MOLECULAR BIOLOGY AND BIOCHEMISTRY OF A TRIBUTYLTIN CHLORIDE RESISTANT MARINE BACTERIUM, *Alcaligenes* sp.

THESIS SUBMITED TO

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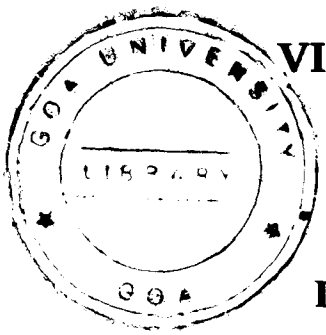
FOR THE DEGREE OF

DOCTOR OF PHILOSOPHY
In
MICROBIOLOGY

by

VIDYA RAMACHANDRAN



Under the guidance of

Dr. Santosh Kumar Dubey
Reader & Head

Department of Microbiology,
Goa University,
Goa, India.

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फॅक्स : +०९१-८३२-२४५११८४/२४५२८८९

Goa University
Taleigao Plateau, Goa - 403 206
Tel : 0832-6519002 / 6519005 / 6519006
Fax : + 091- 832-2451184/2452889
E.mail : registra@unigoa.ac.in
Website: www.unigoa.ac.in
(Accredited by NAAC with a rating of Four Stars)

CERTIFICATE

This is to certify that ***Ms. Vidya Ramachandran*** *has worked on the thesis entitled* ***"Molecular biology and biochemistry of a Tributyltin chloride resistant marine sediment bacteria, Alcaligenes sp."*** *in my Laboratory of Environmental Microbiology & Biotechnology at Goa University, under my supervision and guidance. This thesis, being submitted to Goa University, Taleigao Plateau, Goa, for the award of the degree of Doctorate of Philosophy in Microbiology, is an original record of work carried out by the candidate herself and has not been submitted for the award of any other degree or diploma of this or any other University in India or abroad.*

Certified. Appropriate corrections have been incorporated.

Signature of the Examiner

Date: 30/10/09

Dr. Santosh Kumar Dubey
Research Guide & Associate Professor
Department of Microbiology
Date: 30/10/09.

STATEMENT

*I hereby state that this thesis for the Ph.D. degree on "**Molecular Biology and biochemistry of a Tributyltin chloride resistant bacterium, Alcaligenes sp.**" is my original contribution and the thesis or any part of it has not been previously submitted for the award of any degree/diploma of any University.*

Date: 30/10/09

Vidya Ramachandran
Department of Microbiology
Goa University, Goa

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Vidya

DEDICATED

TO

MY

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PARENTS

ABBREVIATIONS

Abs	Absorbance
APS	Ammonium per sulfate
b.p.	Boiling point
°C	Degree Celsius
Ca^{2+}	Calcium ion
Cd^{2+}	Cadmium ion
D/W	Double distilled Water
DBT	Dibutyltin chloride
EDTA	Ethylene diamine tetra acetic acid
EPS	Exopolysaccharide
Fig.	Figure
Gm	Gram(s)
H	Hour(s)
HCl	Hydrochloric acid
H_2SO_4	Sulphuric acid
Hg^{2+}	Mercuric ion
K^+	Potassium ion
KDa	Kilo Dalton
Kbps	Kilo base pairs
KNO_3	Potassium nitrate
L	Litre
LB	Luria Bertani Broth
LA	Luria Bertani Agar
M	Molar
Mg	Milli gram(s)
MSM	Mineral Salt Medium
MSMA	Mineral Salt Medium Agar
Mg^{2+}	Magnesium ion
Min	Minute(s)
mM	Milli molar
NH_4NO_3	Ammonium nitrate
NH_4Cl	Ammonium chloride
NA	Nutrient Agar
NaOH	Sodium hydroxide
nm	Nanometer
NaCl	Sodium chloride
O.D.	Optical Density
PAGE	Poly-acrylamide gel electrophoresis
rpm	Revolutions per minute
RT	Room temperature
SDS	Sodium dodecyl sulfate
sec.	Second(s)
Sp.	Species
TBT	Tributyltin
TBTCl	Tributyltin chloride
TEMED	Tetra methyl ethylene diamine
UV	Ultra violet
V	Volts
v/v	Volume / Volume
w/v	Weight / Volume
Zn^{2+}	Zinc ion
ZMB	Zobell Marine Broth
µg	Microgram
µM	Micromolar
µL	Microlitre
µ	Micron
λ	Lambda
%	Percentage

