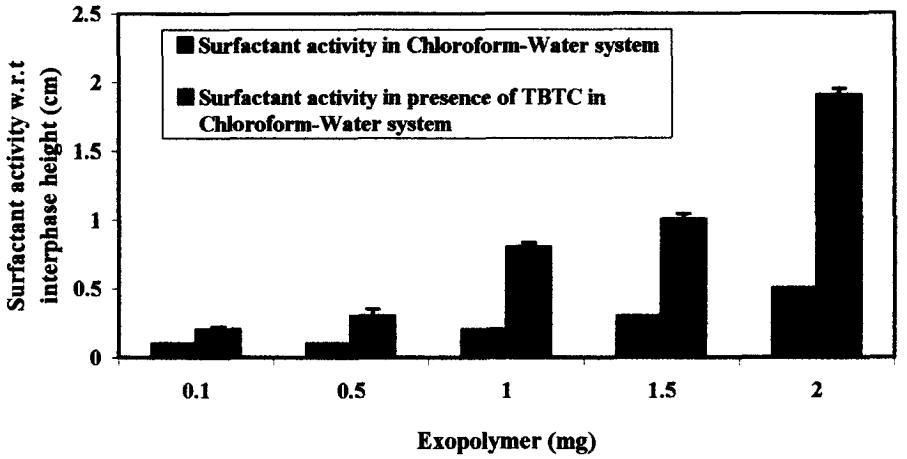


Fig 4.23 Effect of TBTC on surfactant activity of bacterial EPS in Chloroform-Water system

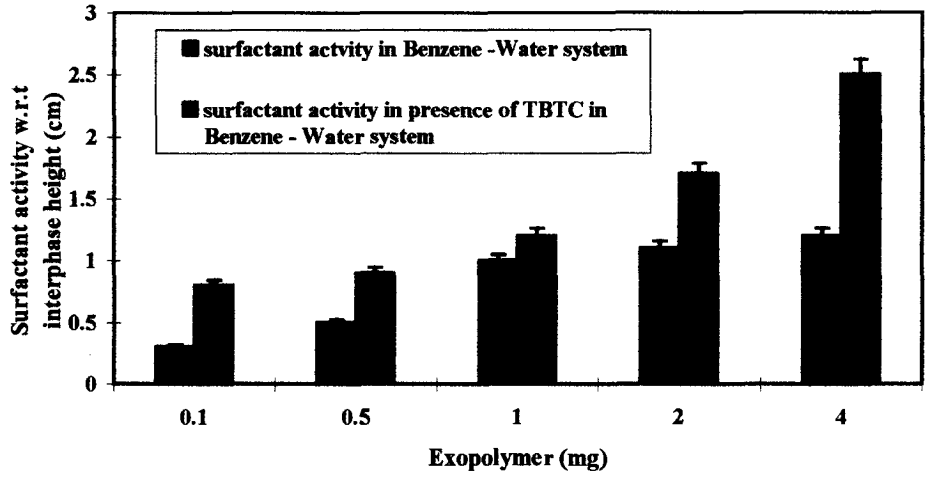


Fig 4.24 Effect of TBTC on surfactant actvity of bacterial EPS in benzene-water system

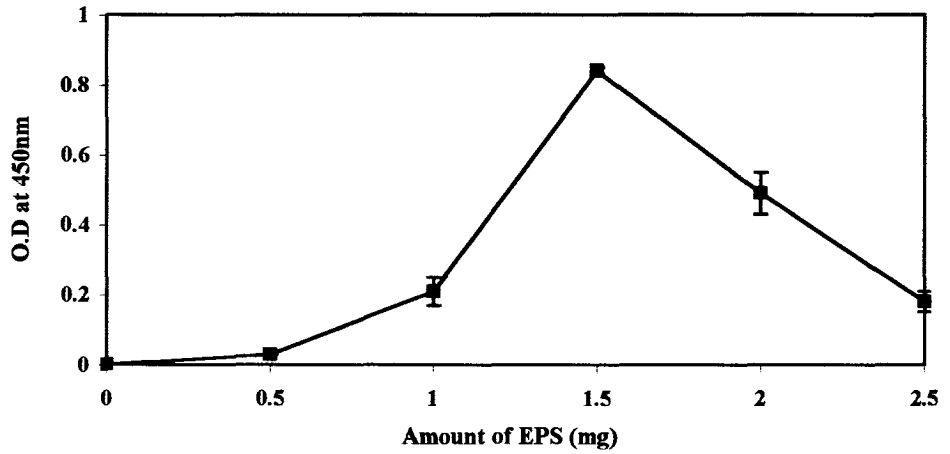


Fig 4.25 - CMC value of EPS

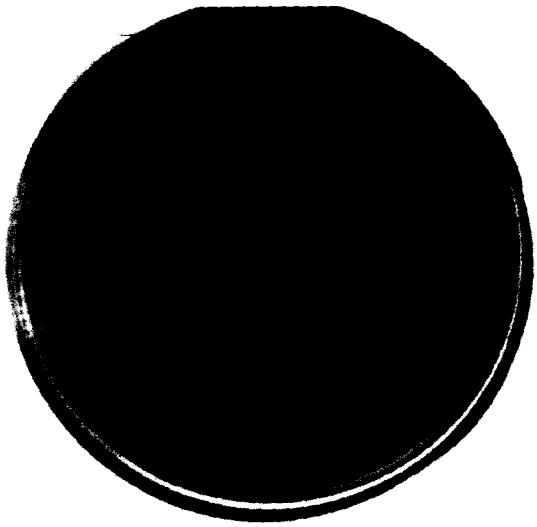


Fig. 4.26 Cells of *Pseudomonas aeruginosa* strain USS25 showing enhanced production of green pigment on MSM Agar supplemented with TBTC

1 2 3

Fig 4.27 Effect of TBTC on green fluorescent pigment production of *Pseudomonas aeruginosa* strain USS25

1. **MSM + glucose(0.5%) – Control (without cells)**
2. **Pigment production by cells grown in MSM+ 0.5% glucose**
3. **Pigment production by cells grown in MSM+ 0.5% glucose + 2 mM TBTC.**

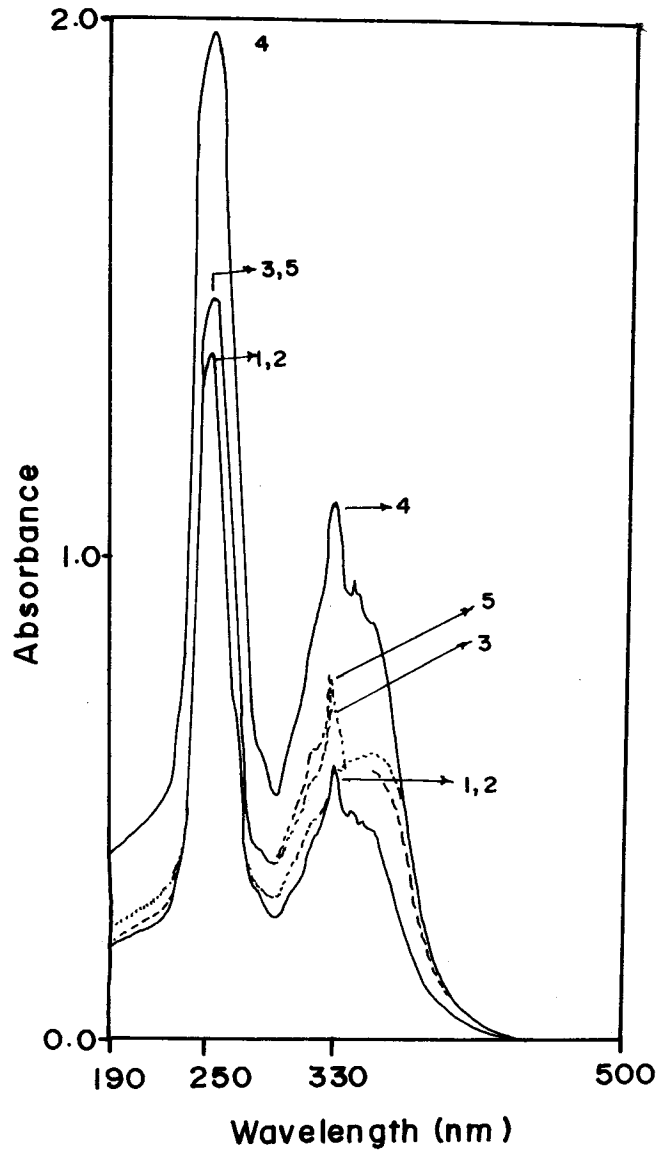


Fig. 4.28 Effect of TBTC on fluorescent pigment produced by Pseudomonas aeruginosa USS 25

1. Control (MSM + 0.5% Glucose)
2. MSM + 0.5 % Glucose + 0.5mM TBTC
3. MSM + 0.5 % Glucose + 1mM TBTC
4. MSM + 0.5 % Glucose + 2mM TBTC
5. MSM + 0.5 % Glucose + 2.5mM TBTC

Fig. 4.29 Fluorescent bacterial pigment of *Pseudomonas aeruginosa* strain USS25 under UV light

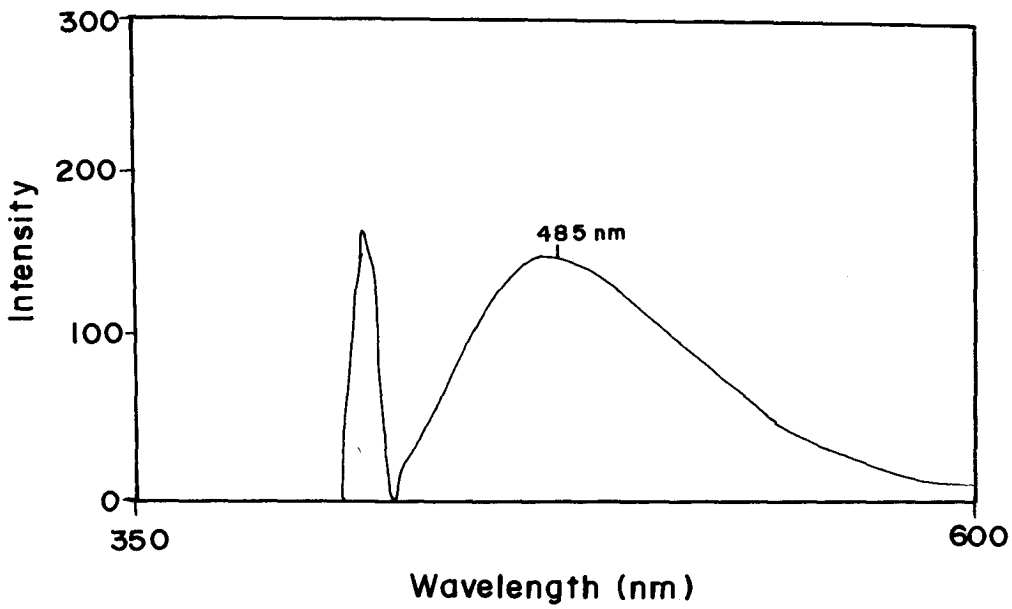


Fig.4.30. Spectrofluorimetric analysis of fluorescent pigment produced by Pseudomonas aeruginosa USS 25.

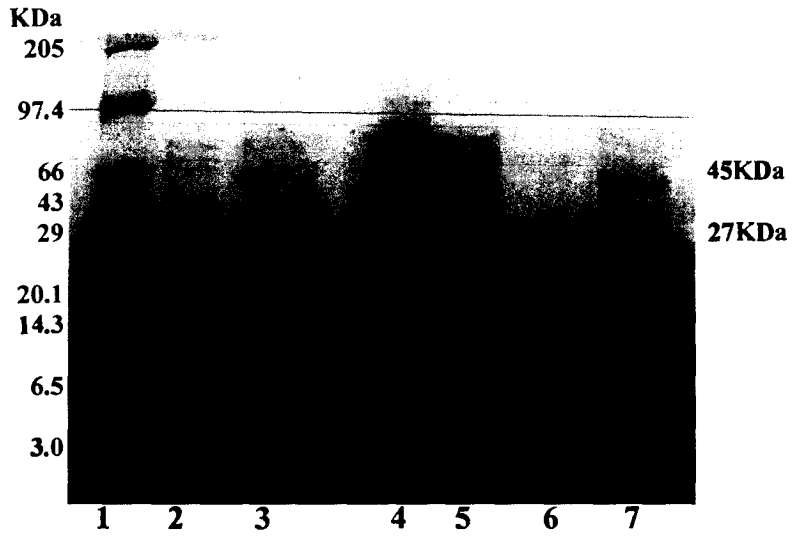


Fig 4.31 Protein profile of *Pseudomonas aeruginosa* strain USS25 (SDS - PAGE)

1. **Broad Range Protein marker (PMW-B)**
2. **Protein sample of cells grown in MSM + 0.1mM TBTC**
3. **Protein sample of cells grown in MSM + 0.5mM TBTC**
4. **Protein sample of cells grown in MSM + 0.5% glucose.**
5. **Protein sample of cells grown in MSM + 0.5% glucose + 2mM TBTC**
6. **Protein sample of cells grown in MSM + 1mM TBTC**
7. **Protein sample of cells grown in MSM + 2mM TBTC**

Chapter V

MOLECULAR BIOLOGICAL AND GENETIC CHARACTERIZATION OF TBTC RESISTANT *Pseudomonas aeruginosa* strain USS25

The TBTC resistant *Pseudomonas aeruginosa* strain USS25 was subjected to molecular biological and genetic analysis to assess their molecular characteristics with reference to TBTC resistance and degradation mechanisms.

5.1 Plasmid profile

5.1.1 Purification of plasmid DNA (Alkaline lysis method) and agarose gel electrophoresis

The plasmid of *Pseudomonas aeruginosa* USS25 was extracted by Alkaline lysis (Birnboim and Doly, 1979) and Boil prep method (Holmes and Quigley, 1981). The yield of the plasmid DNA was better in Alkaline lysis method than Boil prep method, therefore Alkaline lysis method was followed for further experiment to purify and characterize plasmid DNA. Agarose gel electrophoresis of plasmid DNA revealed the presence of a small supercoiled plasmid (Fig 5.1).

5.1.2 Characterization of plasmid DNA (Restriction mapping)

In order to find out the size of the plasmid, the purified plasmid DNA was digested with restriction endonucleases, Bam H1 and Nco1 and DNA samples were analysed on 1% agarose gel. Plasmid DNA bands were compared with molecular marker (λ DNA EcoRI+ Hind III digest and EcoR1 digest). The restriction mapping of the plasmid DNA reveals the total size of the plasmid DNA as several DNA bands are seen (resolved) in the agarose gel (1%). BamHI generates 20.8Kb, 12.8Kb and 7.4Kb bands whereas NcoI generates 20.7Kb and 19.9Kb bands respectively, (Fig 5.2). Restriction mapping profile on agarose gel electrophoresis determines that the size of the plasmid is approximately 41Kb (Fig-5.2).

It has been reported that bacterial strains from toxic chemical contaminated sites, more frequently, contains plasmid DNA. For example, bacteria isolated from Barceloneta regional treatment plant in Barceloneta, Puerto Rico, have shown resistance to several antibiotics along with tributyltin, which was plasmid mediated (Baya et al.1986). *Pseudomonas* sp. has several plasmids which are responsible for degradation of xenobiotics such as pyridine, phenol, *o*-xylene, toluene, 1, 2, 4-trimethylbenzene (Barbieri et al. 1989, Laddy et al. 1995; Yoon, 1998; Mohan et al. 2003) and also exhibit resistance to several heavy metals viz. mercury , cadmium, chromium, copper and silver (Cervantes and Ohtake, 1988,Vasista et al. 1989,Cervantes and Silver 1996, Bruins et al., 2003). In fact, *Pseudomonas aeruginosa* has already been reported to possess plasmid responsible for mercury resistance and chromate mobilization (Trevors, 1986; Cervantis and Ohtake, 1988; Cervantes and Silver1996). It is interesting to note that the test bacterium also possesses a small supercoiled plasmid with three distinct bands (Fig-5.1). Presence of plasmid in *Pseudomonas aeruginosa* strain USS25 reveals a possibility of plasmid mediated TBTC resistance and degradation. It is interesting to note that several new strains of *Sphingomonas* sp., which are very close to *Pseudomonas* sp. (Nishikawa et al. 1998), viz. *S. aromaticivorans, S. stygia* and *S subterranea* also have plasmid mediated degrading capacity for a broad range of aromatic compounds viz. toluene, naphthalene, *o*-xylene, p-cresol, fluorine, biphenyl and dibenzothiophene (Fredrickson et al.1999).

Restriction digestion of the plasmid with BamHI and NcoI confirmed that the size of the plasmid is 41 Kbps. Similarly, *Pseudomonas stutzeri, which* can utilize *o*-xylene as a carbon source, also bears a plasmid of about 80 Kbp which confers resistance to mercuric chloride and organomercurial compounds (Barbieri et al. 1989). In another report *Pseudomonas putida* MT-14 and *Pseudomonas oleovorans* have been found to harbour plasmid encoding both mercury resistance and phenyl acetate or octane degradation (Chakrabarty and Friello, 1974; Pickup et al. 1983).

Therefore we can assume that plasmid mediated organometallic resistance and aromaric hydrocarbon degradation is a universal phenomenon in *Pseudomonas* strains.

5.2 Confirmation of location of gene(s) conferring TBTC resistance and degrading capability

Interestingly, preliminary result show that small plasmid of *Pseudomonas aeruginosa* strain USS25 may be responsible for metals (Hg, Cd, Zn) and organometal (TBTC) resistance and degradation. Therefore, we tried to check these possibilities by curing the plasmid DNA of test organisms.

Acridine orange curing of plasmid DNA

In order to confirm plasmid mediated TBTC resistance and degradation, attempt was made to eliminate the plasmid of *Pseudomonas aeruginosa* USS25 cells by treatment with curing agent, acridine orange. It has been reported that acridine orange inhibits the replication of bacterial plasmids by causing mutation in absence of light at the site of semi-conservative DNA replication (Webb and Hass, 1984; Trevors, 1986). Cells of Pseudomonas *aeruginosa* strain USS25 were grown in presence of increasing concentrations of acridine orange ranging from 25μg/ml to 200μg/ml and their viability was checked. The percentage survival curve indicated that the culture showed 20 percent survival in presence of acridine orange (50 μg/ml) (Fig-5.3). Cells were subcultured 10 times in presence of 50 μg/ml acridine orange. During each subculturing in presence of 50 μg/ml acridine orange, culture was diluted and plated on TBTC containing agar plates to obtain isolated colonies. It is interesting to note that gradual loss of plasmid from acridine orange treated cells was noticed during each subculture, with complete loss after sixth subculture in presence of acidine orange (Fig- 5.4). It is interesting to mention that plasmid cured cells were able to still utilize TBTC as carbon source, therefore we can assure that TBTC resistance and

degradation phenotype of *Pseudomonas aeruginosa* strain USS25 is governed by genes located on chromosomal genome. Similar findings have already been confirmed by Lee et al. (2001) that acridine orange cured cells of *Pseudomonas aeruginosa* and *Pseudomonas putida* still show cadmium resistance as the genes are not plasmid borne.

Genetic studies on TBTC resistant and degrading bacterial strains from aquatic environments are extremely limited with very few reports demonstrating the presence of plasmids, but no correlation with TBTC resistance (Suzuki et al. 1992; Fukugawa and Suzuki, 1993; Miller, et al. 1995; Minchin et al. 1997). Though the TBTC resistance mechanism may be either plasmid mediated or governed by chromosomal genome, generally it has been demonstrated that the genes conferring resistance to metals , organometals and PAH are located on chromosomal genome. One such TBTC resistant bacterial strain *Alteromonas* sp. strain M1 showed TBTC efflux system as a resistance mechanism which is governed by genes on chromosomal genome (Fukagawa and Suzuki,1993). TBTC resistance gene has been successfully cloned in pUC19 and also sequenced. Nucleotide sequence of the shortest 1.8 Kb Hind III fragment revealed an ORF of 324bps (108 amino acids). 48.5% amino acids of this protein were hydrophobic suggesting that encoded polypeptide is a membrane protein of 12 KDa belonging to transglycosylase (Fukagawa and Suzuki, 1993). Recently Kitamura and Suzuki (2003) have reported that SecA is another gene which is also involved in confirring TBTC resistance in *Pseudoalteromonas* sp. Strain M1 (unpublished data). This gene was present on 4.6 Kbp Pst I fragment and possesses an ORF of 2,700 bps. It is interesting to note that this gene shows 70.8% homology with *E coli* SecA and 70.4 % homology with *Vibrio alginolyticus*, SecA (Kitamura and Suzuki, 2003; unpublished data). They have hypothesized that SecA (116KDa) is a TBTC binding cystosolic protein which shows TBTC induced production of this polypeptide. It also appears that SecA-ATPase is also resistant to TBTC, though normally ATPase and other ATPase related enzymes are strongly repressed by TBTC.

Further molecular characterization by cloning and sequencing of gene(s) encoding TBTC resistance and degradation in *Pseudomonas aeruginosa* strain USS25 would clearly reveals the nature of these genes and their involvement in TBTC resistance and utilization. I expected the presence of some genetic determinant which is involved in enzyme mediated utilization of TBTC.

5.3 NTG mutagenesis of *Pseudomonas aeruginosa* strain USS25

In order to isolate NYG-induced TBTC hyper resistant mutants a strong chemical mutagen NTG (N-methyl-N-nitro-Nitrosoguanidine) was used. It is an alkylating agent recognized as a potent mutagen and carcinogen which acts through covalent modification of cellular DNA resulting in various types of primary lesions (Waters, et al. 1990) and it causes insertion mutations (Miyauchi, et al. 2002). NTG is known to induce mutation at the replication point leading to clustering of induced mutations (Guerola, et al. 1971).