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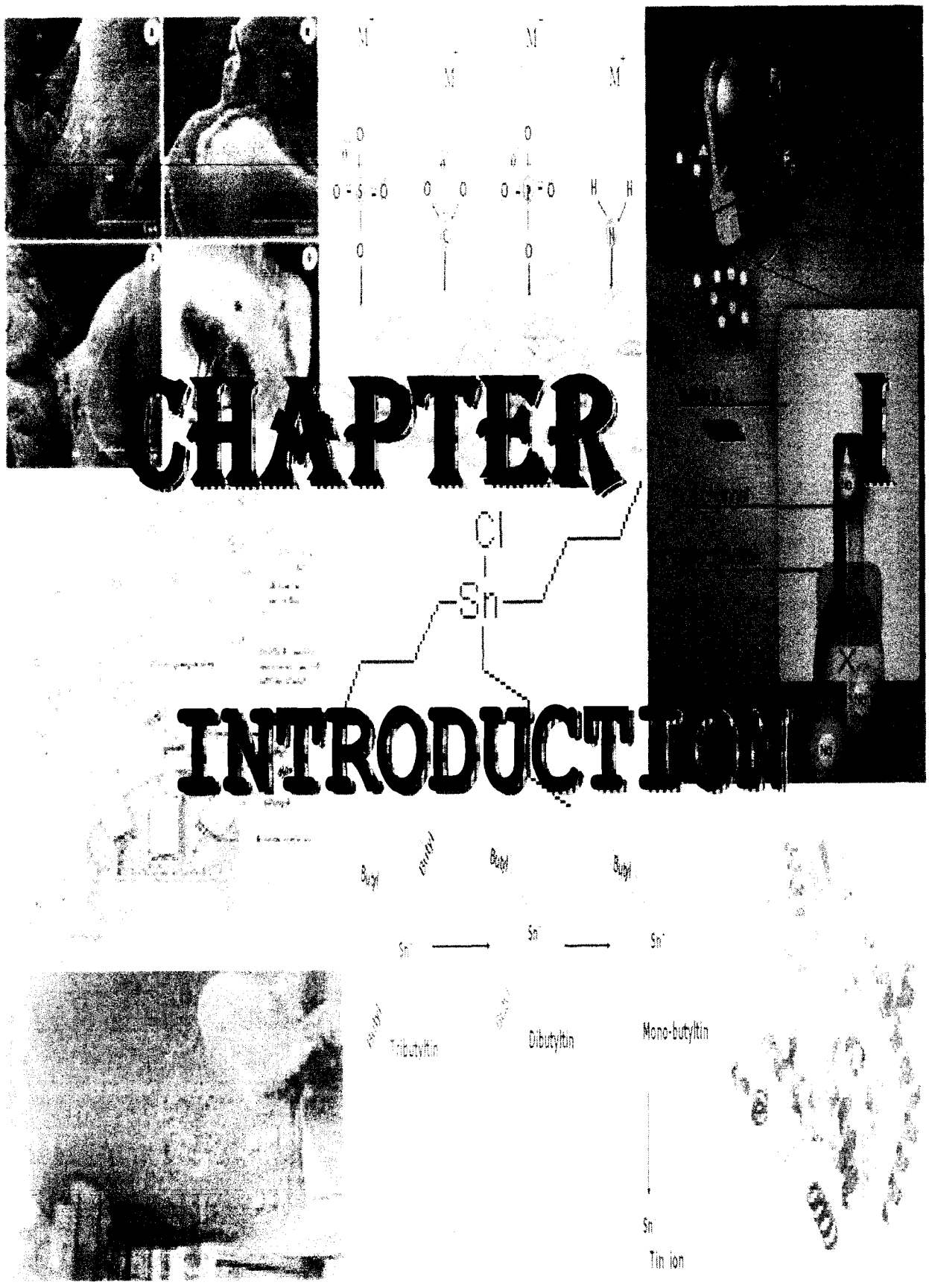

CHAPTER - I
INTRODUCTION
Cl
Sn
Butyl
Tributyltin
Dibutyltin
Mono-butyltin
Sn
Tin ion

1. 1. Organotin compounds

Organotin compounds or stannanes are chemical compounds based on tin with hydrocarbon substituents. Organotin compounds are classified as R_4Sn, R_3SnX, R_2SnX_2, and $RSnX_3$. In compounds of industrial importance, 'R' is usually a butyl, octyl, or phenyl group and 'X', a chloride, fluoride, oxide, hydroxide, carboxylate, or thiolate (Blunden et al., 1983). So far, mono-substituted and di-substituted organotin compounds ($RSnX_3$ and R_2SnX_2) have had a very limited application, and are used in plastic industry, particularly as stabilizers in polyvinyl chloride. They are also used as catalysts in the production of polyurethane foams and in the room-temperature vulcanization of silicones. Trisubstituted organotin compounds (R_3SnX) have biocidal properties that are strongly influenced by the R-groups. The most important of these compounds are the tributyl -, triphenyl - and tricyclohexyl - tin compounds, which are used as agricultural and general fungicides, bactericides, antihelminthics, miticides, herbicides, molluscicides, insecticides, nematocides, ovicides, rodent repellents, and antifouling paints for ships and large boats. The tetra - substituted organotin compounds (R_4Sn) are mainly used as intermediates in the preparation of other organotin compounds (Blair et al., 1982, Blunden et al., 1984; Cooney, 1988; Dubey and Roy, 2003).

Among various organotins, tributyltin (TBT) is one of the most extensively used active antifouling agents in paints for ships and boats. World wide extensive application of this biocide especially in shipping industry has resulted to gradual leaching of TBT into the aquatic ecosystem. This has lead to special environmental concern, since TBT is one of the most toxic compounds adversely affecting non target aquatic biota.

1. 2. Properties of organotin compounds

Organotins (OT) were first synthesised in the 19th century, but their industrial production increased simultaneously to the boom in plastic products after World War II. There are reports that di-octyl and di-butyltin added as PVC stabilizers in these products aided against

decay especially from heat and light. This is connected to the strong affinity of tin for donor atoms, such as oxygen and sulphur. Another important property is the physiological reactions of tri-organotins (TOT) used as pesticides, disinfectants or fungicides, which were marketed in 1950's. There are various industrial applications of organotins (Table 1.1).

Table 1.1 : Applications of various organotin compounds

Organotins	Selected Uses[1]
Triorganotins Triphenyltin (TPT), Tributyltin (TBT)	accelerator in polystyrene, anti-fouling agent, anti-tumor test substance, catalyst and accelators in polymers and epoxy resins, disinfecting agent, insecticide, acrylic glass, molluscicide, spray pyrolysis, wood preservatives, fungicide
Diorganotins Dibutyltin (DBT), Dioctyltin (DOT)	PVC stabilizer, acrylic glass additive, co-polymer in rubber, heat and electro-conductive coatings, cross-linking, stabilizer in polymers
Mono organotins Monobutyltin (MBT)	glass coating, cross linking, stabilizer in polymers

[1]Chemical Abstract Services Index 1995

1. 3. Compound Structure

Organotins are tetra or divalent tin compounds with one or more organic group(s). They form chemically stable compounds with aliphatic as well as aromatic groups. In aqueous phase, the tri-organotins are in equilibrium with anions as Cl^- and OH^-, depending on ion composition and pH (Fig. 1.1).

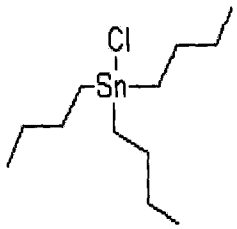


Fig.1.1 Tri-n-butyltin (TBT) as cation.

1. 4. Environmental behaviour of organotins

Sorption of organotin compounds on humic substances is mostly followed by sedimentation. These processes could scavenge most of the organotin compounds to the sediment which serves as an important environmental sink for organotins. According to the scientific reports, degradation of organotins is faster in aqueous phase than in the sediment. Interestingly, degradation of organotins in aerobic sediment is faster than the anaerobic sediment (Stang and Seligmen 1986) . TBT in sediments can accumulate in benthic organisms. Remobilization of organotin compounds can occur, due to tidal and anthropogenic perturbations of the sediments which have possible toxicological implications for the marine environment (Unger et al. 1987). TBT is mainly present as hydroxides in aquatic environments due to its unique chemical characteristics (Table 1.2) and also other inherent properties of environmental relevance (Table 1.3).

Table 1.2. Unique chemical characteristics of TBTCl:

	TBTCl	Reference
CAS No	[1461-22-9]	
Formula	$(C_4H_9)_3SnCl$	
Phase (20°C, 1 atm)	liquid	1
Molecular weight	325 g/mole	1
Melting point	- 16 °C	1
Boiling point	140 °C (13 m bar)	1
Vapour pressure (20°C)	-	1

pKa	6.25	2
Density (20°C)	1.2 kg / L	1
Aqueous solubility (20°C)	75.8 mg / L	1
Organic solvent solubility (20°C)	Good	1

([1] Miljmtyrelsen, 1993; [2] Arnold et al. 1997)

Table 1.3 Environmental properties of TBT:

Property	TBT	Reference
Kd (L/kg)	1(0.34 - 64)x10^3	2
Log K_{ow}	2.3 - 4.4	2
	14.1[1]	3
Log BCF	0.41-3.2	2
Degradation		
(a) sea water (half - life)	4 - 26 days	4
(b) Mediteranean deep seawater (aphotic zone) (half - life)	>years	5
(c) sediments (half - life)	0.9 - 5.2 years	4

[1] Values for the triorganotin hydroxide species, [2] Fent, 1996; [3] Arnold et al., 1997; [4] de Mora, 1996; [5] Averty et al., 1999.