5.3.1 NTG mutagenesis and screening of hyper tolerant TBTC resistant mutant

It is expected that cells of *Pseudomonas aeruginosa* strain USS25 would undergo some mutational changes to give rise mutants when exposed to Nitrosoguanidine, which might tolerate higher concentration of TBTC. So, the strain USS25 was treated with three different concentrations of NTG (50µg/ml,100µg/ml and 200µg/ml) and viability was checked at every 5 min interval upto 2hrs. It was observed that 100µg/ml of NTG causes 90% killing of cells (Fig- 5.5) within 45minutes, therefore it was selected for treatement of cells to induce mutants. It was interesting to note that in case of *E coli* and *Salmonella typhimurium* the concentration and time of exposure of NTG was 50µg/ml for 30min and 20min respectively at 37°C in order to screen NTG induced mutants (Yamada, et al. 1997).

When the *Pseudomonas aeruginosa* strain USS25 was exposed to NTG (100µg/ml) for 45 min, killing of 90% viable cells due to mutagenic effect of NTG, but remaining 10%

viable cells would undergo induced mutation. After serial dilution of the NTG treated culture the cells were plated on MSM agar plates supplemented with 5mM and 10mM TBTC respectively to isolate discrete colonies. Five conspicuous colonies appeared after one week of incubation on both 5mM and 10mM TBTC containing plates. These colonies were then replica plated on 10mM TBTC containing agar plates. Only two colonies appeared on MSM+10mM TBTC agar plates, so these colonies were picked up and characterized further for growth and TBTC degradation. It is very interesting to note that the wild type culture could not grow on MSM+10mM TBTC agar plates, whereas NTG-induced mutant could tolerate even 10mM TBTC (Fig 5.6a, b).

TBTC is one of the organotin compounds which has been reported to be an environmental pollutant showing mutageneic effect on bacteria (Hamaski et al. 1993). It was apperent that mono-n-butyltin oxide, n-butyltin-dichloride, di-n-butyltin-dichloride, tri-n-butyltin dichloride, bis-(tri-n-butyltin)-oxide and dimethyltin-di- chloride have mutagenic effect on *Salmonella typhimurium* and *E coli* (Hamaski et al. 1992).

DNA damaging mutagenic agents produce a variety of DNA adducts that have variable potency with respect to cytotoxicity and mutagenicity. NTG is considered the most efficient cytotoxic agent which causes diverse type of DNA adduct on genome to plasmid (Lonarek and Sori,1998).The bacteria such as *E.coli* , *Salmonella typhimurium* etc are very sensitive to mutagenic action of nitrosoguanidine. In case of *Sphingomonas paucimobilis* SYK-6, it was observed that nitrosoguanidine mutagenesis altered the 5-5- dehydrodivanillic acid degradation pathway and the mutant was able to degrade syringate and vanillate (Nishikawa et al. 1998). The mutant strain of *Pseudomonas fluorescens* showed elevated level of resistance to zinc and cadmium than the wild type strain (Rossbach et al. 2000). In the present investigation, some cells of *Paeudomonas aeruginosa* strain USS25 have under gone mutational change, which helped the cells to tolerate elevated concentration of TBTC

(10mM), whereas the wild type strain could not grow at this level. The molecular mechanism of such mutational change in the test organism is still not clear, but in the other NTG mutagenesis study of pUC19-*lac* ZC ^{141} DNA showed the alteration of codon ($G^{141}CCC^{141}CC$) at position 141 to 143 in the *lacZ* gene and produced a mutant (white) colony in *lacZ*$^-$ cells, as opposed to the dark blue colony (wild type) (Ogawa et al. 1997). As most of the mutagens produces multiple DNA adduct and lesions, it is difficult to identify the potency of these chemicals which are responsible for the specific damage, but the present mutagenesis study revealed that NTG can make the changes in genes of the chromosomal genome which could give rise to hyper TBTC tolerant mutants, capable of utilizing even 10mM TBTC in MSM broth. These mutants were further characterized to check if they transform this result of debutylation of TBTC.

5.3.2 Characterization of mutants with reference to growth behaviour, TBTC tolerance and TBTC degradation

5.3.2.1 Comparison of wild type and NTG induced mutant with reference to growth behaviour at higher level of TBTC

In order to study the comparative growth behaviour of NTG-induced mutant with wild type strain, they were grown separately in MSM broth with 5mM and 10mM of TBTC respectively. In case of MSM+ 5mM TBTC broth, the wild type strain could not multiply with time whereas the mutant strain showed very good growth after lag phase of six hours (Fig 5.6a). Similarly when wild type and mutant type strains (cells) were allowed to grow in MSM broth + 10mM TBTC, wild type strain showed absolutely no growth whereas the mutant strain could grow very well with initial lag phase of 8-9 hrs (Fig 5.6b). It clearly indicated that NTG induced mutant strain is not only resistant to TBTC but can utilize it even at elevated concentration

5.3.2.2 Comparative TBTC degradation profile of wild type and NTG induced mutant

The wild type strain and NTG induced mutant strain of *Pseudomonas aeruginosa* strain USS25 were characterized for TBTC. It was observed that as the mutant strain could grow faster, it could degrade TBTC to a higher more compared to wild type strain. TLC profile of TBTC degradation showed significant production of TBTC derivative in mutant strain (Fig-5.7). This observation clearly confirms that NTG-induced mutant has got higher TBTC degradation capability than the wild type strain of *Pseudomonas aeruginosa* strain USS25.

In the present mutagenesis study, the NTG induced mutant showed better growth and degradation capability at elevated concentrations of TBTC. The mutagenic treatment may have induced some intracellular signaling pathways, which leads to the development of a bacterial isolate which tolerates high level of TBTC. On TLC plate, the degradation spot of mutant strain was more intense than the wild strain. The possible reason could be, in case of NTG mutant biosorption by the cells were more, hence more degradation and more degradation product. This observation confirms that mutant strain has higher TBTC degradation ability than the wild type.

The molecular mechanisms of growth and degradation in mutant and wild type could be numerous. In another study of mutation in *Bacillus* sp. it was observed that mutant exhibited (i) elevated levels of membrane ATPase activity relative to the wild type. (ii) Slightly elevated respiratory rates, with the cytochrome content of the membranes being the same as or slightly lower than those of the wild type. (iii) The most consistent difference in mutant and wild type was reduction in the content of mono-saturated C16 fatty acids in membrane phospholipids (Guffanti et al. 1987). In the case study of TBT resistant spontaneous mutant of *E. coli* K-12 strain, it was found that cytochrome sufficient mutant

was more resistant to TBT than cytochrome deficient mutant. The comparative study of these two strains revealed the presence of TBT-resistant membrane bound ATPase, which may account for their resistance to TBT (Singh and Singh, 1984). The Nitrosoguanidine mutagenesis study of *Pseudomonas paucimobilis,* was to reveal the alteration in 5-5-dehydrodivanillic acid pathway (Nishikawa, et al. 1998).

We need to explore further the exact molecular mechanism of NTG mutagenesis to understand the TBTC utilization by resistant /mutant *Pseudomonas aeruginosa* strain USS25. These natural as well as mutant isolates may be tested to bioremediate TBT contaminated sites.

Lane 1 & 2 – Plasmid DNA

Fig 5.1 Plasmid DNA profile of *Pseudomonas aeruginosa* strain USS25

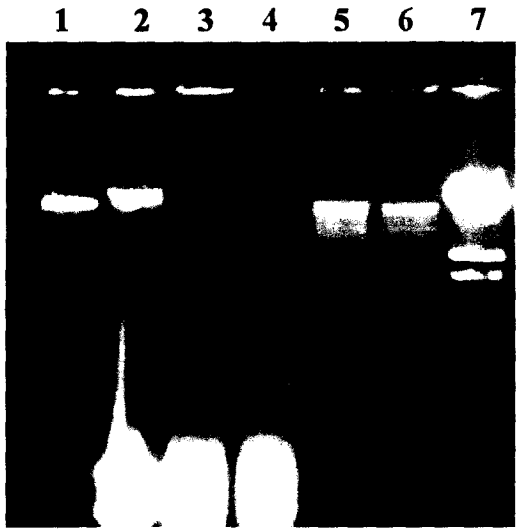


Fig 5.2a Restriction mapping of plasmid DNA of *Pseudomonas aeruginosa* strain USS25

Lane 1 **- Marker – (Lambda DNA Eco RI + Hind III digest),** ***Lane 2*** **- Plasmid DNA (Uncut with endonucleases),** ***Lane 3*** **- Plasmid DNA cut with NcoI,** ***Lane 4*** **- Plasmid DNA cut with NcoI,** ***Lane 5*** **- Plasmid DNA cut with BamHI,** ***Lane 6*** **- Plasmid DNA cut with BamHI.** ***Lane 7*** **- Marker- Lambda DNA (EcoR1 digest)**

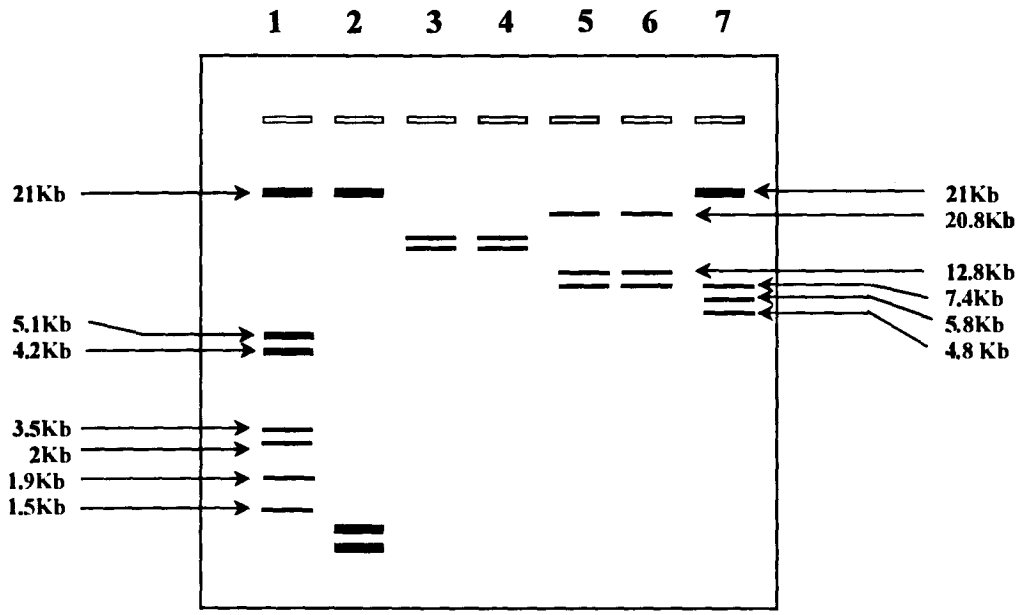


Fig 5.2b Diagramatic sketch of restriction mapping

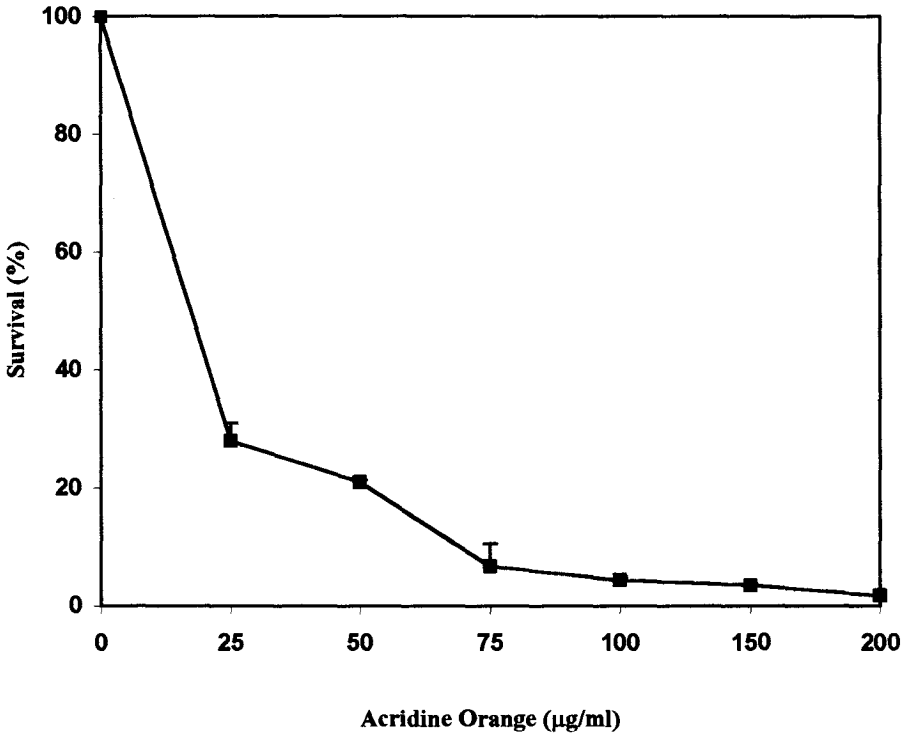


Fig 5.3 Survival of *Pseudomonas aeruginosa* strain USS25 in presence of Acridine orange

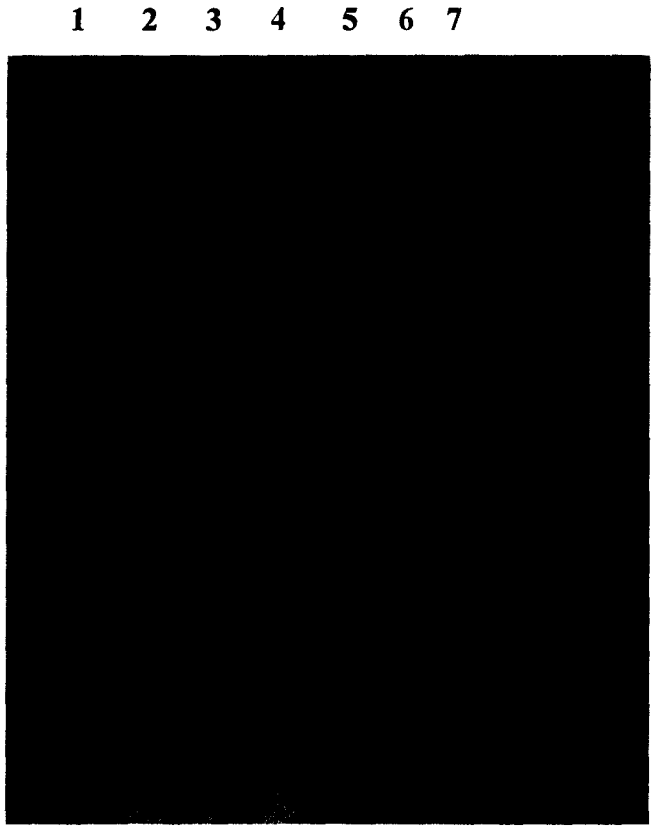


Fig 5.4 Plasmid profile of *Pseudomonas aeruginosa* strain USS25 after subsequent curing with acridine orange

Lane 1 - Marker- Lambda Eco R1 and Hind III digest.
Lane 2 - Plasmid profile of first subculture with acridine orange.
Lane 3 - Plasmid profile of second subculture with acridine orange.
Lane 4 - Plasmid profile of third subculture with acridine orange.
Lane 5 - Plasmid profile of fourth subculture with acridine orange.
Lane 6 - Plasmid profile of fifth subculture with acridine orange.
Lane 7 - Plasmid profile of sixth subculture with acridine orange

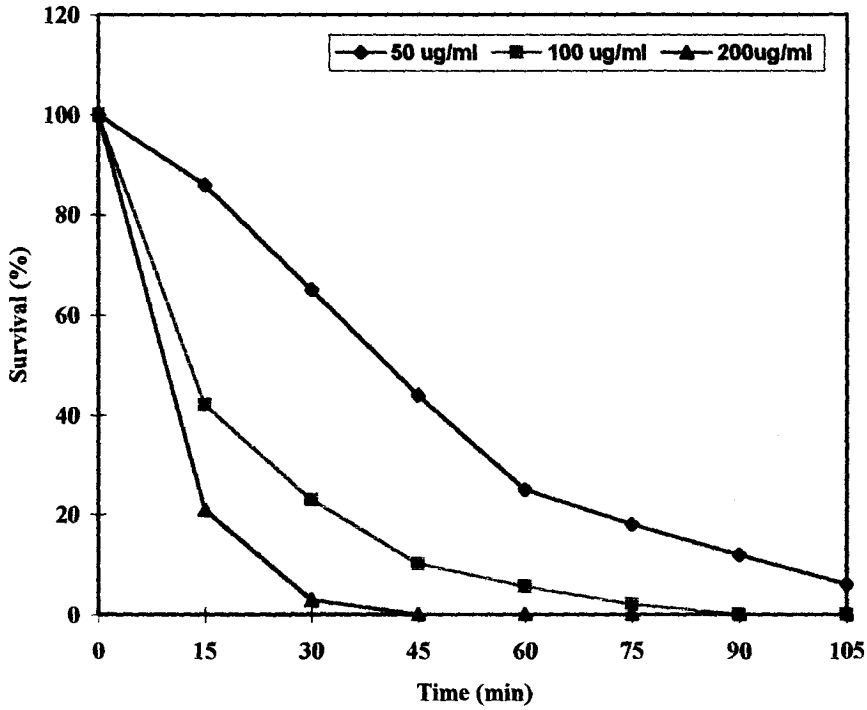


fig 5.5 Survival of *Pseudomonas aeruginosa* strain USS25 in presence of NTG at regular time interval at different concentration

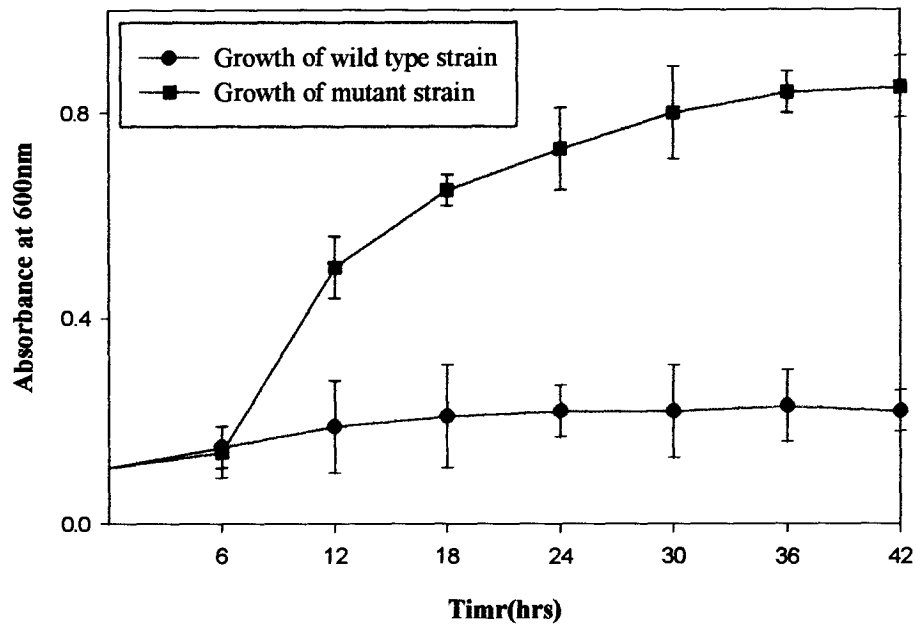


Fig 5.6a Growth behaviour of parent and NTG mutant of *Pseudomonas aeruginosa* strain USS25 in MSM+ 5mM of TBTC

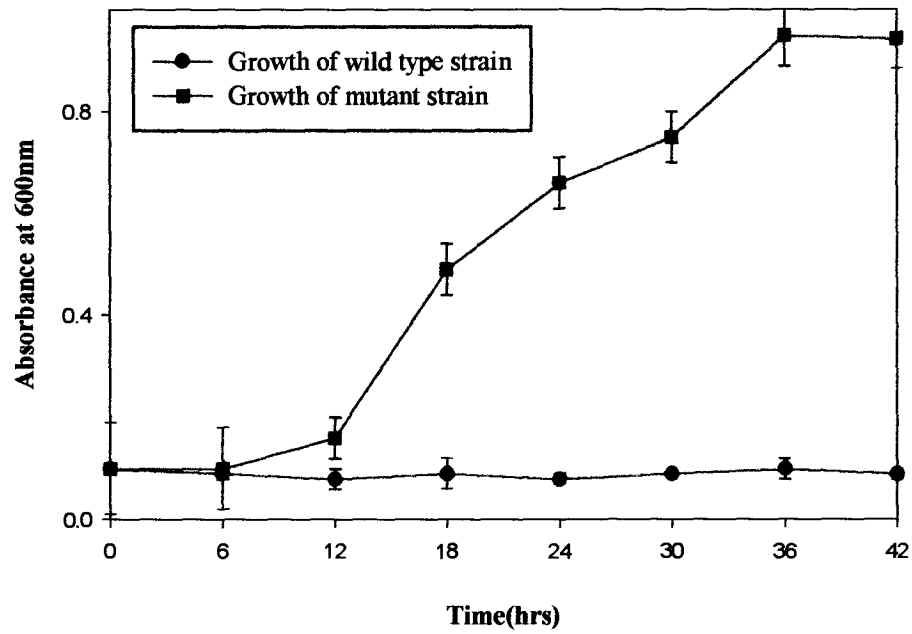


Fig. 5.6b Growth behavour of parent and NTG mutant strain of *Pseudomonas aeruginosa strain* USS25 in MSM + 10mM TBTC

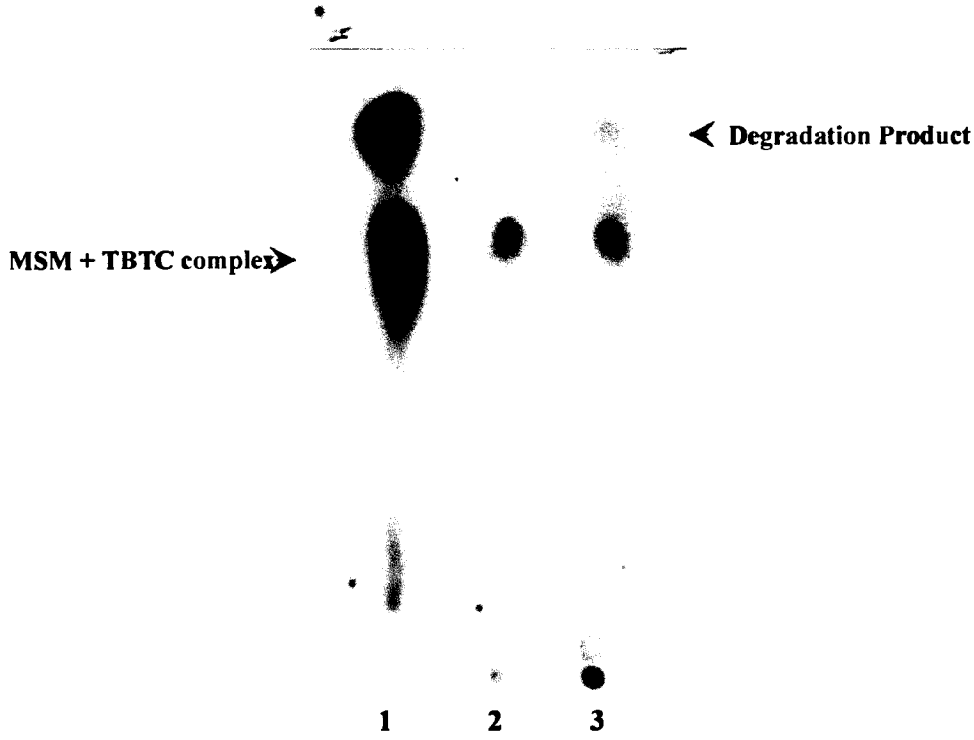


Fig 5.7 Comparison of TBTC degradation profile of *Pseudomonas aeruginosa* strain USS25 (wild type strain) and NTG induced mutant

1. Cell extract of NTG-induced TBTC^R mutant grown in MSM + 5mM TBTC.
2. MSM + 5mM TBTC (Control)
3. Cell extract of wild type strain grown in MSM + 5 mM TBTC.