1. 5. Sources of organotins in the environment

The primary source of organotin compounds to the marine environment is the extensive use of trialkyltin based antifouling paints (TBT and TPT). Di- and mono- organotins occur as a result of degradation of these compounds and also due to use of di- and mono-alkylated (butyl, cyclohexyl and octyl) compounds as stabilisers in PVC as well as catalysts in polymer production (Lawson, 1986) (Table 1.1). The worldwide production of TBT was estimated by

WHO to be around 2 – 3 x 10^3 tonnes per year (WHO report 1990) and later by Davies et al., in 1998 to around 1200 tonnes per year (as TBT).

1. 6. Effects of TBT on marine organisms

The first adverse effects of TBT use as antifouling agent on boats were reported by Dr. Alzieu and his associates at University of Bordeaux, France. The oyster production almost stopped for years because of elevated TBT concentrations in Arcachon Bay, which caused oyster shell anomalies and growth reduction (Alzieu et al., 1984; Alzieu et al., 1986). In 1982, France was the first country to ban TBT use on boats below 25 meters in length. Almost simultaneously, adverse effect of TBT on marine snails was discovered. At TBT concentrations below 1 ng/L, 50 % of a marine snail population (Dogwhelk, *Nucella lapillus*) developed physical deformation of the genitalia named, imposex (Gibbs and Bryan, 1986) (Fig. 1.2). This group at Plymouth Marine Laboratory, UK, associated the sexual malfunction and masculinisation of the dogwhelk, with low levels of TBT in seawater. The worldwide use of TBT is matched by the widespread finding of similar effects throughout the marine environment, even in remote areas (Langston, 1996). These effects on TBT in the marine environment underline the importance of gathering all available data on the environmental impact and fate of organotin compounds.

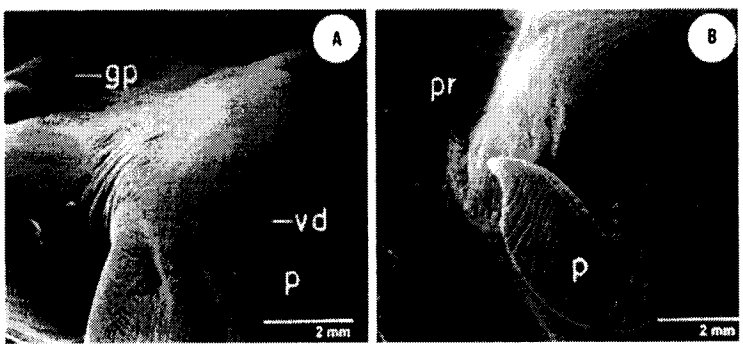


Fig. 1.2 Scanning electron micrographs of *Nucella lapillus* depicting (*A*) imposex stage 3a with a penis and an anterior section of the vas deferens and (*B*) imposex stage 5a with a

fully developed penis, vas deferens, and a prostate gland supplanting the vagina. Abbreviations: gp, genital papilla; p, penis; pr, prostate gland; vd, vas deferens.

1. 7. Early concentration measurements

Measurements of TBT started to develop after the severe effects were discovered (Table1.4). However, the task was very challenging and very costly, due to the low concentrations at which TBT had to be measured. The concentration of TBT was often measured as total organic extractable tin and not specifically as the TBT species. This could lead to erratic results due to the wide use of other organotin compounds than TBT for e.g. laboratory equipment or sampling devices (Sadiki et al., 1996). The very first measurements were from the Arcachon bay in France, where organotin concentration levels in the late 70's and early 80's reached pg/L range. In addition, in the United Kingdom early measurements of organotin concentration levels were in the low pg/L range.

Table 1.4 : Methods for analyzing tributyltin and its degradation products.