Summary

Salient features:

* Surface water samples were collected from different sites of west coast of India viz. Bombay High Oil field area and Goa Ship yard and physicochemical characteristics of water samples were determined as temperature 28°C- 30°C, pH 8-8.3 and salinity 33-35 ‰.
* Viable count of bacteria in these water sample revealed that majority of natural bacterial population (68%) can grow up to 0.4mM of TBTC.
* The five potent TBTC resistant bacterial strains were selected after continuous sub-culturing in MSM in presence of TBTC and were designated as 25W, 25B, 5Y2, 3(4Sub) and 9(3A).
* These five bacterial strain have been identified as *Pseudomonas aeruginosa* strain USS25 (25W), *Pseudomonas aeruginosa* strain 25B, *Pseudomonas aeruginosa* strain $5Y_2$, *Pseudomonas fluorescens* strain 3(4Sub) and *Pseudomonas stutzeri* strain 9(3A).
* All these isolates were found to grow up to 2mM of TBTC in MSM broth as well as MSM agar as they utilize TBTC as carbon source.
* The optimum temperature, pH and salinity for growth of all five isolates were found to be 28°C, pH-7.2 and 2.5 % (25‰) respectively.
* All the five isolates were cross tolerant to heavy metals viz. Hg, Zn and Cd and common antibiotics such as Penicillin, Ampicillin, Tetracyclin, Chloramphenicol and Streptomycin including some other antibiotics viz. Neomycin, Spectinomycin, Rifampicin, Kanamycin, Nalidixic acid etc.
* On the basis of faster utilization of TBTC as carbon source, the strain *Pseudomonas aeruginosa* strain USS25 was selected for further biological characterization with reference to TBTC degradation and its molecular mechanisms.
* It was observed that MSM was best medium for growth and TBTC degradation by

Pseudomonas aeruginosa strain USS25.

* Among the various tested carbon sources viz. glucose, succinate and glycerol, glucose was found to be the best for growth of *Pseudomonas aeruginosa* strain USS25 ,whereas ammonium chloride served as a best nitrogen sources.
* It was observed that uptake of TBTC was more in presence of both succinate and glycerol in MSM.
* The spectrophotometric analysis of chloroform extract of cells grown in TBTC containing medium showed a sharp absorption peak at 241nm.
* Preliminary TBTC degradation studies (TLC analysis) have revealed that TBTC is definitely transformed to different organotin derivatives, but very slowly. Time course study of TBTC degradation has revealed that the bacterial isolate can degrade TBTC within 75 days of incubation.
* It has been observed that both succinate and glycerol together significantly enhance the TBTC degradation process, possibly due to enhanced bioavailability of TBTC as well as cell population.
* The test organism significantly degrades (transforms) the TBTC as 280mg of degradation product is produced out of 1gm of TBTC (28% aprox).
* The IR and NMR analysis of pure TBTC degradation compound has revealed that the molecular formula of the degradation product is Monobutyltin dichloro hydride ($BuSnHCl_2$).
* Mono-thiol (Mercaptoethanol) and chelating agent (EDTA) significantly reduced the toxicity of TBTC to *Pseudomonas aeruginosa* strain USS25, since this isolate could tolerate 4mM and 5mM TBTC in presence of thiol and EDTA respectively.
* *Pseudomonas aeruginosa* strain USS25 showed a significant effect of TBTC on EPS production, as the yield of EPS was recorded 4.9mg/gm of dry cell mass.
* TBTC induced EPS showed enhanced surfactant activity as compared to control, which

is produced only in presence of glucose.

* The surfactant activity of TBTC induced EPS was higher in benzene-water as well as chloroform –water systems.
* The test organism *Pseudomonas aeruginosa* strain USS25 also produces green fluorescent pigment in the growth medium, which has been identified as phenazine and it is interesting to note that TBTC causes significant increase in pigment synthesis.
* SDS-PAGE analysis of protein sample of *Pseudomonas aeruginosa* strain USS25 has revealed expression of an additional a novel protein of 45 K.Da. Further characterization of this polypeptide will be done to explore its involvement in degradation mechanisms.
* TBTC resistant *Pseudomonas aeruginosa* strain USS25 possesses a plasmid of 41Kbps. We have also confirmed that it has no role in TBTC degradation since plasmid cured bacterial cells still show TBTC resistance and degradation capability.
* Therefore we can infer that gene governing TBTC degradation in *Pseudomonas aeruginosa* strain USS25 is located in genomic DNA.
* NTG-mutagenesis studies using *Pseudomonas aeruginosa* strain USS25 revealed that NTG-induced mutants can grow upto 10mM TBTC in MSM broth and also shows significant degradation of TBTC as compared to the wild type strain.

Future prospects of the present study

Microorganisms indigenous to polluted sites often have a limited ability to degrade xenobiotics and toxic pollutants which are highly substituted or which have especially novel chemical structure (Pipke et al. 1992). 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. We have isolated five *Pseudomonas* strains and studied extensively *Pseudomonas aeruginosa* strain USS25 as a potent strain for organotin degradation in marine and estuarine environment of west coast of India. It has novel characteristics to produce EPS which shows significant surfactant activity to emulsify TBTC in the medium. The structural analysis of the EPS may give rise to the exact molecular basis of the TBTC solubilization in the medium and subsequent degradation of the biocides which is a serious threat to marine biota.

As this pigmented isolate can bioaccumulate TBTC on the cell surface, therefore this culture can be used for immediate removal of TBTC from the contaminated marine and estuarine site. Role of EPS in emulsification and biosorption of TBTC needs to be explored in detail in order to answer various questions related to TBTC biodegradation and bioremediation.

The molecular biological and genetic studies have confirmed that the TBT degradation gene(s) is located on chromosomal genome. We have planned to find out the gene(s) responsible for degradation. Further characterization of the gene(s) may certainly reveal exact molecular mechanism of TBTC degradation and resistance. We can explore and examine these TBTC inducible genes by cloning these genes in *lux* reporter plasmid pUCD615 (Gift from Dr. Kado) using standard techniques (Sambrook et al. 1989).

Therefore, we suggest that much focussed research is required to elucidate the mechanism of TBTC bioaccumulation, biodegradation and bioremediation in TBTC resistant marine bacteria employing molecular biological and genetic tools (Sambrook et al. 1989). This study would ultimately enable us to check the potential of these natural as well as genetically engineered bacterial strains for bioremediation of TBTC contaminated marine and estuarine sites of west coast of India.

Appendices

APPENDIX

A Media composition

A.1 Mineral Salt media (single strength) for 1L : (Seubert, 1960; Mahtani and Mavinkurve, 1979)

Ferrous sulphate (green crystals)	0.06 gm
Dipotassium hydrogen ortho phosphate (12.6%)	100ml
Potassium dihydrogen ortho phosphate (18.2%)	20ml
Ammonium nitrate (10%)	20ml
Magnesium sulphate (1%)	20ml
Manganese sulphate (0.6%)	0.2 ml
Sodium molybdate (0.6%)	0.2 ml
Calcium chloride (dihydrate) (1%)	15 ml
Double Distilled Water	1000 ml

For use: 10 ml of double-strength media made to 20 ml with Double Distilled Water and sterilized for 10 min at 120°C temperature and 15 lbs pressure. To prepare MSM agar, MSM broth is mixed with agar (1.5%) and autoclaved accordingly.

A.2 Nutrient Broth

Peptone	10.0 gm
Beef extract	3.0 gm
Sodium-chloride	5.0 gm
Double Distilled Water	1000 ml

pH was adjusted to 7.0 with 0.1 N NaOH

For nutrient agar, 1.5 gm of agar added to 100 ml nutrient broth. Digested in water-bath and sterilized accordingly.

A. 3 Luria Bertani (L.B) Broth (Gerhardt et al., 1994)

Tryptone	10.0 gm
Yeast extract	5.0gm
Sodium Chloride	10.0gm
Double Distilled Water	1000 ml

Adjusted to pH 7.0 with 0.1 N NaOH

For L.B agar, 1.5 gm of agar is added to 100 ml L.B broth. Digested in water-bath and sterilized accordingly.

A.4 Zobell marine Broth (Zobell, C.E. 1941)

Peptic digest of animal tissue	5gm
Yeast Extract	1gm
Ferric citrate	0.1gm
Sodium chloride	19.45gm
Magnesium chloride	8.8gm
Calcium chloride	1.8gm
Potassium chloride	0.55gm
Sodium sulfate	3.24gm
Sodium bicarbonate	0.16gm
Potassium bromide	0.08gm
Strontium chloride	0.034gm
Boric acid	0.22gm
Sodium silicate	0.004gm
Sodium fluorate	0.0024gm
Ammonium nitrate	0.0016gm
Disodium Phosphate	0.008gm
Agar	15gm
Double Distilled Water	1lit

For agar plates add 1.5% agar to Zobell marine broth and sterilized (autoclaveed).

B Reagents for Estimation of nitrite, nitrate, phosphate and salinity:

B.1 Reagents for nitrite estimation

1. Sulphanilamine : Sulphanilamine (1 gm) was dissolved in 10 ml concentrated HCl and the solution was made up to 100ml with Double Distilled Water.
2. N- (1- naphthyl) ethylene diamine dihydrochloride: In 100ml Double Distilled Water, 0.1 gm of reagent was dissolved.
3. Standard $NaNO_2$ solution: Pure analytical grade $NaNO_2$ (0.1725 gms) was dissolved in 250ml of Double Distilled Water (1ml contains 10 μg atom of NO_2-N).
4. Working solution A: Above mentioned standard $NaNO_2$ (2.5ml) solution was diluted to 250ml with Double Distilled Water (1ml contains 0.1 μg atom of NO_2-N).
5. Working solution B: Working solution A (50ml) was diluted to 500ml with distilled water. (1ml contains 0.01μg atom of NO_2 - N).

B.2 Reagents for nitrate estimation

1. Concentrated Ammonium chloride (NH_4Cl): NH_4Cl (62.5 gm) was dissolved in a 250ml volumetric flask with distilled water.
2. Diluted Ammonium chloride (NH_4Cl): The above concentrated NH_4Cl (5ml) was diluted to 200ml with distilled water.
3. Amalgamated Cadmium granules : This reagent (100gm) is treated with 500ml of 2% solution of $CuSO_4$. Then the amalgamated Cd granule are washed several times with distilled water and is stored in diluted NH_4Cl.
4. Sulphanilamide: The reagent (1g) was dissolved in 10ml of concentrated HCl and the volume was made to 100ml with distilled water.
5. N-(1-naphthyl)-ethylene diaminedihydrochloride: The reagent (0.1gm) was dissolved in 100ml of distilled water to prepare the stock solution of the reagent.
6. Standard KNO_3 solution: KNO_3 (0.1g) was dissolved in 100ml distilled water .
7. Working solution of KNO_3 (standard): The above solution (2.5ml) was diluted to 250ml with distilled water.

B.3 Reagents for phosphate estimation

1. 9N H_2SO_4: Concentrated H_2SO_4 (25ml) was added to 75ml Double Distilled Water.
2. Ammonium molybdate solution: Ammonium molybdate (9.5gm) was dissolved in 100ml of Double Distilled Water.
3. Ascorbic acid: Ascorbic acid (7gm) was dissolved in 100ml of Double Distilled Water.
4. Potassium Antimonyl Tartarate: Potassium antimonyl tartarate (3.25gms) was dissolved in 100ml Double Distilled Water.
5. Mixed Reagent: Ammonium molybdate (22.5ml) solution, 100ml of H_2SO_4 and 2.5ml of Potassium Antimonyl tartarate solution was mixed together.
6. Standard phosphate solution - Potassium-di-hydrogen-ortho phosphate (KH_2PO_4) (0.1361gms) was dissolved in 100ml of Double Distilled Water.
7. Working Phosphate solution: The above solution (2.5ml) was diluted to 250ml of Double Distilled Water (1ml = 0.1 μg atom).

B.4 Reagents for salinity estimation

1. $AgNO_3$ solution: Pure crystallized Silver nitrate (27.25 gm) was dissolved in 1L of Double Distilled Water. This solution was mixed and stored in amber coloured bottle.
2. Potassium chromate indicator solution: Pure Potassium chromate (10 gm) was dissolved in 100ml of Double Distilled Water.

C. Preparation of stock solutions

C.1 Tributyltin chloride (TBTC)($C_{12}H_{27}ClSn$)(F.W-325.49g/mol),obtained from MERCK ,Germany.

Preparation of 1M stock of TBTC

Absolute ethanol (filter sterilized) (72.2ml) + TBTC (27.8 ml)(3.7M) was mixed

make up the final volume of 100ml. The solution was kept in amber coloured bottle in cold and dark condition. The experiments were performed by remove the required amount stock solution in sterile condition.

C.2. Dibutyltin chloride (DBT)($C_8H_{18}C_{l2}Sn$)(F.W-303.83 g/mol) from MERCK, Germany.

C.3 Metal stock solution

1. Mercuric chloride ($HgCl_2$)(F.W-271.50)

Stock solution (1M) - $HgCl_2$ (6.78gm) was dissolved in 25ml of De-ionized Double Distilled Water. The solution was filter sterilized and stored at 4°C in a dark place.

2. Cadmium chloride ($CdCl_2$) (F.W-183.31)

Stock solution (1M) - $CdCl_2$ (4.6gm) was dissolved in 25ml of De-ionized Double Distilled Water. The solution was filter sterilized and stored at 4°C in a dark place.

3. Zinc sulphate ($ZnSO_4$) (F.W-287.54)

Stock solution (1M) - $ZnSO_4$ (1.4gm) was dissolved in 25ml of De-ionized Double Distilled Water. The solution was filter sterilized and stored at 4°C in a dark place.

C.4 Antibiotics

Antibiotic (10mg) was weighed and dissolved in 1ml of sterile De-ionized Double Distilled Water and filter sterilized. Antibiotic used were Chloramphenicol, Rifampicin, Tetracyclin, Kanamycin, Ampicillin, Nalidixic Acid, Streptomycin, Neomycin, Streptomycin, Penicillin, Antimycin, Amikacin, Nystatin, Novobiocin, Antimycin.

C.5 β mercapto ethanol (C_2H_6OS) (F.W-78.13) (SIGMA)

β mercapto ethanol was used v/v in medium to obtain the required concentration.

C.6 EDTA (F.W-372.24)

EDTA	20gm
Double Distilled Water	50ml

Required amount of concentrated NaOH was added to dissolve EDTA and final volume was made up to 50ml.

C.7 Glycerol

Sterile glycerol was added to the medium (v/v) to obtain the required concentration.

C.8 Succinate

Succinate	10gm
Double Distilled Water	100ml

The stock solution was autoclaved and used for experimental purpose.

C.9 Glucose

D-Glucose	20gm
De-ionized Double Distilled Water	100ml

The stock solution was filter sterilized and used for experimental purpose.

C.10 Ammonium nitrate, Potassium nitrate and Ammonium chloride

Salt	10gm
De-ionized Double Distilled Water	100ml

The stock solution was autoclaved and used for experimental purpose.

C.11 Acridine orange

Acridine orange (50mg) was dissolved in 100ml of double distilled water and filter sterilized. The solution was kept in amber coloured bottle at 4°C in a dark place. (The final stock concentration was 500 μg/ml).

D. Other Reagents, buffers and stains

D.1 Reagents for protein separation by PAGE/SDS-PAGE (Laemmli. 1970)

(i) Monomer Solution (30%T, 27% C)

Acrylamide	29.2 gm
Bis-acrylamide	0.8 gm
Double Distilled Water	100 ml

Stored at 4°C in dark conditions

(ii) Separating gel buffer (1.5 M, pH 8.8)

Tris (base)	18.15 gm
Double Distilled Water	100 ml

pH was adjusted to 8.8 with 6 N HCl, stored at 4°C in dark condition.

(iii) Stacking gel buffer (0.5 M, pH 6.8)

Tris (base)	3 gm
Double Distilled Water	50 ml

pH was adjusted to 6.8 with 6 N HCl and stored at 4°C

(iv) 10% SDS

Sodium dodecyl sulphate	1 gm
De-ionized Double Distilled Water	10 ml

(v) 10% ammonium per sulphate (Initiator) (APS)

APS	0.5 gm
Double Distilled Water	5 ml

(vi) Dye solution

Bromophenol blue	0.1 gm
Sucrose	50 gm
Double Distilled Water	100 ml

(vii) Tank buffer (0.025 M Tris, 0.192 M glycine, pH 8.3)

Tris-Base	3 gm
Glycine	14.4 gm
SDS (10%)	10 ml
Double Distilled Water	1L

pH was adjusted with acid and base to 8.3

and Store at 4°C.

(viii) Sample buffer

0.5M Tris HCl , pH 6.8	1.25 ml
SDS (10%)	2 ml
Glycerol	2.5 ml
Bromophenol blue (W/V)(0.5%)	0.2ml
Double Distilled Water	3.55 ml

Add 50 µl of β-mercaptoethanol was added to 950µl sample buffer prior to use.

Sample was diluted at 1:2 with buffer and heated at 95°C for 4min.

D.2 Gel preparation

Solution	Separating Gel		Stacking gel	
	Big gel	Mini gel	Big gel	Mini gel
Monomer solution	10 ml	2.5 ml	1.33 ml	0.66 ml
Separating gel buffer	7.5 ml	1.875 ml	-	-
Stacking gel buffer	-	-	2.5 ml	1.25 ml
SDS (10%)	0.3 ml	0.075 ml	0.1 ml	0.05 ml
APS (10%)	150 µl	37.5 µl	50 µl	25 µl
TEMED	10 µl	2.5 µl	5 µl	2.5 µl
Double Distilled Water	12 ml	3 ml	6.1 ml	1.05 ml

D.3a Staining of PAGE/SDS-PAGE gels with Coomassie brilliant blue

(i) Staining solution

Coomassie Brilliant Blue R-250	600 mg
Methanol	240 ml
Acetic acid	100 ml
Double Distilled Water	260 ml

(ii) Destaining solutions

	I	II or	II
Methanol	500 ml	50 ml	-
Acetic acid	100 ml	70 ml	7 ml
Double Distilled Water	400 ml	1000 ml	100 ml

D.3b Silver staining of SDS-PAGE gels (Sparnins and Chapman., 1976)

(i) Fixative solution

Ethanol	25 ml
Acetic acid	10 ml
D/W	100 ml

(ii) Dithiothreitol (5mg/ml)

Dithiothreitol	0.5 mg
Double Distilled Water	100 ml

(iii) Silver nitrate solution (0.1%)

$AgNO_3$	0.1 gm
Double Distilled Water	100 ml

(iv) Formaldehyde solution in 3% Na_2CO_3

40% Formaldehyde	0.1 ml
Na_2CO_3	6 gm

Double Distilled Water	200 ml

Na_2CO_3 was dissolved in D/W and then formaldehyde was add and mixed.

(v) 2.3M Citric acid

Citric acid	24.15 gm
Double Distilled Water	50 ml

E. Reagents for plasmid extraction

E.1 Alkaline Lysis Method (Brinboim and Doly, 1979)

i) Solution I (pH 8.0)

Glucose	0.9 gm
Tris-chloride	0.394 gm
EDTA	0.292 gm
Double Distilled Water	100 ml

ii) Solution II

SDS	1.0 gm
0.2N NaOH	100 ml

iii) Solution III (pH 5.0)

5 M Potassium acetate	60.0 ml
Glacial acetic acid	11.5 ml
Double Distilled Water	28.5 ml

E.2 Boil Prep method (Holmes and Quigley, 1981)

i) STET buffer (pH 8.0)

NaCl	0.584 gm
Tris-chloride	0.158 gm
EDTA	0.037 gm
Triton X-100	5.0 ml
Double Distilled Water	100 ml

ii) Lysozyme solution

Lysozyme	100 mg
Tris-chloride (pH 8.0)	10 ml

iii) 2.5 M Na-acetate

E.3 TE buffer (pH 8.0)

Tris-chloride	0.156 gm
EDTA	0.029 gm
Double Distilled Water	100 ml

E.4 Electrophoresis

i) Agarose gel

Agarose	0.8 gm
TAE buffer	100 ml

ii) Tris acetate EDTA (TAE) buffer (pH 8.0) (1X)

50x: Tris base	2.42 gm
0.5M EDTA	1ml

Tris base and 0.5M EDTA was dissolved in 10ml of Double Distill water and pH was adjusted to 8.0 with glacial acetic acid (0.57ml) and the final volume was made upto 500ml.

iii) Ethidium bromide solution

Ethidium bromide	10 mg
Distilled water	1 ml

Stock solution was prepared and kept in cool and dark place. The final concentration used for agarose gel was 5μg/ml.

E.5 Tracker dye

Bromophenol blue	0.25 gm
Sucrose	40 gm
0.1M EDTA	10ml
1% SDS	10ml
Distilled water	100 ml

The dye was stored at 4 °C.

F. Reagents for mutagenesis study

F.1 NTG (1-Methyl-3-Nitro-Nitrosoguanidine)(F.W-147.09)(MERCK)

NTG	1mg
0.1M Citrate Buffer (pH 5.5)	1ml

Freshly stock solution was prepared and kept in amber coloured bottle in cold and dark place.

F.2 Citrate buffer (pH 5.5)

Citric acid	10.5gm
NaOH	4.4 gm
Double Distilled Water	500ml

pH was adjusted to 5.5 and autoclaved.

F.3 Phosphate buffer (pH 7.0)

Potassium dihydrogen phosphate	6.8gm
NaOH	1.16 gm
Double Distilled Water	500ml

pH was adjusted to 7.0 with 2N NaOH and autoclaved.

G. Reagents for Protein estimation (Lowry, et al., 1951)

G.1 Reagent A: Sodium Carbonate reagent

Na_2CO_3	2 g
NaOH (0.1 N)	100 ml

G.2 Reagent B: Copper Sulphate Solution

Sodium potassium tartarate	1 g
Copper Sulphate	0.5 g
Double Distilled Water	100 ml

G.3 Reagent C: Alkaline Copper Sulphate Soultion

(i) Reagent A	50 ml
(ii) Reagent B	1 ml

Reagent C was prepared fresh at the time of estimation.

G.4 Reagent D – Folin Ciocalteau (FC) reagent

FC reagent (commercial grade)	10 ml
Double Distilled Water	20 ml

Freshly prepared at the time of estimation.

H. Reagents for sugar estimation (Phenol Sulphuric acid method) (Dubois, et al., 1956)

H.1 Reagent A

Phenol (Reagent agent)	5gm
Double Distilled Water	100ml

Phenol (5gm) dissolved in water and diluted up to 100ml.

H.2 Reagent B

Sulphuric acid (96%) (Reagent grade)

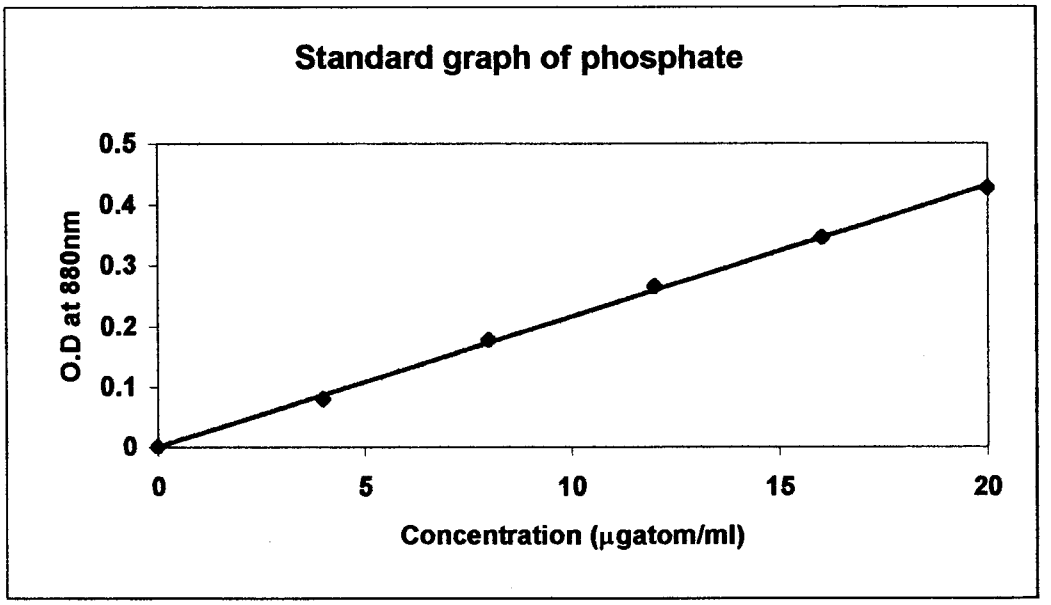
H.3 Reagent C

Glucose	100mg
D/W	100ml

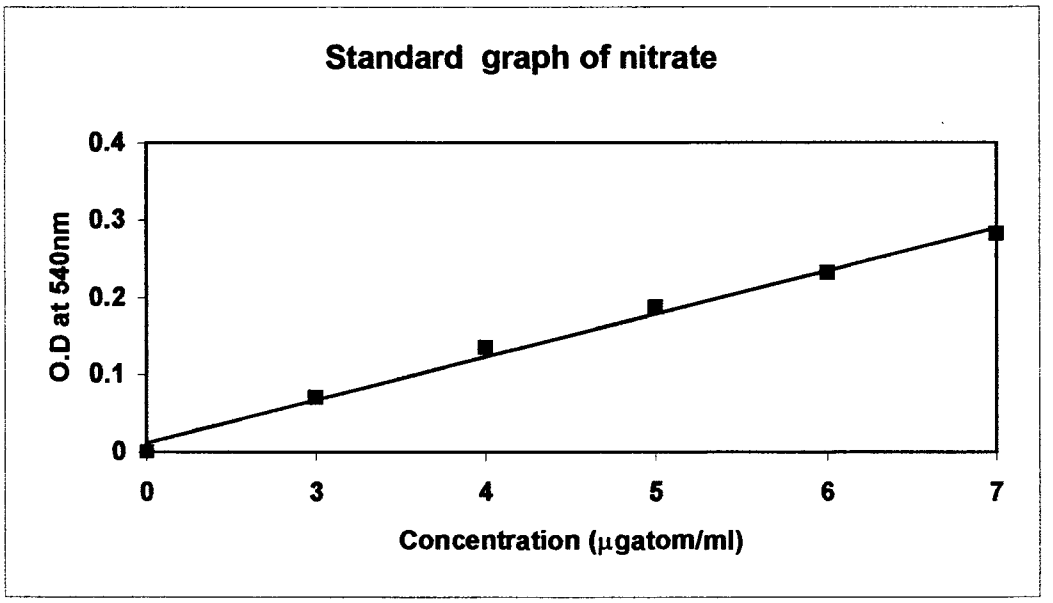
Working standard- 10ml stock diluted to 100ml with distill water.

L. Standard graphs

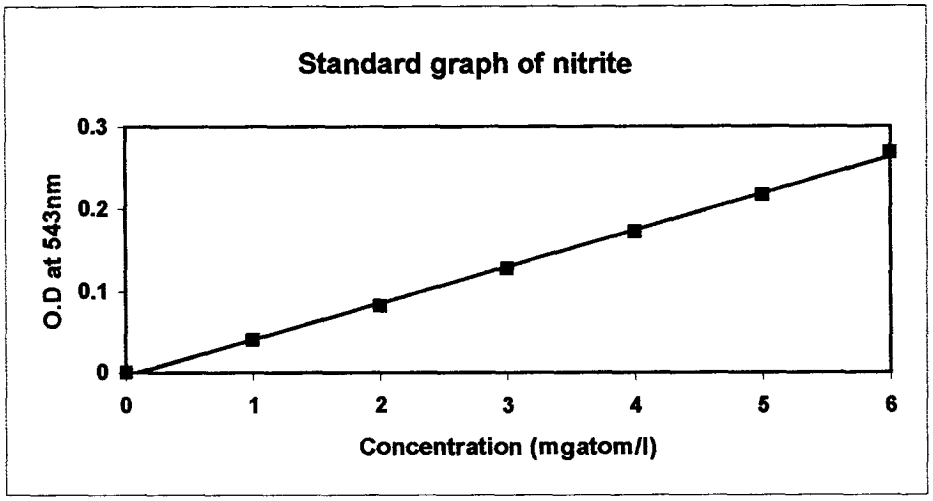
a. Standard graph of phosphate



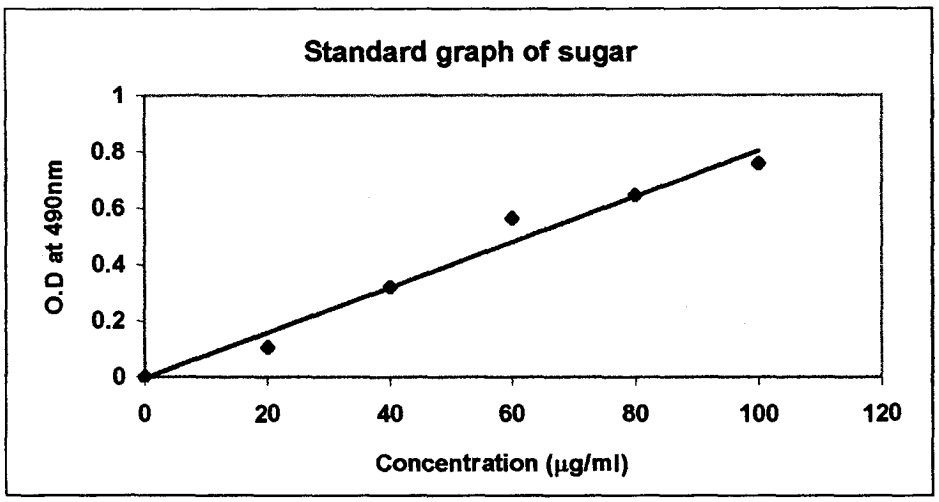
b. Standard graph of nitrate



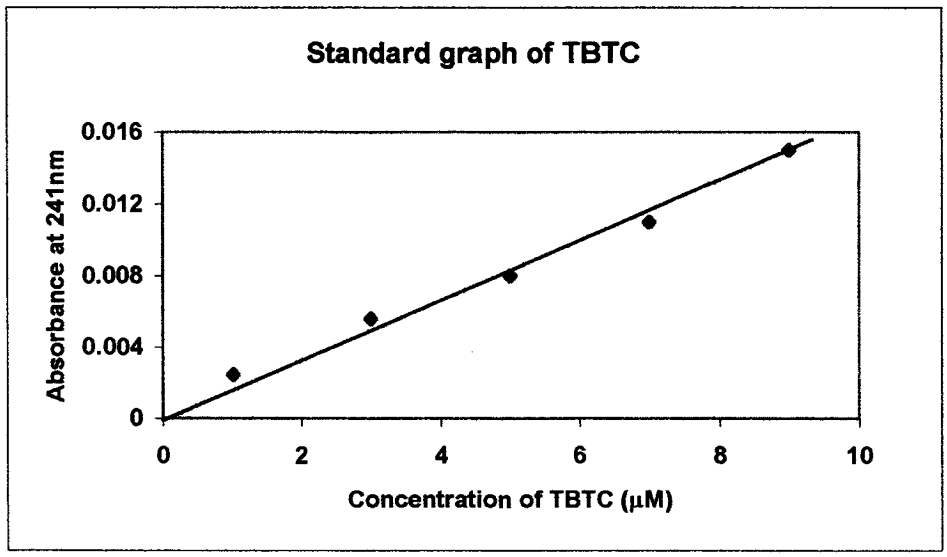
c. Standard graph of nitrite



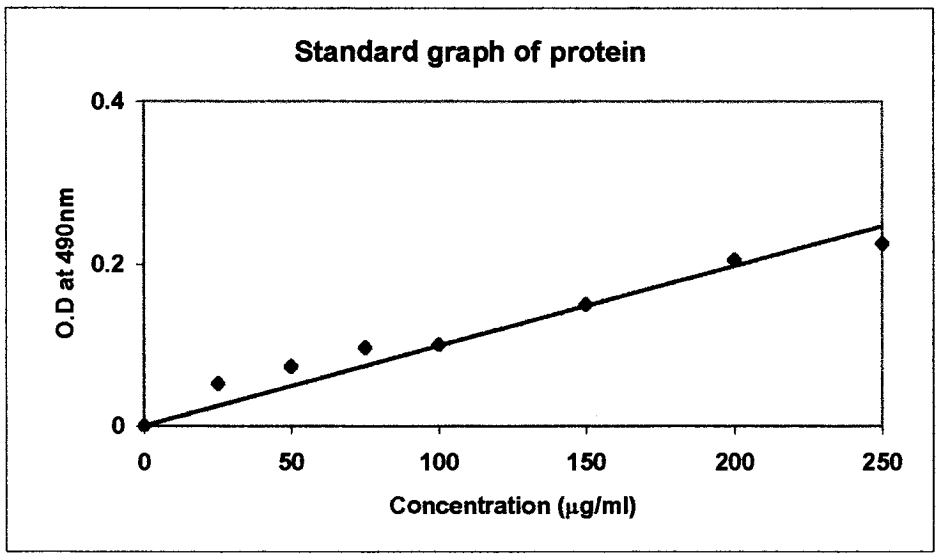
d. Standard graph of sugar

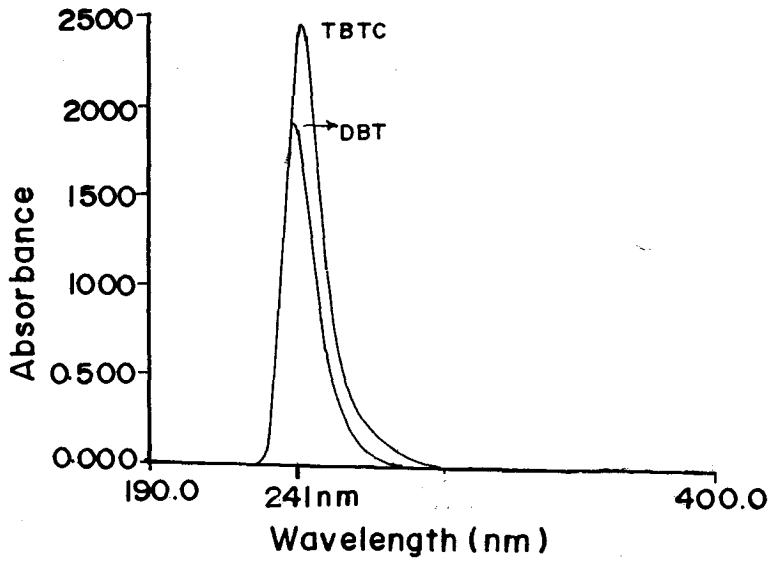


e. Standard graph of TBTC



f. Standard graph of protein





J.1. Spectrophotometric analysis of TBTC (2mM) and DBT(2mM) dissolved in MSM

Bibliography

Alzieu, C.L., Heral, M. 1984. Ecotoxicological effects of organotin compounds on oyster culture. *In:* "Ecotoxicological testing for the marine environment" (Ed. G. Persoone et al) Vol.II. State University of Ghent, Ghent and Institute of Marine Science Research Bredence, Belgium, pp. 187-196.

Alzieu, C.L., Sanjuan, J., Deltreil, J.P., Borel, M. 1986. Tin contamination in Arcachan Bay" Effect on oyster shell anomalies. Mar. Pollu. Bull. 17:494-498.

Alzieu, C.L., Sanjuan, J., Michel, P., Borel, M., Dreno, J.P. 1989. Monitoring and assessment of butyltin in Atlantic coastal water. Mar. Pollu. Bull. 20:22-26.

Alzieu, C. 2000. Impact on tributyltin on marine invertebrate.Ecotoxicol.9:71-76.

Ansari, A. A., Singh, I.B. and Tobschall, H.J. 1998.Organotin compounds in surface and pore waters of Ganga Plain in the Kanpur-Unnao Industrial Region, India . The Science of Total Environment.223:157-166.

Ashtaputre, A. A. and Shah, A.K. 1995a. Studies on the exopolysaccharide from *Sphingomonas paucimobilis* GS1: Nutritional requirement and precursor-forming enzyme. Current Microbiol. 31: 234-238.

Ashtaputre, A.A. and Shah, A.K. 1995b. Studies on a viscous, gel-forming exopolysaccharide from *Sphingomonas paucimobilis* GS1. Appl. Environ. Microbiol.61. (3): 1159-1162.

Aston, S.R., 1980. "Nutrients, Dissolved Gases and General Biogeochemistry in Estuaries", (Eds. E. Olausson and Cato, I) John Wiley & Sons, New York. pp. 233 – 257.

Atireklap, S.K., Tanabe, S., Sanguansin, J. 1997. Contamination of butyltin compounds in sediments from Thailand. Mar. Pollu. Bull. 34: 894-899.

Atkinson, A., Rodriguez, M.D., Shewmaker, T.E. and Walmsley, J.A. 1999. Synthesis and characterization of compounds of di- and tributyltin chloride with adenine and guanine mononucleotides. Inorganica Chemica Acta. 285: 60-69.

Avery, S.V., Codd, G.A. and Gadd, G.M.1993. Biosorption of tributyltin and other organotin compounds by cyanobacteria and microalgae. Appl. Microbiol. Biotechnol.39:812- 817.

Barbieri, P., Galassi, G., and Galli, E. 1989. Plasmid encoded mercury resistance in a *Pseudomonas stutzeri* strain that degrades *o* -xylene. FEMS Microbiol. Ecol. 62: 375-384.

Barkay, T., Miller, S.M. and Summers, A.O. 2003. Bacterial mercury resistance from atoms to ecosystem. FEMS Microbiol. Rev. (article in press).

Barug, D. 1981. Microbial degradation of bis (tributyltin) oxide. Chemosphere. 10: 1145-1154.

Barug, D.and Vonk, J.W. 1980. Pesticide Science. 11: 77.

Bates, S. S., Tessier, A., Campbell, P.G.C., and Letourneau, M. 1985. Zinc-phosphate interaction and variation in zinc accumulation during growth of *Chlamydomonas variabilis* (*Chlorophyceae*) in batch culture . Can. J. Fish. Aquatic. Sci. 42: 86-94.

Baya, A.M., Brayton, P.R., Brown, V.L., Grimes, D.J., Russek-Cohen, E. and Colwell, R. R.1986. Coincident plasmid and antimicrobial resistance in marine bacteria isolated from polluted and unpolluted Atlantic Ocean Samples. Appl. Environ. Microbiol. 51: 1285-1289.

Belliveau, B.H., Starodub, M.E. , Cotter, C and Trevors, J.T. 1987. Metal resistance and accumulation in bacteria. Biotechnol. Advances.5(1):101-127.

Bertrand, J.C.,Bonin, P, Goutx, M and Mille, G.1993.Biosurfactant production by marine microorganisms: Potential application to fighting hydrocarbon marine pollution. J. Mar. Biotechnol. 1:125-129.

Beveridge, T.J. and Doyle, R.J. 1989. "Metal ions and bacteria" (Eds. Beveridge, T.J. and Doyle, R.J.) John Wiley, New York.

Birnboim, H.C. and Daly, J. 1979. A rapid alkaline extraction procedure for screening recombinant plasmid DNA. Nucleic. Acids Res. 7: 1513 – 1523.

Blair, W.R, Olson, G.J., Brinckman, F.E and Iverson, W.P. 1982. Accumulation and fate of tri-n-butyltin cation in estuarine bacteria. Microbiol. Ecol. 8: 241-251.

Blencowe, D.K. and Morby, A.P. 2003. Zn(II) metabolism in prokaryotes. FEMS Microbiol. Rev. (article in press).

Blunden, S.J., Chapman, A.H. 1983. A review of the degradation of organotin compounds in the environment and their determination in water. I.T.R.I. publication. 626: 1-7.

Blunden, S.J., Hobbs, L.A. and Smith, P.J.1984. The environmental chemistry of organoitn compounds. *In* : Environmental chemistry. Edited by H.J.Bowen Soc. Chem., London. pp: 46-77.

Bognolo, G. 1999. Biosurfactants as emulsifying agents for hydrocarbons. Colloids and Surfaces A: Physicochemical and Engineering Aspects.152: 41-52.

Boopathy, R. and Daniels, L., 1991. Pattern of organotin inhibition of methanogenic bacteria. Appl. Environ. Microbiol. 57: 1189-1193.

Bruins, M.R., Kapil, S., Oehme, F.W. 2003. Characterization of a small plasmid (pMBCP) from bovine *Pseudomonas pickettii* that confers cadmium resistance. Ecotoxicol. and Environ. Saf. 54 (3): 241-248.

Bunz, P.V., Buck, M. Hebenbrock, S. and Fortnagel , P. 1999. Stability of mutation in a *Sphingomonas* strain. Can. J. Microbiol. 45. 5: 404-407.

Cai, M and Xun, L. 2002. Organization and regulation of pentachlorophenol-degrading genes in *Sphingobium chlorophenolicum* ATCC 39723. J. Bacteriol. 184(17): 4672-4680.

Callow, M. E. and Willingham., G. L. 1996. Degradation of antifouling biocides. Biofouling 10(1-3): 239-249.

Cardwell, R.D. and Sheldon, A.W. 1986. A risk assessment concerning the fate and effect of triybutyltin in the aquatic environment. *In* : *Proceedings of the Symposium,"Oceans"*. (Ed. Champ, M. A.). pp- 1127-1129.

Celis, H., Escobedo, S. and Romero, I. 1998.Triphenyltin as an inhibitor of membrane bound pyrophosphate of *Rhodospirillum rubrum.* Arch. Biochem. Biophy. 358:157-163.

Cervantes, C and Silver, S. 1996. Metal resistance system in *Pseudomonas* . Revista Latinoamericana De Microbiologia. 38(1): 45-64.

Cervantes, C. and Ohtake, H. 1988. Plasmid-determined resistance to chromate in *Pseudomonas aeruginosa* . FEMS Microbiol. Lett. 56: 173-176.

Chakrabarty, A. M. and Friello, D.A. 1974. Dissociation and interaction of individual components of a degradative plasmid aggregate in *Pseudomonas*. Proc. Natl. Acad. Sci. U.S.A. 71: 3410-3414.

Champ, M.A. 2000. A review of organotin regulatory strategies: Pending actions, related costs and benefits. The Sci. of the Total Environ. 258: 21-71.

Champ, M.A. 2001. Pollution prevention from ships and shipyards. In: "Proceedings of International Oceanology symposium-2001". Vol.1. Miami, Florida, U.S.A. p. 250.

Champ, M.A. and Seligman, P.P. 1997. "Organotin: Environmental fate and effects". Chapman and Hall Publishers, U.K. pp. 1-64.

Chandramohan, D. 1997.Recent advances in marine microbiology: The Indian scenario. J. Mar. Biotechnol. 5: 73-81.

Chang, J.S., Law, R. and Chang , C.C. 1997. Biosorption of lead, copper and cadmium by biomass of *Pseudomonas aeruginosa* PU21. Wat. Res. 31(7): 1651-1658.

Chau, Y.K., Maguire, R.J., Brown, M., Yang, F. and Batchelar, S.P. 1997. Occurrence of organotin compounds in the Canadian aquatic environment. Water Qual. Res. J (Canada). 32: 453- 521.

Chaumery,C.J.and Michel, P. 2001.Seasonal variation in degradation of tributyltin in the Brest Naval Harbour water in relation to water temperature and instolation. Environ. Technol.22: 175-181.

Christie, A.O. 1972. In: "Proceedings of 3rd International Congress of Marine Corrosion and Fouling", Gaithersburg, M.D., U.S.A. p. 676.

Cieglar A., 1964. Microbial carotenogenesis. Advances in Applied Microbiology. 7: 1- 34

Clark, E.A., Sterritt, R.M and Lester, J.N. 1988. The fate of tributyltin in the aquatic environment, a look at the data. Environ. Sci. Technol. 22: 600-604.

Cleary, J.J., Stebbing, A.R.D. 1987. Organotin in the surface microlayer and subsurface waters of Southwest England. Mar. Pollu. Bull. 18:238-246.

Cooney, J.J. 1988. Microbial transformation of tin and tin compounds. J. Ind. Microbiol. 3: 195-204.

Cooney, J.J. and Wuertz, S. 1989. Toxic effect of tin compounds on microorganisms. J. Indus. Microbiol. 4: 375-402.

Cooney, J.J., de Rome, L., Laurence, O.S. and Gadd, G.M., 1989. Effect of organotin and organolead compounds on yeast. J. Ind. Microbiol. 4: 279-288.

Cooney, J.J., Wuertz, S. 1989. Toxic effect of tin compounds on microorganisms. J. Ind. Microbiol. 4: 375-402.

Cooney, J.J. 1994. Tri, Di and Monobutyltin on heterotrophic nitrifying bacteria from marine sediments. Arch. Environ. Contam. & Toxicol. 27: 501-506.

Cooney, J.J. 1995. Organotin compounds and aquatic bacteria - a review. Helgoland Meeresunter. 49: 663-677.

Cooper, D.G. and Zagic, J.E. 1980. Surface active compounds from microorganisms. Adv. Appl. Microbiol. 26: 229-253.

Corsini, E., Viviani, B., Marinovich, M and Galli, C.L.1997. Role of mitochondria and calcium ions in tributyltin –induced gene regulatory pathways. Toxicol. Appl. Pharmacol. 145(1): 74-81.

Craig, P.J. 1982. Environmental Chemistry of Organotins. In: "Comprehensive organometallic chemistry" (Ed. G. Wilkinson) Vol. II, Pergamon Press, Oxford, p. 979.

Cruickshank,R., Duguid, J.P. and Swain, R.H.A.1972. Test employed in Bacterial Identification . *In*: Medical Microbiology, A Guide to the Laboratory Diagnosis and control of Infection, Part-V, Technical Methods, Chapter-49, English Language Book Society and Churchill Livingstone, Great Britain. pp-724-729.

Danese, P.N., Pratt, L.A. and Kolter, R. 2000. Exopolysaccharide production is required for development of *Escherichia coli* K-12 biofilm architecture. J. Bacteriol. 182 (12): 3593-3596.

Davis, M. T., Newell., P.F. and Quinn, N.J. 1999. TBT contamination of an Artisanal subsistence fishery in Suva harbour, Fiji. Ocean and Coastal Management. 42: 591- 601.

de Mora. S.J., King, N.G. and Miller, M.C.1989. TBT in total marine sediments. Environ. Technol. 10: 901-908.

de Mora, S.J., Stewart, C. and Phillips, D.1995. Sources and rate of degradation of Tri(n-butyl) tin in marine sediments near Auckland , New Zealand. Mar. Pollut. Bull. 30: 50-57.

de Mora, S.J. 1996. 'Tributyltin-case study of a environmental contaminant". Cambridge University Press, Cambridge, U.K.

de Mora, S.J. and Pelltier, E. 1997. Environmental tributyltin research: past, present and future. Environ. Technol. 18: 1169-1177.

De, J., Ramaiah, N., Mesquita, A., and Verlekar, X.N. 2003.Tolerance to various toxicants by marine bacteria highly resistant to mercury. Mar. Biotechol. 5: 1-9.

Decho, A.W. 1990. Microbial exopolymer secretions in ocean environments: Their role(s) in food webs and marine process. Oceanogr. Mar. Biol. Annu. Rev. 28: 73-153.
Deley J. 1964. *Pseudomonas* and related genera. Annl. Rev. Microbiol. 18: 28-32.

Deley, J.1964. *Pseudomomas* and related genera. Ann. Rev. Microbiol.18:28-32.

Diez, S., Abols, M. and Bayona J.M. 2000. Organotin contamination in sediments from the Western Mediterranean enclosures following 10 years of TBT regulation. Water Res.36(4): 905-918.

Dowson, P.H., Bubb, J.M., Lester, J.N. 1993. Temporal distribution of organotins in the aquatic environment: five years after the 1987 UK retail ban on TBT based antifouling paints. Mar. Pollu. Bull. 26: 487-494.

Dowson, P.H., Bubb, J.M. and Lester, J.N. 1996. Persistence and degradation pathways of tributyltin in freshwater and estuarine sediments. Estuarine, Coastal and Shelf Science. 42: 551-562.

Dubey, S.K. and Rai, L.C. 1987. Effect of chromium and tin on survival, growth, carbon fixation, heterocyst differentiation, nitrogenase, nitrate reductase and glutamine synthetase activities of *Anabaena doliolum*. J. Plant Physiol. 130: 165-172.

Dubey, S.K. and Rai, L.C. 1989a. Toxicity of chromium and tin to *Anabaene doliolum*: Interaction with bivalent cations. Biology of Metals. 3: 8-13.

Dubey, S.K. and Rai, L.C. 1989b. Protective effects of certain natural and synthetic complexans on the toxicity of chromium and tin to nitrogen-fixing cyanobacterium, *Anabaena doliolum*. Biomed. And Environ. Scien. 2: 271-281.

Dubey, S.K. and Rai, L.C. 1990a. Impact of chromium and tin on a nitrogen-fixing cyanobacterium, *Anabaena doliolum*: Regulation of toxicity by pH, salinity, extracellular metabolites and organic acids. Biomed. And Environ. Scien. 3: 240-249.

Dubey, S.K. and Rai. L.C.1990b. Heavy metal toxicity in N_2 fixing Cyanobacterium, *Anabaena doliolum:*Regulation of toxicity by certain environmental factors. Biomedical and Environ. Scien. 3: 240-249.

Dubey, S.K., Rai, L.C. and Mallick, N. 1992. Influence of chromium on some physiological variables of *Anabaena doliolum*: Interaction with metabolic inhibitors. Biometals. 5: 13-16.

Dubey, S.K. Holmes, D.S. and Gangolli, S. S. 1993. Development of biosensor to measure metal ion bioavailability in environmental samples. Northeastern Geology.15: 188-194.

Dubey, S. K. and Roy, U. 2003. Biodegradation of Tributyltins (organotins) by Marine Bacteria. Appl. Organometal. Chem.17:1-6.

Dubois, M., Gilles, K.A., Hamilton, J.K, Roberts, P.A and Smith, F.1956. Colorimetric method for determination of sugars and related substances. Anal. Chem. 26: 350-356.

Esiobu, N., Armenta, L., Ike, J.and Esiobu, J.N. Antibiotic resistance in soil and water environments. Int. J. Environ. Heath Res. 12 (2): 133-144.

Evans, C.J., Smith, P.J. 1975. Organotin based antifouling systems. J. Oil. Col. Chem. Assoc. 58: 160-168.

Evans, S.M. 1999. TBT or no TBT? that is the question. Biofouling. 14(2):117-129.

Fein, J.B. and Delea, D.1999. Experimental study of the effect of EDTA on Cadmium adsorption by *Bacillus subtilis*: a test of the chemical equilibrium approach. Chem. Geol. 161: 375-383.

Fent, K. 2003. Ecotoxicological problems associated with contaminated sites. Toxicol. Lett.140-141: 353-365.

Ferrao, V and Mavinkurve, S.S. 1977. *Pseudomonas aeruginosa* in infantile Gastroenteritis. Arogya - Journal of Heath Science (India).3: 85-87.

Fishman, M. L., Cescutti, P., Fett, W. F., Osman, S. F., Hoagland, P. D. and Chau, I. K. (1997) Screening the physical properties of novel *Pseudomonas* exopolysaccharides by HPSEC with multi angle light scattering and viscosity detection. Carbohyd. Polymers 32: 213-221.

Foster, P.L.1982. Metal resistance of *Chlorophyta* from rivers polluted by heavy metals. Freshwater Biol. 12: 41-61.

Foster, T.J. 1983. Plasmid-determined resistance to antimicrobial drugs and toxic metal ions in bacteria. Microbiol. Rev. 47: 361- 409.

Foster, T.J. 1987. CRC Critical Reviews in Microbiology. 15: 117-139.

Fredrickson, J.K., Blackwill, D.L., Romine, M.F. and Shi, T. 1999. Ecology, physiology and phylogeny of deep subsurface *Sphingomonas* sp. J. Indus. Microbiol Biotechnol. 23(4-5): 273-283.

Freedman, B. 1995.Environmental Ecology: The ecological effects of pollution, Disturbance and other stresses. Academic Press, San Deigo,CA.

Fukagawa, T., Suzuki, S., Fukagawa, K, Suzuki, T. and Takama, K. 1992. Isolation and characterization of tributyltin chloride resistant marine *Vibrio*. FEMS Microbiol. Lett. 93: 83-86.

Fukagawa, T. and Suzuki, S. 1993. Cloning of gene responsible for tributyltin chloride (TBTCl) resistance in TBTCl - resistant marine bacterium, *Alteromonas sp.* M-1. Biochem. Biophys. Res. Commun. 194: 733-740.

Fukagawa, T., Konno, S., Takama, K. and Suzuki, S. 1994. Occurrence of tributyltin (TBT) and methyl mercury tolerant bacteria in natural seawater to which TBT was added. J. Mar. Biotechnol. 1: 211-214.

Gaber, N.N.1973. Microbial Phenazines. *In* CRC Handbook of Microbiology, Vol- III, Microbial products. Ed. Laskin A. I., Lechevalier, H.A. CRC Press. Ohio.U.S.A. pp-329-332.

Gabrielides, G. P., Alzieu, C., Readman, J. W., Bacci, E., Abouldahab, O. and Salihoglu, I.1990. MED POL survey of organotins in the Mediterranean. Mar. Poll. Bull. 21: 233-237.

Gadd, G. M. 1986a. Fungal responses towards heavy metals In : Microbes in Extreme Environments, (eds. Herbert, R.A. and Codd, G.A.) Academic Press, New York: pp-83-110.

Gadd, G.M. 1986b. Fungal responses towards heavy metals. *In* Microbes in Extreme Environments, (eds. Herbert, R.A., Jones, C.W. and Watson-Craik, I.A.) Cambridge University Press. Cambridge.U.K. pp: 59-88.

Gadd, G.M., Gray, D.J. and Newby, P. J. 1990. Role of melanin in fungal biosorption of tributyltin chloride. Appl. Microbiol. Biotechnol. 34: 116-121.

Gadd, G. M. 1992. Microbial control of heavy metal pollution. *In*: Microbial control of pollution, (Eds. Frig, J.C.; Gadd,G.M., Herbert, R.A., Jones, C.W. and Watson, I.W.) Cambridge University Press, Cambridge.U.K. pp : 59-88.

Gadd, G.M. 1993. Microbial formation and transformation of organometallic and organometalloid compounds. FEMS Microbiol. Rev. 11: 297-316.

Gadd, G.M. and White, C.1993. Microbial treatment of metal pollution – a working biotechnology. TIBTECH,11:353-359.

Gadd, G.M. 2000 Microbial interaction with tributyltin compounds, detoxification, accumulation and environmental fate. Sci.Total Environ. 258: 119-127.

Gerhardt, P., Murray, R.G.E., Wood, W.A., Krug, N.R.1989 "Gene transfer in Gram positive bacteria" *In*: Methods for General and Molecular Bacteriology, Chapter-15, American Society for Microbiology.pp-353-402.

Gichner, T. and Veleminsky, J. 1988. Inhibitors of N-nitroso-compounds induced mutagenecity. Mutation Resarch / Rev. in Genetic Toxicol.195 (1): 21-43.

Gomez-Ariza, J.L., Giraldez, I., Morales, E., Ariese, F., Cofino, W. and Quevauviller, P. 1999. Stability and storage problem in organotin speciation in environmental sample. J. Environ. Monit. 1: 197-202.

Goodfellow, M. 1977. Numerical Taxonomy. *In:* CRC Hand Book of Microbiology, Vol-1. Bacteria. (Eds. Laskin, A.I. and Lechevalier, A.H.) 2nd Ed. CRC press, Ohio,U.S.A. pp: 579-596.

Grasshoff, K.1983. Determination of salinity. *In* Methods of seawater analysis. 2nd Ed., (Eds. Grasshoff, K., Ehrhardt. M., Kremling, K.),Verlag chemie publication. Germany.pp :31-59.

Gray, B. H., Porvaznik, M., Flemming , C. and Lee, L.H. 1987. Tri-n-butyltin: a membrane toxicant. Toxicol. 47: 35-47.

Guerola, N., Ingraham, J.L. and Olmedo-Cerda, E. 1971. Induction of closely linked multiple mutations by nitrosoguanidine. Nature. 230: 122.

Guffanti, A.A., Clejan, S., Falk, L.H., Hicks, D.B. and Krulwich, T.A. 1987. Isolation and characterization of uncoupler-resistant mutants in *Bacillus* sp. J. Bacteriol. 169 (10): 4469-4478.

Gui-bin, J. Qun-fang, Z., Lui-Ji, Y. and De-Jing, W. 2001.Occurence of butyltin compounds in the water of selected lakes, rivers and coastal environments from china. Environ. Pollu. 115(1):81-87.

Hallas, L.E. and Cooney, J.J. 1981a. Tin and Tin resistant microorganisms in Chesapeake bay. Appl. Env. Microbiol. 41:466-471.

Hallas, L.E.and Cooney, J.J. 1981b. Effects of stannic chloride and organotin compounds on estuarine microorganisms. Dev. Ind. Microbiol. 22:529-535.

Hallas, L.E., Means, J.C., Cooney, J.J. 1982a. Methylation of tin by estuarine microorganisms. Science. 215: 1505-1507.

Hallas, L.E., Thayer, J.S., Cooney, J.J. 1982b. Factors affecting the toxic effect of tin on

estuarine microorganisms. Appl. Environ. Microbiol. 44:193-197.

Hamasaki, T., Sato, T., Nagase, H. and Kita, H.1992. The genotoxicity of organotin compounds in SOS chromotest and rec-assay. Mutation Res/ Genetic Toxicol. 280 (3): 195 -203.

Hamasaki, T., Sato, T., Nagase, H. and Kito, H. 1993. The mutagenicity of organotin compounds as environmental pollutants. Mutation Res/ Genetic Toxicol. 300 (1-4): 265-271.

Hamer, D.H. 1986. Metallothioneines. Annl. Rev. Biochem. 55:913-957.

Hamilton, R and Hamilton, S. 1987. *In*: " Thin Layer Chromatography" John Wiley and Sons Publication.U.S.A. pp : 1-62.

Hanahan, D. 1993. Studies on transformation of *E.coli* with plasmids. J Mol. Biol. 166. 557.

Hashimoto, S., Watanabe., M., Noda. Y., Hayashi. T., Kurita., Y., Takasu, Y., and Otsuki., A. 1998. Concentration and distribution of butyltin compounds in a heavy tanker route in the strait of Malaca and in Tokyo Bay. Mar. Environ. Res. 45:169-177.

Henshaw, B.G., Laidlaw, R.A., Orsler, R.J., Carey, J.K. and Savory, J.G. 1978. *In*: "Record of the 1978 Annual Convention of B.W.P.A." Cambridge, U.K. p. 19.

Higham, D.P., Sadler, P. J. and Scawen, M.D.1984. Cadmium resistant *Pseudomonas putida* synthesises novel cadmium proteins. Science. 225: 1043-1046.

Hirasawa, T., Wachi, M. and Nagai, K.2001. L- Glutamate production by lysozyme sensitive *Corynebacterium glutamicum its*A mutant strains. BMC Biotechnol. 1: 9-13.

Hoch, M. 2001. Organotin compounds in the environment – an overview. Appl. Geochem. 16: 719-743.

Hohn, B and Korn, D. 1969. Co-seggregation of a sex factor with the *Escherichia coli* chromosome during curing by acridine orange. J. Mol. Biol. 45: 385- 395.

Holden, P.A., La Montagne, M.G., Bruce, A.K., Miller, W.G. and Lindow, S.E.2002.Assessing the role of *Pseudomonas aeruginosa* surface active gene expression in hexadecane biodegradation in sand. Appl. Environ. Microbiol. 68(5): 2509-2518.

Holmes, D.S. and Quigley, M. 1981.A rapid method for the preparation of plasmid. Anal. Biochem.114: 193-197.

Hsia and Liu.2003. Accumulation of organotin compounds in pacific Oysters, *Crassostrea gigas*, collected from aquaculture sites in Taiwan. The Science of Total Environ. 313(1-3):41-48.

Hu, X. and Boyer, G.L. 1996. Siderophore-mediated aluminum uptake by *Bacillus megaterium* ATCC 19213. Appl. Environ. Microbiol. 62(11): 4044-4048.

Inaba, K., Shiraishi, H and Soma, Y. 1995. Effect of salinity, pH and temperature on aqueous solubility of four organotin compounds . Water Res. 29(5): 1415-1417.

Inoue, H. Takimura, O., Fuse, H., Murakami, K., Kamimura, K and Yamaoka, Y. 2000. Degradation of triphenyltin by a fluorescent *Pseudomonad.* Appl. Environ. Microbiol. 66(8): 3492-3498.

Inoue, H., Tikamura, O., Kawaguchi, K., Nitoda, T., Fuse, H., Murakami, K. and Yamaoka, Y. 2003. Tin-carbon cleavage of organotin compounds by pyoverdine from *Pseudomonas chlororaphis*. Appl. Environ. Microbiol. 69(2): 878-883.

Iwabuchi, N. Sunairi, M., Urai, M., Chiaki, I., Anzai, H. Nakajima, M. and Harayama, S. 2002. Extracellular polysaccharides of *Rhodococcus rhodochrous* S-2 stimulate the degradation of aromatic components in crude oil by indigenous marine bacteria. Appl. Environ. Microbiol. 68: 2337-2343.

Jayachandran, R. and George, V.K.1991. Zinc toxicity and its alleviation by calcium salt in alga *Scenedesmus acutus* 273. J. Ecotoxicol. Environ. Monitoring.1(3): 180-184.

Ji, G. and Silver, S. 1995. Bacterial resistance mechanisms for heavy metals of environmental concern. J. Indust. Microbiol. 14: 61-75.

Jonas, R. B., Gilmour, C. C., Daphne, L.S., Margaret, M. W. and Tuttle, J. H. 1984. Comparison of methods to measure acute metal and organometal toxicity to natural aquatic microbial communities. Appl. Environ. Microbiol. 47: 1005 – 1011.

Kachlany, S.C., Levery, S.B., Kim, J.S., Reuhs, B.L., Lion, L.W. and Ghiorse, W.C. 2001. Structure and carbohydrate analysis of the exopolysaccharide capsule of *Pseudomonas putida* G7. Environ. Microbiol. 3(12): 774-784.

Kado, C.I. and Liue, S.T. 1981. Rapid procedure for detection and isolation of large and small plasmid. J. Bacteriol. 145: 1365-1373.

Kan-Atireklap, S., Tanabe, S. and Sanguansin, J. 1997. Contamination by butyltin compound in sediments from Thailand . Mar. Poll. Bull. 34: 894-899.

Kanthak, D. I. J., Bernstorff, A. and Jayaraman. N. 2001. Ship for scrap: Steel and toxic wastes for Asia. Green Peace. 5: 26.

Khare, S., Ganguli, A. and Tripathi, A.K. 1997. Responses of *Pseudomonas aeruginosa* to chromium stress. Env. J. Soil. Bio. 133: 153-158.

Kimmel, E.C., Fish, R.H., and Casida, J.E.1977. Bioorganotin chemistry: Metabolism of organotin compounds in microsomal monooxygenase system and in mammals. J. Agric. Food Chem.25:1-9.

King, B.M.and Adler, D. L. 1964. A previously undescribed group of *Enterobacteriaceae*. Amer. J. Clin. Pathol. 41: 230-232.

Kitamura, S.I. and Suzuki, S.2003. SecA causes TBT(Tributyltin) resistance in Pseudoalteromonas sp. Strain M1 (unpublished data).

Ko, M. M.C., Bradley, G.C., Neller, A.H. and Broom, M.J. 1995.Tributyltin contamination of marine sediments of Hong Kong. Mar. Poll. Bull.31:249-253.

Kochkin, D.A. 1976. Relationship of biological activity of organotin (or lead) compounds from chemical structure (including cis- and trans- isomerism). Proceedings of the 4th International Congress on Marine Corrosion and Fouling. pp.281-284.

Koroleff, F.1983. Determination of nutrients. *In*: Methods of sea water analysis. 2nd Ed. (Eds. Grasshoff, K., Ehrhardt. M., Kremling, K). Verlag chemie publication,Germany. pp- 125-187.

Krieg, N. R. and J. G. Holt (Ed).1984. Bergey's manual of systemic bacteriology, 1,Section - 4,The Williams & Wilkins Co., Baltimore, U.S.A. pp.140-309.

Krone, C. A., Stein, J.E. and Varanasi, U.1996. Butyltin contamination of sediments and benthic fish from the east gulf and pacific coast of the United States. Environ. Montor. Assess. 40:75-89.

Kuch. P. J. 1986. Survey of antifouling paints at boatyard and shipyard. Proceedings of the Ocean 86. Organotin Symposium, Vol-4, Marine Technology Society. Washington, DC. pp-1114-1116.

Leahy, Joseph, G., and Colwell, Rita, R., 1990. Microbial degradation in the environment. Microb. Rev.54(3): 305-315.

Laddy, M.B., Phipps, D.W. and Ridgway, H.F. 1995. Catabolite-mediated mutations in alternate toluene degradative pathways in *Pseudomonas putida*. J. Bacteriol. 177 (16): 4713-4720.

Laemmli, U. K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature. 227: 680-685.

Langston, W. J., Burt, G.R. and Mingjiaang, Z. 1987. Tin and organotin in water, sediments and benthic organisms of Pool harbour. Mar. Poll. Bull.18: 634-639.

Lau, M. M. 1991. Tributyltin antifoulings: a threat to Hong Kong marine environment. Archives of Environ. Conta. And Toxicol. 20:299-304.

Laughlin, R. B., French, W. and Guard, H.E. 1986. Accumulation of bis(tributyltin) oxide by the marine mussel, *Mytilus edulis*. Environ. Sci. Technol. 20: 884-890.

Laurence, O.S Cooney, J.J., and Gadd, G.M. 1989. Toxicity of organotins towards the marine yeast, *Debaryomyces hansenii*. Microb. Ecol. 17: 275-285.

Lee, S.W. Glickmann, E. and Cooksey, D. A. 2001. Chromosomal locus for cadmium resistance in *Pseudomonas putida* consisting of a cadmium transporting ATPase and a Mer R family response regulator. Appl. Environ. Microbiol. 67(4): 1437- 1444.

Leonard, A., Gerber, G.B. and Leonard, F. 1986. Mutagenicity, carcinogenecity and teratognicity of zinc. Mutation Res. 168 (3): 343-353.

Lo, S., Allera, A., Albers, P., Heimbrecht, J., Jantzen, E., Klingmuller, D. and Steckelbroeck, S. 2003. Dithiothyretol (DTE) prevents inhibitory effects of triphenyltin (TPT) on the key enzymes of the human sex steroid hormone metabolism. J. of Steroid Biochem. Mol. Biol. 84: 569-576.

Lobban, C.S., Harrison, P.J. and Duncan, M.J. 1985. Nutrients, Chapter 6, *In:* The physiological ecology of sea weeds. Cambridge University Press, London. pp: 75–110.

Lonarek, J. and Sori, J. 1998. Bacterial O^6 – methylguanine –DNA methyltransferase reduces N-methyl –N-nitro- N-nitrosoguanidine induction of plasminogen activator in Mer- human glioblastoma A1235 cell line. Mutation Research/DNA repair. 408 (1): 47-54.

Lowry, O. H., Rosebrough, N. J., Farr, A. L. and Randall, R. J. 1951. Protein measurement with the Folin phenol reagent. J. Biol. Chem. 193: 265-275.

Luisi, P.L. and Majid L.J. 1986 .CRC Crit. Rev. Biochem.20.409.

Ma, H., Dai, S. and Huang, G. 2000. Distribution of tributyltin on laboratory simulated estuarine microcosms. Water Res. 34: 2829-2841.

Maguire, R.J., Chau, Y.K., Bengert, G.A., Hale, E.J., Wong, P.T.S. and Kramar, O. 1982. Occurrence of organotin compounds in Ontario lakes and rivers. Environ. Sci. Technol. 16: 698-702.

Maguire, R.J. 1984. Butyltin compounds and inorganic tin in sediments in Ontario. Environ. Sci. Technol. 18: 281-294.

Maguire, R. J., Tkacz, R. J., Chau, Y.K., Bengert, G.A. and Wong, P.T.S.1986. Occurrence of organotin compounds in water and sediment in Canada. Chemosphere.15: 253-274.

Maguire, R.J. 2000. Review of the persistence, bioaccumulation and toxicity of tributyltin in aquatic environments in relation to Canada's toxic substances management policy. Water Qual. Res. J. (Canada). 35(4): 633-679.

Mahtani, S. and Mavinkurve, S.1979. Microbial purification of longifolene - Asesquiterpene. J. Ferment. Technol. 57: 529-533.

Maier, R.M. and Soberon, C.G.2000. *Pseudomonas aeruginosa* Rhamnolipids: biosynthesis and potential applications. Appl. Microbiol. Biotechnol. 54 (5): 625-633.

Makker, N.S., Kronick, A.T. and Cooney, J. J.1989. Butyltins in sediments from Boston Harbor, U.S.A..Chemosphere.18: 2043-2050.

Matthias, C.L. and Bellama, J.M., Olson, G.I., and Brinckman, F.E.1986a.Determination of di and tributyltin in sediment and microbial biofilms using acidified methanol extraction and sodium borohydride derivatization and GC with flame photometric detection . Int. J. Environ. Analyt. Chem.35: 61-68.

Matthias, C.L., Beluga, J.M., Olson, G.J. and Brinckman, F.E. 1986b. Comparative methods for determination of aquatic butyltin and butylmethyltin species at ultratrace levels using simultaneous hybridization / extraction with gas chromatography - flame photometry detection. Environ. Sci. Technol. 20: 609-615.

Maureen, E.G., Willingham, G.L. 1996. Degradation of antifouling biocides. Biofoul. 10: 239-249.

Mavinkurve, S.S. and Ferrao, V.1979. Microbiological studies on gastroenteritis in Hospitalized children in Goa. Indian Pediatrics (India).16 (1): 27-31

Mawatari, S. 1972. In: "Proceedings of 3rd International Congress of Marine Corrosion and Fouling" Gaithersburg, M.D., U.S.A., pp: 956.

McDonald, L. and Trevors, J. T. 1988. Review of tin resistance, accumulation and transformation by microorganisms. Water. Air Soil. Pollu. 40:215-221.

McGee, B.L., Schlekat, C.E., Boward, D.M. and Wade, T.L.1995. Sediments contamination and biological effects in a Chesapeake Bay marina. Ecotoxicology.4: 39-59.

Mengistu, Y., Edwards, C. and Saunders, J. R. (1994) Continuous culture studies on the synthesis of capsular polysaccharide by *Klebsiella pneumoniae* K1. J. Appl. Bacteriol. 75: 424-430.

Meyers, J. A., Sanchez, D., Elwell, P. L. and Falkow, S. 1976. Simple agarose gel electrophoretic method for the identification and characterization of plasmid deoxyribonucleic acid. J. Bacteriol. 127: 1529-1537.

Miller, M.E. and Cooney, J.J. 1994. Effect of tri, di and monobutyltin on heterotrophic nitrifying bacteria from marine sediments. Arch. Environ. Cont. and Toxicol. 27: 501-506.

Miller, C.E., Wuertz, S., Cooney, J.J and Pfister, R.M. 1995. Plasmid in tributyltin-resistant bacteria from fresh and estuarine water. J. Ind. Microbiol. 14: 337-342.

Miller, R.M. 1995. Biosurfactant -facilitated remediation of metal contaminated soil. Environ. Health. Prospect. 103: 59-62.

Minchin, D., Bauer. B., Oehlmann, J., Oehlmann and Duggan, C.B., 1997. Biological indicator used to map organotin contamination from a fishing port. Killeybegs, Ireland, Mar. Pollu. Bull. 34: 235-243.

Miyauchi, K., Lee, H.S., Fukuda, M., Masamichi, T., Nagata, Y. and Ku., B. 2002. Cloning and characterization of linR, involved in regulation of the downstream pathway for gamma-hexachloro-cyclohexane degradation in *Sphingomonas paucimobilis* UT26. . Appl. Environ. Microbiol. 68 (4): 1803-1807.

Moat, A.G. and Foster , J.W. 1988."Scope of Microbial physiology"Chapter-1, *In* : "Microbial Physiology" ,2nd Ed. A Weley Inter science publication.U.S.A. pp: 1-50.

Mohan , S. V., Sisrla, S., Guru, R. K., Prasad,K. K., Kumar, C.S., Ramakrishna, S.V., Sarma, P.N.and Sarma, P. N.2003.Microbial degradation of pyredine using *Pseudomonas* sp and isolation of plasmid responsible for degradation. Waste Management. 23(2):167-171.

Moreno, J., Vargas-Garcia C., Lopez, M. J. and Sanchez-Serrano (1999) Growth and exopolysaccharide production by *Azotobacter vinelandii* in media containing phenolic acids. J. Appl. Microbiol. 86: 439-445.

Neu, T.R. 1996. Significance of bacterial surface active compound in interactions of bacteria with interface. *Microbial Rev.* 60(1): 151 - 166.

Neis, D.H. and Silver, S. 1989. Plasmid – determined inducible efflux is responsible for resistance to Cd, Zn and Co in *Alkaligenes eutrophus*. J. Bacteriol. 171: 896-900.

Neis, D.H. 1992. Resistance to cadmium, cobalt , Zinc and nickel in microbes. Plasmid. 27: 17-28.

Nies, D.H. and Silver, S.1995.Ion efflux system involved in bacterial metal resistance . J. Indus. Microbiol. 14: 186-199.

Nies, D. H. 1999. Microbial heavy metal resistance. Appl. Microbiol. Biotechnol.51: 730-750.

Nishikawa, S., Sonoki, T., Kasahara, T., Obi, T., Kubota, S. and Kawai, S. 1998. Cloning and sequencing of the *Sphingomonas* (*Pseudomonas*) *paucimobilis* gene essential for the O demethylation of vanillate and syringate. Appl. Environ. Microbiol. 64(3): 863-842.

Noordman, W.H., Johann, H.J.W., Geert, J., de, B and Janssen, D.B. 2002. The enhancement by surfactant of hexadecane degradation by *Pseudomonas aeruginosa* varies with substrate availability. J. Bacteriol. 94: 195-212.

O' Halloran, T.V. 1993.Transition metal in control of gene expression. Science. 261: 715-725.

Ogawa, H., Ohyama, T., Katsura, E and Katoh, Y. 1997. Characterization of the pUC19- *lac* ZC141 reversion system for assaying chemical mutagenesis. Mutation Research / Genetic Toxicity and Environmental Mutagenesis. 394 (1-3): 141-151.

Olafson, R.W., Abel, K., Sim, R.G. 1979. Prokaryotic metallothionine: Priliminary characterization of blue green algal heavy metal binding protein.. Biochem. Biophyc. Res. Commun.89:36-43.

Ohtake, H., Cervantes, C. and Silver, S. 1987. Decreased chromate uptake in *Pseudomonas fluorescens* carrying a chromate resistance plasmid. J. Bacteriol. 169 (8): 3853-3856.

Orsler, R. J. and Holland, G.E. 1982. Degradation of tributyltin oxide by fungal culture filtrates. Int. Biodeterio. Bull.16(4): 95-98.

Ozdemir, G., Ozturk, T., Ceyhan, N., Isler, R. and Cosar, T.2003. Heavy metal biosorption by biomass of *Ochrobacterium anthropi* producing exopolysaccharide in activated sludge. Biosensor Technol. 90(1): 71-74.

Paca, J., Barta, J., Bajpai, R. and Stiborova, M. 2002. Aerobic biodegradation of 2,6 – Dinitrotoluene in aqueous solution by a mixed culture. Poster presentation 3-64, 24th Symposium on Biotechnology for Fuels and Chemicals. 28th April -1st May, 2002. Park Vista Resort , Hotel Gatlinburg , USA.

Page, S.D. 1989. An analytical method for butyltin species in shellfish. Mar. Pollu. Bull. 20: 129-133.

Page. D.S., Ozbal, C.C. and Lanphear, M.E.1996.Concentration of butyltin species in sediments associated with shipyard activity. Environ. Poll. 91:237-243.

Pain, A and Cooney, J.J. 1998. Characteriazation of organotin resistant bacteria from Boston Harbor sediment. Arch. of Environ. Contamin. and Toxicol. 35(3): 412-416.

Parfett, C.L. and Pilon, R.1993. Tri-n-butyltin chloride promotes morphological transformation and induces proliferin expression in C3H10T1/2 cells. Cancer Lett. 71 (1-3): 167-176.

Parkinson, M. 1985. Bio-surfactants. Biotechnology Advances. 3: 65-83.

Patt, J.R, Neiderlehner, B.R., Bowers, N.J and Cairns, J.(Jr.). 1987. Effect of zinc on microbial communities. *In* : Proceedings of International Conference on Heavy Metals in the Environment. (eds. Lindberg, S.E. and Hutchinson, T.C.), Edinburgh, U.K. pp.324-329.

Paulsson, M., Nystrom, B. and Blanck, H. 2000. Long term toxicity of zinc to bacteria and algae in periphyton communities from the river Gota Alv, based on a microcosm study. Aquatic Toxicol. 47: 243-257.

Pazirandeh,M., Chrisey, L.A., Mauro, J.M., Campbell, J.R. and Gaber, B.P. 1995. Expression of the *Neurospora crassa* metallothionein gene in *Escherichia coli* and its effect on heavy metal uptake. Appl. Microbiol. Biotechnol. 43: 1112-1117.

Pazirandeh, M., Welles, B.M. and Ryan, R.L. 1998. Development of bacterium – based heavy metal biosorbents: Enhanced uptake of cadmium and mercury by *Escherichia coli* expressing a metal binding motif. Appl. Environ. Microbiol.64: 4068-4072.

Pettibone, G.W and Cooney, J.J. 1986. Effect of organotins in fecal pollution indicator organisms. Appl. Environ. Microbiol. 52: 562-566.

Pettibone, G.W., and Cooney, J.J. 1988. Toxicity of methyltin to microbial populations in estuarine sediments. J. Ind. Microbiol. 2: 373-378.

Phillip, A.T. 1973. In: "Australian OCCA Proceedings and News" (July, 1973) p. 17.

Pickup, R.W., Lewis, R. J. and Williams, P. A. 1983. *Pseudomonas* MT14, a soil isolate which contains two large catabolic plasmid, one a TOL plasmid and one coding for phenyl acetate catabolism and mercury resistance. J. Gen. Microbiol. 129: 153-158.

Pipke, B., Dobler, I.W., Timmis, K.N. and Dwyer, D. F. 1992.Survival and function of a genetically engineered *Pseudomonad* in aquatic sediment microcosms. Appl. Environ. Microbiol. 58(4):1259-1265.

Pollock, T.J., Thorne, L.Y., Amazaki, M., Mikolajczak, M.J. and Armentrout, R.W. 1994. Mechanism of bacitracin resistance in gram-negative bacteria that synthesizes exopolysaccharides. J. Bacteriol. 176 (20): 6229-6237.

Potter, A.A. and Loutit, J.S. 1983. FP2 plasmid curing in *Pseudomonas aeruginosa.* Can. J. Microbiol. 29: 732-733.

Quasim, S.Z. and Sengupta, R., 1981. Environmental characteristics of the Mandovi- Zuari estuarine system in Goa. Estuarine, Coastal and Shelf Science. 13: 557- 578.

Rai, L.C. and Dubey, S.K. 1988.Chromium toxicity to *Cyanobacterium:* Possible role of carbon sources in toxicity amelioration. Microbios. 55: 165-172.

Rai, L.C. and Dubey, S.K. 1989. Impact of chromium and tin on a nitrogen-fixing cyanobacterium, *Anabaena doliolum*: interaction with bivalent cations. Ecotoxicol. and Environ. Saf. 17: 94-104.

Rai, L.C., Riazada, M., Mallick, N., Husainy, Y., Singh, A.K. and Dubey, S.K. 1990. Multiple toxicity of heavy metals in a nitrogen fixing cyanobacterium, *Nostoc muscorum*. Biol. of Metals. 3: 229-234.

Rani, D.B.R. and Mahadevan, A. 1989. Plasmid encoded metal resistance in bacteria. J. Sci. Indus. Res. 48: 338-345.

Read, R.R and Costerton, J.W 1987. Purification and characterization of adhesive exopolysaccharides from *Pseudomonas putida* and *Pseudomonas fluorescens*. Can. J. Microbiology.33: 1080-1090.

Rehberger, T.G. and Glatz, B.A. 1990. Characterization of *Propionibacterium* plasmid. Appl. Environ. Microbiol. 56: 864-871.

Riley, J.P. and Chester, R. 1980. "Introduction to marine chemistry", Academic Press, London. 152-179.

Robinson, J.B. and Tuovinen, O.H. 1984. Mechanisms of microbial detoxification of mercury and organomercury compounds: Physiological, Biochemical and Genetic analysis. Microb. Rev. 48: 95-124.

Ron, E.Z. and Rosenberg, E.2002. Biosurfactant and oil bioremediation. Curr. Opinion in Biotech. 13: 249-252.

Rosato, N.; Mei, G.; Savini, I.;Del Bolgia, F.; Finazzi-Agro, A.; Lommen, A. and Canters, G.W.1991. Intrinsic fluoresces of the bacterial copper containing protein amicyanin. Archi. Biochem. Biophy.284: 112-115.

Rosenberg, E., Zuckerberg, A., Rubinovitz, C. and Gutnick, D. L.1999. Emulsifier of *Arthrobacter* RAG-1: Isolation and emulsifying properties. Appl. Environ. Microbiol. 37: 402-408.

Rossbach, S., Wilson, T.L., Kukuk, M.L. and Carty, H. A. 2000. Elevated zinc induces siderophore biosynthesis gene and a *znt*A like gene in *Pseudomonas fluorescens*. FEMS Microbiol. Lett. 191(1): 61-70.

Royan, S., Parulekar, C. and Mavinkurve, S. 1999. Exopolysaccharides of *Pseudomonas mendocina* P_2 d. Lett. Appl. Microbiol.29: 342-346.

Roy, 0B., Laughlin, J.C., Cobet, A.B. and Guard, H.E.1988. Mechanisms of triorganotin toxicity to macro-invertebrates and role of biofilm as a controlled release medium. *In*: Marine Biodeterioration. (Eds. Thomson, M.F., Sarojini, R., Nagabhushanam, R.) Oxford & IBH Publishing Co. Pvt. Ltd. U.K.pp-757-767.

Sader, H.S., Jones, R.N., Silve, J.B. and Sader, H.S. 2002. Skin and soft tissue infection in Latin American medical center: four year assessment of the pathogen frequency and antimicrobial susceptibility patterns. Diagonostic Microbiol. and Infectious Disease. 44 (3): 281-288.

Salisbury, V., Hedges, R. W. and Datta, N. 1972. Two modes of curing transmissible plasmid. J. Gen. Microbiol. 70: 443-452.

Sambrook, J., Fritsch,E.F. and Maniatis, T. 1989.Molecular cloning : A laboratory Manual. Cold Spring Harbor Laboratory press. Cold Spring Harbor. N.Y.

Samuel, P. M., Roy, S., Jaiswal, A. and Rao, V. 1998. Differential effect of organometallic tin compounds on Na^+/K^+ -ATPase activity. J. Appl. Toxicol. 18:383-386.

Santroni, A. M., Fedeli, D., Gabbianelli, R., Zolese, G. and Falcioni, G. 1997. Effect of organotin compounds on trout haemoglobins. Biochem. Biophy. Res. Commun. 238: 301-304.

Sardessai, S. and de Souza, S.N. Deep Sea Research Part-II: Topical Studies in Oceanography. 2001. 48: 3353-3361.

Sarradin, P.M., Lapaquellerie, Y., Astruc, A., Latouche, C. and Astruc, M. 1995. Long term behaviour and degradation kinetics of tributyltin in a marine sediment. The Sci. Total Environ. 170: 59-70.

Schmid, A., Kollmer, A. and Wutholt, B. 1998. Effect of biosurfactant and emulsification on two–liquid phase *Pseudomonas oleovorans* cultures and cell–free emulsions containing n-decane. Enzyme and Microbial Technol. 22: 487- 493.

Seffernick, J.L ; Mc Tavish, H; Osborne, J.P, De Suza , M. L.; Sadowsky, M. J.; Wackett, L.P. and L.P, Wackett. 2002. Atrazine chlorohydrolase from *Pseudomonas* sp. strain ADP is a metalloenzyme. Biochem.41(1): 4430-14437.

Seligman, P.F., Valkris, A.O. and Lee, R.F. 1986. Degradation of tributyltin in San Deigo Bay, California. Environ. Sci. Technol. 20: 1229-1235.

Seubert, W. 1960. Degradation of isoprenoid compounds by microorganisms. I. Isolation and characterization of an isoprenoid degrading bacterium, *Pseudomonas citronellolis.* J. Bacteriol. 79. 426-434.

Sheldon, A.W. 1975. Effect of organotin antifouling coating on man and his environment. J. Paint. Technol. 47: 54-58.

Sheldon, A.W. 1978. *In*: "Proceedings of 18th Annual Marine Coatings Conference Monterey, California, U.S.A.

Shriner., R.L., Fuson, R.C. and Curtin, D.Y.1964. Qualitative Analysis for the elements. *In* : The Systematic Identification of Organic compounds, A Laboratory Manual. Chapter-5. 5th Ed . John Wiley & Sons. Inc. New York. USA. pp. 62-66.

Shuttleworth, K.L., and Unz, R.F. 1993. Sorption of heavy metals to the filamentous bacterium *Thiothrix* strain A1. Appl. Environ, Microbiol. 59: 1274-82.

Silver, S and Laddaga, R.A. 1990. Molecular genetics of heavy metal resistance system in *Staphylococcus aureus* plasmids. *In:* Molecular Biology of the *Staphylococci.* (ed. Novick, R.P.), YCH Publications, New York. pp. 531-349.

Silver, S. 1992. Plasmid-encoded metal resistance mechanisms: range and overview. Plasmid. 27:1-3.

Silver, S. and Kinscherf, T.G. 1987. Genetic and biochemical basis for microbial transformations and detoxifications of mercury and mercurial compounds. In: Biodegradation and Detoxification of Environmental Pollutants . (Ed. Chakraborty. A.M.), CRC Press. Inc., Boca Raton. Florida. pp: 85-103.

Singh, A.P. and Singh, K. 1984. Isolation and characterization of tributyltin resistant mutant of *Escherichia coli* . Zeitschrift Fur Naturforschung. 39(3-4): 293-299.

Singh, K and Singh A.P. 1985. Effect of medium on trybutyltin toxicity of *Escherichia coli* K-12. Adv. Biol. Res. 3 (2): 50-54.

Singh, K. 1987. Biocidal effect of organotin compounds. In: "Frontiers in Applied Microbiology", Vol II (Eds. K.G. Mukherjee, V.P. Singh, K.L. Garg). Publication Print House, New Delhi, India, pp. 297-316.

Singh, K. 1989. Effect of tributyltin on *Escherichia coli*: Role of modifying factors. Biomed. Environ. Science. 2: 366-375.

Sinha, M.P. 1997. Recent advances in Ecological research. A.P.H. Publishing Corporation. pp: 435 – 441.

Skinner, C.E. 1971. In: "Proceedings of antifouling symposium", CIBA-GEIGY, Marienberg, West Germany.

Smith, P.J. 1978. Toxicological data on organotin compounds, I.T.R.I Publications. 538.

Smith, P.J. 1980. Structure activity relationships for di- and tri-organotin compounds. I.T.R.I. Publications, 569.

Smith, P.J. 1998. "Chemistry of Tin" -II edition, Blackie academic and professionals, London, U.K.

Sparnins, V.L. and Chapman, P.J. 1976. Catabolism of L-Tyrosine by homoprotocatechuate pathway in Gram positive bacteria. J. Bacteriol.127:362-366.

Stang, P.M. and Seligman, P.F. 1986. Distribution and fate of butyltin compounds in the sediments of San Diego Bay, CA. *In Proceedings of Organotin Symposium Oceans '86 conference*, Washington, DC, U.S.A. 23-25 September,1986, Vol-4. IEEE Service Centre, Piscataway, NJ, U.S.A. , pp: 1256-1261.

Strand., J., Jacobson, J.A., Pedersen, B., Granmo, A. 2003. Butyltin compounds in sediments and molluscs from shipping strait between Denmark and Sweden. Environ. Pollu.124: 7-15.

Stridh, H., Fava, E., Single, B., Nicotera, P., Orrenius, S. and Leist, M. 1999. Tributyltin–induced apoptosis requires glycolytic adenosine triphosphate production . Chem. Res. Toxicol. 12(10): 874-882.

Stronkhors, J., Hattum, B.V., Bowmer, T. 1999. Bioaccumulation and toxicity of tributyltin to a heart urchin and an amphipod in spiked, silty marine sediments. Environ. Toxic. Chem. 18:2343-2351.

Subramanyam, N.S. and Sambamurty, A.V. S. S. 2000. Marine Ecology. *In*: Ecology (ed. Subramanyam, N.S. and Sambamurty, A.V. S. S.) Narosa Publishing House, New Delhi. pp: 244 - 253.

Summers, A.O. and Silver, S. 1978. Microbial transformation of metals. Ann. Rev. Microbial. 32: 637-672.

Suzuki, S., Fuagawa, T.and Takma, K. 1992. Occurrence of tributyltin tolerant bacteria in tributyltin or cadmium containing sea water. Appl. Environ. Microbiol. 58: 3410-3412.

Suzuki, S. Tsukamoto, K.K. and Fukagawa, T. 1994. The 16S rRNA sequence and genome sizing of tributyltin resistant marine bacterium, strain M-1. Microbiol. 101: 101-109.

Suzuki, S. and T. Fukagawa. 1995. Tributyltin resistant bacteria : a summary of recent work. J. Indus. Microbiol. 14: 154-158.

Sweet, P.S., Mac Farlane, T.W. and Samaranayake, L. P. 1987. Determination of the cell surface hydrophobicity of oral bacteria using a modified hydrocarbon adhearance method. FEMS Microbiol. Lett. 48: 159-163.

Takamura , N., Kasai, F., Watanabe, M.M. 1989. Effect of Cu, Cd and Zn on photosynthesis of freshwater benthic algae. J.Appl. Phycol. 1: 39-52.

Tamburini, C., Garcin, J., Ragot, M. and Bianchi, 2002.A. Deep Sea Research Part-II: Topical Studies in Oceanography. 49: 2109-2123.

Tisa, L.S. and Rosen, B.P. 1990. Transport system encoded by bacterial plasmids. J. Bioenerg. Biomembr. 22: 493-507.

Tisdale, W.H. 1943. British Patent No.578. 312. U.K.

Trevors, J.F., Oddie, K.M. and Belliveau, B.H. 1985. Metal resistance in bacteria. FEMS Microbiol. Rev. 32: 39-54.

Trevors, J.T. 1986. Plasmid curing in bacteria. FEMS Microbiol. Lett. 32:149-157.

Tsing, R. K. and Cooney, J.J. 1995. Action of Tributyltin on enzymes on four bacteria. Environ. Toxicol. Chem. 14 (7): 1113-1121.

Turner, J.S. and Robinson, N.J. 1995. Cyanobacterial metallothioneins: biochemistry and molecular genetics. J. Indus. Microbiol. 14: 61-75.

Valkirs., A.O., Seligman., P.F., Stang, P.M., Hemer., V., Lieberman, S.H., Vafa., G. and Dooley., C.A. 1986. Measurement of butyltin compounds in San Diego Bay. Mar. Pollu. Bull. 17: 319-124.

Vandevivre, P. and Kirchman., D.L.1993.Attachment stimulates exoploysaccharide synthesis by a bacterium. Appl. Environ .Mocrobiol.59: 3280-3286.

Vanhaecke, E. and Pijck, J. 1988. Bioluminescence assay for measuring the number of bacteria adhering to the hydrocarbon phase in the BATH test. Appl. Environ. Microbiol. 54(6): 1436-1439.

Vasishta, R., Chhibber, S. and Saxena, M. 1989. Heavy metal resistance in clinical isolates of *Pseudomonas aeruginosa.* Folia Microbiologica. 34(5): 448-452.

Voleski, B.1994. Advances in biosorption of metals: selection of biomass type. FEMS Microbiol. Rev.14: 291-302.

Waters, M.D., Brady, A.L., Stack, F. and Brockman, H.E. 1990. Antimutagenicity profile for some model compounds. Mutation Research / Reviews in Genetic Toxicol. 238(1): 57-85.

Webb, R. B. and Hass, B. S. 1984. Biological effects of dyes on bacteria VI mutation induction by acridine orange and methylene blue in the dark with special reference to *Escherichia coli* WP6 (*pol* A1). Mutation Research / Genetic Toxicology. 137(1): 1-6.

Wechsler, J. and Kline, B.C. 1980. Mutation and identification of the F-plasmid locus determining resistance to acridine orange curing. Plasmid. 4: 276-280.

Weiner, R.M., 1997. Biopolymers from marine prokaryotes. Trends in Biotech. 15: 390 – 394.

White, J.S., Tobin, J.M. and Cooney, J.J. 1999. Organotin compounds and their interaction with microorganisms. Can. J. Microbiol. 45(7): 541-554.

Wilkinson, R.R., 1984. Techno-economic and environmental assessment of industrial organotin compounds. Neurotoxicol. 5:141-158.

Williams, R.P.1973. Biosynthesis of prodigiosin, a secondary metabolite of *Serratia mercescens.* Appl. Microbiol. 25:396-402.

Wuertz, S. Miller, C.E., Pfister, R.M. and Cooney, J.J. 1991.Tributyltin-Resistant bacteria from estuarine and freshwater sediments. Appl. Environ. Microbiol. 57:2783-2789.

Yamada, J., Tatsuguchi, K. and Watanabe, T., 1978. Effect of trialkyltin chlorides on microbial growth. Agric. Biol. Chem. (Tokyo). 42: 1167-1172.

Yamada, J., Tatsuguchi, K., Watanabe, T. 1979. Cell lysis and leakage of cell constituents by tripropyltin chloride. Agri. Biol. Chem. (Tokyo). 43: 125-130.

Yamada, M., Matsui, K., Sofuni, T. and Nohmi, T. 1997. New tester strains of *Salmonella typhimurium lacking* O^6- methylguanine DNA methyltransferases and highly sensitive to mutagenic alkylating agents. Mutation Research/ Fundamental and Molecular Mechanisms of Mutagenesis. 381(1):15-24.

Yamaoka, Y.,Inoue, H., Takimura, O. and Oota, S. 2001. Degradation of triphenyltin compounds in sea water by pyoverdins from *Pseudomonas chlororaphis.* Appl. Organometal. Chem.15: 757-761.

Yamaoka , Y., Inoue, H., Takimura, O. and Oota, S.2002. Effect of iron on the degradation of triphnyltin by pyoverdins isolated from *Pseudomonas chlororaphis.* Appl. Organometal. Chem.16: 277-279.

Yomoda, S. Takahashi, A., Okubo,T., Murakami, M. and Iyobe, S. 2003. Isolation of carbapenem resistance *Pseudomonas putida* and its genetic background. Japanese. J. Chemotherapy. 51(1): 8-12.

Yoon, K.P. 1998. Isolation and characterization of *Pseudomonas* sp KM10, a cadmium and mercury resistant and phenol degrading bacterium. J. Microbiol. Biotechnol. 8(4): 388-398.

Zobell, C.E. 1941. Studies of marine bacteria I .The cultural requirements of heterotrophic aerobes. J. Mar. Res. 4: 42-67.

ZoBell, C.E., and Conn, J. E. 1940. Studies on the thermal sensitivity of marine bacteria. J. Bacteriol. 14: 223 - 238.

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Review

Biodegradation of tributyltins (organotins) by marine bacteria

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Many marine bacterial strains have an inherent capability to degrade toxic organotin compounds, especially tributyltins (TBTs), that enter into the environment in the form of insecticides, fungicides and antifouling paints as a result of anthropogenic and industrial activities. Significant degradation of these compounds in the ambient environment may take several years, and it is necessary to consider methods or strategies that can accelerate the degradation process. There have been few demonstrations of biological degradation of these organotin biocides exclusively in laboratory-scale experiments. Compared with the few bench-scale degradation processes, there are no reports of field-scale processes for TBT bioremediation, in spite of its serious environmental threat to non-target organisms in the aquatic environment. Implementation of field-scale biodegradation of TBT requires inputs from biology, hydrology, geology, chemistry and civil engineering. A framework is emerging that can be adapted to develop new processes for bioremediation of toxic environmental wastes. In the case of TBT bioremediation, this framework incorporates screening and identification of natural bacterial strains, determination of optimal conditions for growth of isolates and TBT degradation, establishment of new metabolic pathways involved in TBT degradation, identification, localization and cloning of genes involved in degradation and in TBT resistance, development of suitable microbial strains using genetic manipulation techniques for practical applications and optimization of practical engineering processes for bioremediation of organotin-contaminated sites. The present review mainly addresses the aspect of TBT biodegradation with special reference to environmental sources of TBT, chemical structure and biological activity, resistant and degrading bacterial strains, possible mechanisms of resistance and degradation and the genetic and biochemical basis of TBT degradation and resistance. It also evaluates the feasibility and potential of natural and genetically modified TBT-degrading bacterial strains in field-scale experiments to bioremediate TBT-contaminated marine sites, and makes recommendations for more intensive and focused research in the area of TBT bioremediation mediated by marine bacterial strains.

KEYWORDS: biodegradation; tributyltin (TBT); microorganism; bioremediation; resistance mechanisms; toxicity.

ORGANOTINS IN THE ENVIRONMENT

Organotin compounds remained of purely scientific interest for a long time, since their discovery around 1850. Though the first mention of a practical application of organotin compounds was made in a patent taken out in 1943, which indicated their potential in antifouling systems,[1] commercial production only started in 1960s. All organotin compounds are toxic, but the effect varies according to the number and the type of organic moiety present, as propyl- and butyl-group-bearing organotins are more toxic to fungi and bacteria.[2] Extensive use of organotins worldwide has

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provoked scientific interest on the toxic effect of organotin compounds on aquatic and terrestial biota.[3–5] Tributyltin (TBT) has been in use as a paint additive since the 1970s to prevent bio-fouling on ship hulls, marine platforms and fishing nets. By the mid 1980s, researchers in France and the UK had confirmed that TBT present in antifouling paints adversely affects non-target organisms. In 1982, France was the first country to ban the use of organotin-based antifouling paints on boats less than 25 m long.[6,7] Similar regulations have also been imposed in North America, Australia, New Zealand, South Africa, Hong Kong and most European countries since the late 1980s.[8–14] Subsequently, worldwide monitoring programmes have shown reduced concentrations of TBT in the water column, sediments and tissues of marine animals. The International Maritime Organization (IMO) has repeatedly expressed concern about the harmful effects of TBT-based paints.[12] It has also been shown that TBT may be responsible for the weakening of oyster and mussel shells, as well as of retardation of growth of various species of aquatic snails.[15,16] Two widely published events in the 1980s, viz the near-collapse of oyster farming in Arcachon bay, Western France, and the demise of populations of dogwhelk, *Nucella lapillus*, close to boating activity in southwest England, have been attributed to severe TBT contamination. These studies resulted in a number of surveys of TBT pollution worldwide and also indicated that the problem was global.[12,17–20] TBT concentrations in the aquatic environment have been monitored for many years at many locations throughout the world, including the North Sea, Black Sea, Atlantic Ocean, Pacific Ocean and Japanese waters.[7,12,21–23] Noticeable concentrations of organotins reported so far include: 38 $\mu g\ g^{-1}$ TBT in Suva Harbour, Fiji; 10.780 ng g^{-1} Hexyltin in Vancouver, Canada; 518 ng g^{-1} TBT in Boston Harbour, USA; 400 ng g^{-1} TBT in Lake Lucerne, Switzerland; and up to 380 ng g^{-1} TBT in Puget Sound, USA.[9,10,18] The IMO has passed a resolution to ban the application of TBT-based antifouling paints on ships and boats and has also proposed to establish a mechanism to prevent the potential future use of other harmful substances in antifouling systems.[13] Triorganotins, such as tributyltin oxide (TBTO), tributyltin chloride (TBTCl), triphenyltin chloride (TPTCl), tributyltin fluoride (TBTF), tributyltin hydroxide (TBTH), tributyltin naphthenate (TBTN) and tris(tributylstannyl) phosphate (TBTP), are used extensively as biocides in antifouling paints on ship hulls, boats and docks, as slimicides in cooling towers, as fungicides, bactericides and insecticides, as preservatives for wood, textiles, papers and leather, as stabilizing material in PVC pipes, electrical equipment and as catalysts for synthesis of polyurethane foam and silicone rubber.[24–26] Trisubstituted organotins have wide-ranging toxicological properties, and their biocidal uses have been reported to have detrimental environmental impacts.[27] In the UK, under the Control of Pollution Act, 1974, the retail sale of organotin paints was restricted to co-polymer paints containing <7.5% tin and free association paints containing <2.5% tin in the dry film.[8] During the 1990s, the USA alone produced 10000 t of organotin compounds each year.[28] Recent estimates show that the annual world production of organotins may be close to 50000 t per year.[27] Commercial ships, in particular, consume about 75% of total TBT used as antifouling paints.[29] In Suva Harbour, Fiji, water blasting of relatively large vessels has caused severe contamination of near-shore sediments and shellfish. A British survey revealed that unregulated dry dock practices clearly result in the release of large quantities of TBT into marine environment.[9] Non-point sources of environmental exposure include the discarding and sanitary landfill disposal of plastic and the direct release of biocides to the aquatic and marine environments. Other dissipative uses of organotins that pose potential risk to humans include PVC food wrappings, bottles and rigid potable water pipes, although long-term human health hazards due to low-level exposure to organotins are not known. Toxic metal cycling in the environment, including biomethylation of inorganic tin by naturally occurring bacteria, is also of concern.[30,31] *In situ* measurement of TBT-based antifouling paint leachates have shown that TBT is the principal compound released in water. It has been shown that different forms of TBTs, such as the hydroxide, chloride, and various carboxylate forms are released into the aqueous environment from different types of paint as a result of leaching.[24]

CHEMISTRY OF TBT (ORGANOTIN) COMPOUNDS

In view of the diversity of organotins used industrially, knowledge of their environmental chemistry is of fundamental importance, and some aspects have already been reviewed.[30] The TBT compounds are a subgroup of the trialkyl organotin family. It is interesting to note that the alkyls tend to be more toxic than the aryls, and that triorganotins are more toxic than di-, mono- or tetra-organotins. Generally, the toxicity of the organotin is influenced more by the alkyl substituents than the anionic substituents. Progressive introduction of organic groups to the tin atom in any member of the R_3SnX_{4-n} series produces maximal biological activity against all species, when $n = 3$, for R_3SnX.[3,4,32,33] Generally, trisubstituted (R_3SnX) organotins, where R = butyl or phenyl, are more highly toxic than di- and mono-substituted organotin compounds, and the anion (X) has little influence on the toxicity.[34,35] It is interesting to note that they can provide antifouling cover for 5 years or more and have been acclaimed widely as the most effective antifoulants ever devised. TBT in such paints is chemically bonded in a copolymer resin system via an organotin–ester linkage, but there is a slow and controlled release of the biocide, as the link becomes hydrolysed when sea water comes into contact with the paint surface.[12]

BIOLOGICAL ACTIVITY OF ORGANOTINS

Though tin in its inorganic form is considered to be non-toxic, the toxicological pattern of the organotin compounds is complex.[20] TBT, tripropyltin and triphenyltin are highly effective biocides against several marine fouling organisms, including bacteria and fungi, whereas tricyclohexyltin compounds exhibit miticidal properties. In general, organotin toxicity to microbes decreases in the following order: $R_3SnX > R_2SnX_2 > RSnX_3 > R_4Sn$. Since, microorganisms accumulate organotins in the cell wall envelope by a non-energy requiring process, organotins such as tripropyl-, tributyl- and triphenyl-tin seem to be highly toxic to bacteria and fungi.[36–38] It is interesting to note that increased total surface area and lipid solubility of the trisubstituted tin correlates well with the toxic effects observed and confirms that triorganotins exert toxicity through their interaction with membrane lipids. 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 towards Gram negative bacteria. Among the trialkyltin series, the most active compounds inhibiting growth of the Gram positive species at 0.1 mg l^{-1} belong to the type R_3SnX. Gram positive bacteria are less sensitive to triethyl and tripropyltin acetate or chloride than Gram negative bacteria, whose growth is inhibited at concentrations of 20–50 μg ml^{-1}. TBTCl and TBT acetate have stronger growth inhibitory effects on Gram positive bacteria than on Gram negative bacteria.[39,40]

TBT is a membrane-active lipophilic compound known to exhibit the same inhibitory mechanisms in bacteria as seen in mitochondria and chloroplasts by acting as an ionophore-facilitating halide–hydroxyl ion exchange by interfering with the energy transduction apparatus. In addition, TBT can inhibit a variety of energy-linked reactions in *Escherichia coli*, including growth, solute transport, biosynthesis of macromolecules and activity of transhydrogenase.[33] Boopathy and Daniels[28] have also tested the toxic effects of several organotins and tin chloride on the methanogenic bacteria *Methanococcus thermolithotrophus, Methanococcus deltae* and *Methanosarcina barkeri* 227. These methanogens were strongly inhibited by triethyltin, tripropyltin and monophenyltins generally below the 0.05 mM level. Less inhibition was observed for TBT at 0.1 mM, but there was complete inhibition of growth at 1 mM concentration. Virtually all organotin toxicological studies have been conducted using aerobic microorganisms, viz. bacteria and yeast.[36,37,41–45] In addition, biocidal effects of organotins against other marine fouling organisms viz. algae (*Enteromorpha, Ectocarpus* and *Ulothrix*), barnacles, tubeworms and shrimps have also been studied.[46–49]

TBT-RESISTANT BACTERIA

Several reports have been documented on the isolation and characterization of TBT-resistant bacteria from soil, marine and estuarine environments.[25,34,50–55] The isolation and characterization of the TBT-tolerant (-resistant) marine bacterium *Alteromonas* sp. M-1 is the first of its kind. It is interesting to note that addition of TBT to natural sea water specifically enriched TBT-tolerant bacteria.[55,56] These resistant bacteria can tolerate high levels of TBT biocide due to their inherent capability: (i) to transform it into less toxic compounds, viz. dibutyltin (DBT) and monobutyltin (MBT) by a dealkylation mechanism; or (ii) to exclude/effuse these toxicants outside the cell, mediated by membrane proteins; or (iii) to degrade/metabolically utilize it as a carbon source mediated by enzymes; or (iv) to bioaccumulate the biocide without breakdown using metallothionein-like proteins.[56,57]

Little is known about the incidence of organotin resistance in natural microbial populations, or the resistance mechanism by which microorganisms tolerate high levels of organotins.[54] Therefore, the list of potential organotin-resistant bacteria includes *E. coli, Pseudomonas fluorescens, Pseudomonas aeruginosa, Proteus mirabilis, Serratia marcescens* and *Alcaligenes faecalis*, which are Gram negative, and *Staphylococcus aureus, Staphylococcus epidemidis, Bacillus subtilis, Mycobacterium phlei* and *Vibrio sp.*, which are Gram positive.[26,35,54,56]

DEGRADATION OF TBT BY ABIOTIC AND BIOTIC FACTORS

Organotin degradation involves sequential removal (dealkylation) of alkyl groups from the tin atom, which generally results in a toxicity reduction.[38,57,58] This can be achieved by biotic and abiotic factors, with UV and chemical cleavage being the most important abiotic factors in aquatic and terrestrial ecosystems.[51,58] Although the degradation of organotins has been shown to be mediated by microorganisms, information is still severely limited in relation to the mechanism of degradation the tolerance mechanisms of microbes and their relative significance and also the role of anionic radicals in the degradation process in natural habitats.[35,58,60] Biotic processes have been demonstrated to be the most significant mechanisms for TBT degradation both in soil and in fresh water, marine and estuarine environments.[51,61]

The lack of knowledge on the environmental fate of TBT in coastal waters stimulated research interest on the biodegradation and bioaccumulation of TBT in water columns, sediments and also by higher marine organisms. Environmental surveys from different locations throughout the world have shown that TBT is present in three main compartments of the aquatic ecosystem: the surface microlayer, the water column and the surface layer of the bottom sediments.[24] The result of these studies indicates that TBT can be degraded rapidly in the marine water column to DBT and MBT with a half-life of several days. TBT degradation by photolysis alone proceeds slowly, with a half-life of >89

days.[54] Half-lives from a clean water site (0.03 μg l^{-1} of TBT) were 9 days and 19 days for light and dark treatments respectively,[62] but photolysis probably is not a significant breakdown process for TBT.[24] In the case of TBT present in sediments, a first-order multi-step kinetic model for the sequential degradation of TBT to form DBT, MBT and tin(IV) has been proposed which indicated that the half-life of TBT, DBT and MBT was 2.1 years, 1.9 years and 1.1 years respectively.[63] The principal degradation product in all experiments was DBT with lesser amounts of MBT. Complete mineralization of TBT, measured by the formation of $^{14}CO_2$, proceeded slowly, with a half-life of 50–75 days. Rates of TBT degradation may be influenced by several biotic and abiotic factors, such as the nature and density of microbial populations, TBT solubility, dissolved/suspended organic matter, pH, salinity, temperature and light. Sheldon[64] has reported that ^{14}C-labelled TBTO, TBTF and triphenyltin fluoride in soil was degraded faster in aerobic conditions than in anaerobic conditions. However, persistence does not necessarily equate to a compound being toxic, because it may not be bioavailable.[12] Interaction of microorganisms with organotins is significantly influenced by environmental conditions. In aquatic ecosystems, both pH and salinity can determine organotins speciation/bioavailability and, therefore, biological activity. In one study Pottassium (K^+) release was used as an index of toxicity, as both the rate and the extent of K^+ release was affected by salinity. Increased NaCl concentration reduced the toxic effect of TBT, with the possible effects being due to Na^+ and Cl^- moieties, as well as by possible osmotic responses of the organisms, which included changes in intracellular compatible solute and membrane composition.[44] These environmental factors may also alter selectively the resistance of microorganisms in polluted aquatic systems.[45] Biological and chemical degradation of TBT in marine and freshwater sediments has been reported to be slow,[54] as the half-life of TBT in marine water has been found to be about a week, whereas in sediments it was about 2.5 years.[29] This clearly indicates that sorption of TBT in the silty sediments strongly reduced the bioavailability of the biocide to microorganisms.[65] Because of the low water solubility, TBT preferably binds to suspended organic matter released from marine sediments. The extent of binding to bottom sediments varies with location, organic matter content and particle size.[16]

Abiotic degradation processes have also been put forward as possible pathways for the removal of TBT from soil sediments and water columns, as the Sn—C bond could be broken by four different abiotic processes, viz. UV irradiation, chemical cleavage, gamma irradiation and thermal cleavage.[66] Because gamma irradiation rarely occurs and the Sn—C bond is stable up to 200 °C, gamma irradiation and thermal cleavage have a negligible effect on the environmental breakdown of TBT. Only the near-UV spectrum (300–350 nm) is likely to cause direct photolysis of TBT, and, owing to the low transmittance of UV light, this breakdown process is expected to occur only in the upper few centimetres of the water column.[24]

The numerous studies undertaken on the fate of TBT have indicated that it degrades by a stepwise debutylation mechanism to the less toxic DBT and MBT, which have also been detected in the aquatic environment.[8,35] Maureen and Willingham[67] have reported that the TBT degradation process may be explained as a sequential loss of an alkyl group from TBT to form non-toxic inorganic tin ultimately in the following manner: $R_3Sn^+ \rightarrow R_2Sn^{2+} \rightarrow RSn^{3+} \rightarrow Sn(IV)$. Complicating the issue of organotin persistence is the possibility of other degradation pathways for TBT species, including a number of possible redistribution reactions catalysed by environmental molecules such as amines, sulfides or other reactants. The possibility of environmental methylation of butyltins has been raised by recent reports of the presence of mixed butylmethyltin species in sediments, presumably arising by biological methylation of anthropogenic butyltin in the aquatic environment. A few of the possible reactions of Sn—C include the following:

$$2Bu_3Sn+ \rightarrow Bu_2Sn^{2+} + Bu_4Sn \quad (1)$$

$$Bu_2Sn^{2+} + Bu_3Sn^+ \rightarrow BuSn^{3-} + Bu_4Sn \quad (2)$$

$$Bu_3Sn^{\cdot} + Me^{\cdot} \rightarrow Bu_3MeSn \quad (3)$$

At present, the source of the methyl moiety is unknown, but it may be due to redistribution and biogenesis of methyltin species.[68]

There are few reports on the biodegradation of TBT mediated by microorganisms viz bacteria, fungi, cyanobacteria and green algae in terrestrial and aquatic environment.[35,50,58,60,64] Barug[51] has reported that Gram negative bacteria, viz. *P. aeruginosa* and *A. faecalis*, and fungi, viz. *Tramatis versicolor* and *Chaetomium globosum*, could degrade TBTO via a dealkylation process. Pure cultures of the wood-rotting fungi, *Coniophora puteana* and *Coriolus versicolor* can also degrade this biocide to form OBT and MBT derivatives.[69] It is interesting to note that some *Pseudomonas* sp. have been reported to bioaccumulate TBT up to 2% of dry weight.[35,57] It has also been reported by Barug[51] that several other Gram negative bacteria possess the capability to accumulate TBTO without its breakdown. The high lipid-solubility of organotins ensures cell penetration and association with intracellular sites, and cell wall components also play an important role.[35] It is evident that the site of action of organotins may be both at the cytoplasmic membrane and at the intracellular level. Consequently, it is not known whether cell surface adsorption, accumulation within the cell, or both is a prerequisite for toxicity. TBT biosorption studies in fungi, cyanobacteria and microalgae indicate that cell surface binding alone occurred in these organisms, while studies on the effect of TBT on certain bacterial strains indicated that it can also interact with cytosolic enzymes.[45] The elimination of such hydrophobic compounds is facili-

tated by their biotransformation to water-soluble polar compounds. Thus, the metabolism of a compound generally reduces persistence, increases removal or elimination and results in a reduction of toxicity. Therefore, microbial degradation is probably the most predominant process for the breakdown of TBT in near-shore waters with DBT as the major degradation product.[70]

BIOCHEMICAL AND GENETIC BASIS OF TBT (ORGANOTIN) RESISTANCE IN BACTERIA

Genetic studies on TBT-resistant and -degrading bacterial strains from terrestrial and aquatic environments are limited with a few reports demonstrating the presence of plasmids but no correlation with TBT resistance.[26,33,71-73] In most cases, it has been demonstrated that the resistance-conferring genes are located on a chromosomal genome.[26,74] Fukagawa and Suzuki[72] reported the presence of genes conferring TBT resistance in *Alteromonas* sp. strain M1. They have successfully isolated, cloned and sequenced the gene that seems to be involved in the efflux of TBT employing a membrane-bound TBTCl-induced transport protein, possessing 108 amino acid residues encoded by an open reading frame of 324 nucleotides. This membrane protein has 48.5% of hydrophobic residues and shows more homology with transglycosylases of *E. coli* and other bacterial strains.[72] Therefore, this membrane protein has been predicted to be the most prominent resistance mechanism in this marine bacterial strain. Suzuki *et al.*[74] have further confirmed the taxonomic position of this strain by 16S rRNA sequencing and genomic sizing by pulse field gel electrophoresis using a contour clamped homogeneous electric field technique. These studies revealed that *Alteromonas* sp. M1 possesses a genome of 2240 kb. It is interesting to note that this strain is devoid of any plasmid, suggesting the exclusive presence of TBT-resistance genes on the chromosomal genome.[72]

FUTURE PROSPECTS FOR A MICROBIAL BIOREMEDIATION PROCESS

The application of natural microbial populations for bioremediation of organotin-contaminated sites is far away from a real large-scale field/commercial process, since little work has been done to explore the exact mechanism of biodegradation and the genes involved in the process. Therefore, we suggest that research is required to elucidate the basic mechanism of TBTCl bioaccumulation and biodegradation, employing molecular biologically and biochemical tools. These studies would ultimately enable use of natural and genetically modified bacterial strains for bioremediation of TBT-contaminated sites.

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REFERENCES

1. Tisdale WH. *British Patent No.* 578 312 1943.
2. Evans CJ, Smith PJ. *J. Oil Colour Chem. Assoc.* 1975; **58**: 160.
3. Smith PJ. *Toxicological Data on Organotin Compounds.* International Tin Research Institute Publications, 538; 1978.
4. Smith PJ. *Structure Activity Relationships for Di- and Tri-organotin Compounds.* International Tin Research Institute Publications, 569; 1980.
5. Smith PJ. *Chemistry of Tin*, 2nd edition. Blackie Academic and Professional: London, 1998.
6. Alzieu CL, Sanjuan J, Deltreil JP, Borel M. *Mar. Pollut. Bull.* 1986; **17**: 494.
7. Alzieu CL, Sanjuan J, Michel P, Borel M, Dreno JP. *Mar. Pollut. Bull.* 1989; **20**: 22.
8. Dowson PH, Bubb JM, Lester JN. *Mar. Pollut. Bull.* 1993; **26**: 487.
9. De mora SJ, Stewart C. Phillips D. *Mar. Pollut. Bull.* 1995; **30**: 50.
10. De mora SJ. *Tributyltin – Case Study of an Environmental Contaminant.* Cambridge University Press: Cambridge, 1996.
11. Minchin D, Bauer B, Oehlmann J, Oehlmann US, Duggan CB. *Mar. Pollut. Bull.* 1997; **34**: 235.
12. Evans SM. *Biofouling* 1999; **14**(2): 117.
13. Champ MA. *Sci. Total Environ.* 2000; **258**: 21.
14. Champ MA. *Pollution prevention from ships and shipyards.* In *Proceedings of International Oceanology Symposium – 2001*, vol. I, Miami, FL, USA; 2001; 250.
15. Alzieu CL, Heral M. *Ecotoxicological effects of organotin compounds on oyster culture. In Ecotoxicological Testing for the Marine Environment*, (eds), Persoone G *et al.* vol. II, State University of Ghent, Ghent, and Institute of Marine Science Research, Bredence, Belgium; 1984; 187-196.
16. Laughlin RB, French W, Guard HE. *Environ. Sci. Technol.* 1986; **20**: 884.
17. Maguire RJ, Chau YK, Bengert GA, Hale EJ, Wong PTS, Kramar O. *Environ. Sci. Technol.* 1982; **16**: 698.
18. Maguire RJ, Tkaez RJ, Chau YK, Bengert GA, Wong PTS. *Chemosphere* 1986; **15**: 253.
19. Champ MA, Seligman PF. *Organotin: Environmental fate and effects.* Chapman and Hall: UK, 1997.
20. Hoch M. *Appl. Geochem.* 2001; **16**: 719.
21. Cleary JJ, Stebbing ARD. *Mar. Pollut. Bull.* 1987; **18**: 238.
22. Maguire RJ. *Environ. Sci. Technol.* 1984; **18**: 281.
23. Maguire RJ. *Water Qual. Res. J. Can.* 2000; **35**(4): 633.
24. Clark EA, Sterritt RM, Lester JN. *Environ. Sci. Technol.* 1988; **22**: 600.
25. Fukagawa T, Suzuki S, Fukunaga K, Suzuki T, Takama K. *FEMS Microbiol. Lett.* 1992; **93**: 83.
26. Suzuki S, Fukagawa T. *J. Ind. Microbiol.* 1995; **14**: 154.
27. Inoue H, Takimura O, Fuse H, Murakami K, Kamimura K, Yamaoka Y. *Appl. Environ. Microbiol.* 2000; **66**: 3492.
28. Boopathy R, Daniels L. *Appl. Environ. Microbiol.* 1991; **57**: 1189.
29. Atireklap SK, Tanabe S, Sanguansin J. *Mar. Pollut. Bull.* 1997; **34**: 894.
30. Craig PJ. Environmental chemistry of organotins. In *Comprehensive Organometallic Chemistry*, vol. II, Wilkinson G (ed.). Pergamon Press: Oxford, 1982; 979.
31. Hallas LE, Means JC, Cooney JJ. *Science* 1982; **215**: 1505.
32. Blunden SJ, Hobbs LA, Smith PJ. The environmental chemistry of

organotin compounds. In *Environmental Chemistry*, Bowen HJM (ed.). The Royal Society of Chemistry: London, 1984.
33. Singh K. Biocidal effect of organotin compounds. In *Frontiers in Applied Microbiology*, vol. **II**, Mukherjee KG, Singh VP, Garg KL (eds). Publication Print House: New Delhi, India, 1987; 297–316.
34. Pain A, Cooney JJ. *Arch. Environ. Contam. Toxicol.* 1998; **35**: 412.
35. Gadd GM. *Sci. Total Environ.* 2000; **258**: 119.
36. Cooney JJ, Wuertz S. *J. Ind. Microbiol.* 1989; **4**: 375.
37. Laurence OS, Cooney JJ, Gadd GM. *Microb. Ecol.* 1989; **17**: 275.
38. Cooney JJ. *Helgol. Meeresunters.* 1995; **49**: 663.
39. Yamada J, Tatsuguchi K, Watanabe T. *Agric. Biol. Chem. (Tokyo)* 1978; **42**: 1167.
40. Yamada J, Tatsuguchi K, Watanabe T. *Agric. Biol. Chem. (Tokyo)* 1979; **43**: 125.
41. Hallas LE, Cooney JJ. *Dev. Ind. Microbiol.* 1981; **22**: 529.
42. Hallas LE, Thayer JS, Cooney JJ. *Appl. Environ. Microbiol.* 1982; **44**: 193.
43. Pettibone GW, Cooney JJ. *J. Ind. Microbiol.* 1988; **2**: 373.
44. Cooney JJ, de Rome L, Laurence OS, Gadd GM. *J. Ind. Microbiol.* 1989; **4**: 279.
45. White JS, Tobin JM, Cooney JJ. *Can. J. Microbiol.* 1999; **45**: 541.
46. Skinner CE. In *Proceedings of Antifouling Symposium*, CIBA-GEIGY, Marienberg, West Germany 1971.
47. Christie AO. In *Proceedings of 3rd International Congress of Marine Corrosion and Fouling*, Gaithersburg, MD, USA; 1972; 676.
48. Mawatari S. In *Proceedings of 3rd International Congress of Marine Corrosion and Fouling*, Gaithersburg, MD, USA, 1972; 956.
49. Phillip AT. In *Australian OCCA Proceedings and News* (July): 1973; 17.
50. Barug D, Vonk JW. *Pestic. Sci.* 1980; **11**: 77.
51. Barug D. *Chemosphere* 1981; **10**: 1145.
52. Hallas LE, Cooney JJ. *Appl. Env. Microbiol.* 1981; **41**: 466.
53. McDonald L, Trevors JT. *Water Air Soil. Pollut.* 1988; **40**: 215.
54. Wuertz S, Miller CE, Pfister RM, Cooney JJ. *Appl. Environ. Microbiol.* 1991; **57**: 2783.
55. Suzuki S, Fukagawa T, Takama K. *Appl. Environ. Microbiol.* 1992; **58**: 3410.
56. Fukagawa T, Konno S, Takama K, Suzuki S. *J. Mar. Biotechnol.* 1994; **1**: 211.
57. Blair WR, Olson GJ, Brinckman FE, Iverson WP. *Microb. Ecol.* 1982; **8**: 241.
58. Cooney JJ. *J. Ind. Microbiol.* 1988; **3**: 195.
59. Blunden SJ, Chapman AH. *A Review of the Degradation of Organotin Compounds in the Environment and their Determination in Water*. International Tin Research Institute Publication, 626; 1983; 1–7.
60. Gadd GM. *FEMS Microbiol. Rev.* 1993; **11**: 297.
61. Dowson PH, Bubb JM, Lester JN. *Estuarine Coastal Shelf Sci.* 1996; **42**: 551.
62. Seligman PF, Valkris AO, Lee RF. *Environ. Sci. Technol.* 1986; **20**: 1229.
63. Sarradin PM, Lapaquellerie Y, Astruc A, Latouche C, Astruc M. *Sci. Total Environ.* 1995; **170**: 59.
64. Sheldon AW. In *Proceedings of 18th Annual Marine Coatings Conference*, Monterey, CA, USA 1978.
65. Stronkhors J, Hattum BV, Bowmer T. *Environ. Toxiol. Chem.* 1999; **18**: 2343.
66. Sheldon AW. *J. Paint. Technol.* 1975; **47**: 54.
67. Maureen EC, Willingham GL. *Biofouling* 1996; **10**: 239.
68. Matthias CL, Beluga JM, Olson GJ, Brinckman FE. *Environ. Sci. Technol.* 1986; **20**: 609.
69. Henshaw BG, Laidlaw RA, Orsler RJ, Carey JK, Savory JG. In *Record of the 1978 Annual Convention of B.W.P.A.*, Cambridge; 1978; 19.
70. Page SD. *Mar. Pollut. Bull.* 1989; **20**: 129.
71. Singh AP, Singh K. *Z. Naturforsch.* 1984; **39**: 293.
72. Fukagawa T, Suzuki S. *Biochem. Biophys. Res. Commun.* 1993; **194**: 733.
73. Miller CE, Wuertz S, Cooney JJ, Pfister RM. *J. Ind. Microbiol.* 1995; **14**: 337.
74. Suzuki S, Tsukamoto KK, Fukagawa T. *Microbios* 1994; **77**: 101.

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 x 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 x 10^2 to 408 x 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 x 10^2 to 85 x 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 x 10^2 and 383 x 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* P2d 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).

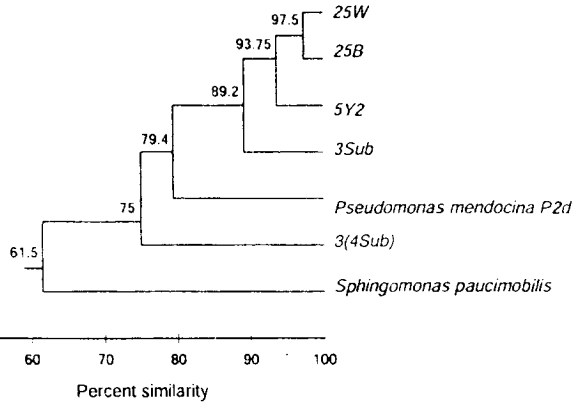

Figure 1. Phenogram showing similarity among different isolates.

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].

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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