Extraction and/or derivatization	Speciation	Mode of detection	Detection limits (of TBT)	Reference
Trololon-Sep-PAK C cartridge: diethyl ether as elutant followed by ethylation	GC using capillary column	FPD / MS	1 ng/ L	1
Simultaneous hydridization-dichloromethane extraction	GC using packed column	FPD / MS	1-2 ng/L (as Sn)	2
Purge of generated hydrides into cryogenic trap (- 196 °C)	Boiling point separation	AAS	< 1 ng/L	3
Hydrochloric acid digestion, then	GC using	FPD	5 µg/Kg (dry	4

tropolone- benzene extraction, followed by pentylation	packed column		weight)	
GC = gas chromatography; FPD = flame photometric detection; MS = mass spectrometry; AAS = atomic adsorption spectrometry.				

[1] Fent, 1996; [2] Arnold et al., 1997; [3] de Mora, 1996; [4] Avery et al., 1993.

1. 8. Legislation to control TBT in the environment

As the result of the implementation of European Union directives (1991) TBT was banned to be sold or used in antifouling paints for boats < 25 m length, fishing nets, marine constructions and buoys. Prior to this regulation, France had already banned TBT use on boats < 25 meters in 1982 and UK issued a similar ban in 1987. This regulation is now implemented in many countries. The sources in the water column are therefore recent contamination and especially in marine desorption from the polluted harbour sediments are expected. The use of TBT in wood preserving paints is being phased out by a boat sales and production issued in June 1999.This regulation includes a total phase out in the middle of year 2000 (Nielsen, 1999). Internationally, the regulation of TBT in antifouling paints for ships > 25 meters is an issue for the United Nations organ, the International Maritime Organisation (MO). They have proposed a regulation on marine use regarding the sale and paint applications in 2003 and a total ban in 2008. The process is ongoing and negotiations are continuing in the IMO at present. Implementation of this ban is dependent on ratification processes by the member countries during 2001. In the EU membership countries, the antifouling agents in general, are due to be regulated by the so-called biocides directive, issued by the EU parliament in 1998. This regulation applies for all biocides, including antifouling agents, which after May 2000 all new substances need to be approved prior to use. All biocides all ready on the market need to pass the approval process before a 10-Year period (Nielsen, 1999).

1. 9. 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 as it adversely affects the growth and metabolic pathways in these organisms (Table 1.5). In general, hierarchy of organotin toxicity to microbes is 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. Tributyltin chloride or acetate had a strong growth inhibitory effect on Gram-positive bacteria than on Gram negative bacteria (Yamada et al., 1978, 1979).

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 (Fig. 1.3). 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

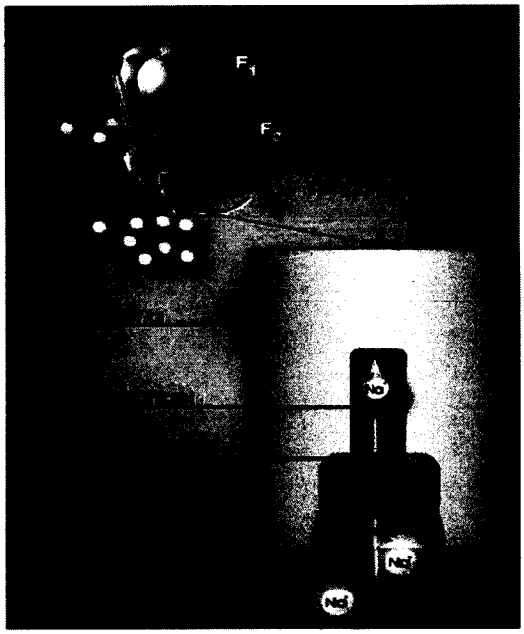
toxic effects of several organotins and tin chloride on the methanogenic bacteria, *Methanococcus thermolithotrophus, Methanococcus deltae* and *Methanosarcina barkeri* 227. These methanogens were strongly inhibited by triethyltin, tripropyltin and monophenyltin generally below 0.05 mM level. Less inhibition 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 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).

Table 1.5 Toxic effect of TBT on microbes

Process affected	Organisms	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.



(Source: von Ballmoos *et al.* PNAS August 3, 2004,vol. 101 , no. 31, 11239–11244)

Fig. 1.3 Model for the interaction of organotin compounds with F-ATP synthases. ATP synthesis from ADP and Pi is coupled to the down hill flux of ions across the membrane-bound F_0 portion. The lower part shows a section through the subunit a channel along the membrane normal. During ATP synthesis hydrated ions enter the mouth of the channel and strip off part of their hydration shell at the selectivity filter (only Na^+ ions can pass the filter). If hydrophobic organotin compounds are present, they accumulate within the membrane and easily penetrate into the entrance of the channel. Here, they interact with a site near the selectivity filter, which disables incoming ions to shed their hydration shell. As a consequence, the ions do not proceed through the channel and ATP synthesis is blocked.

1. 10. 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-I 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).

1. 11. 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

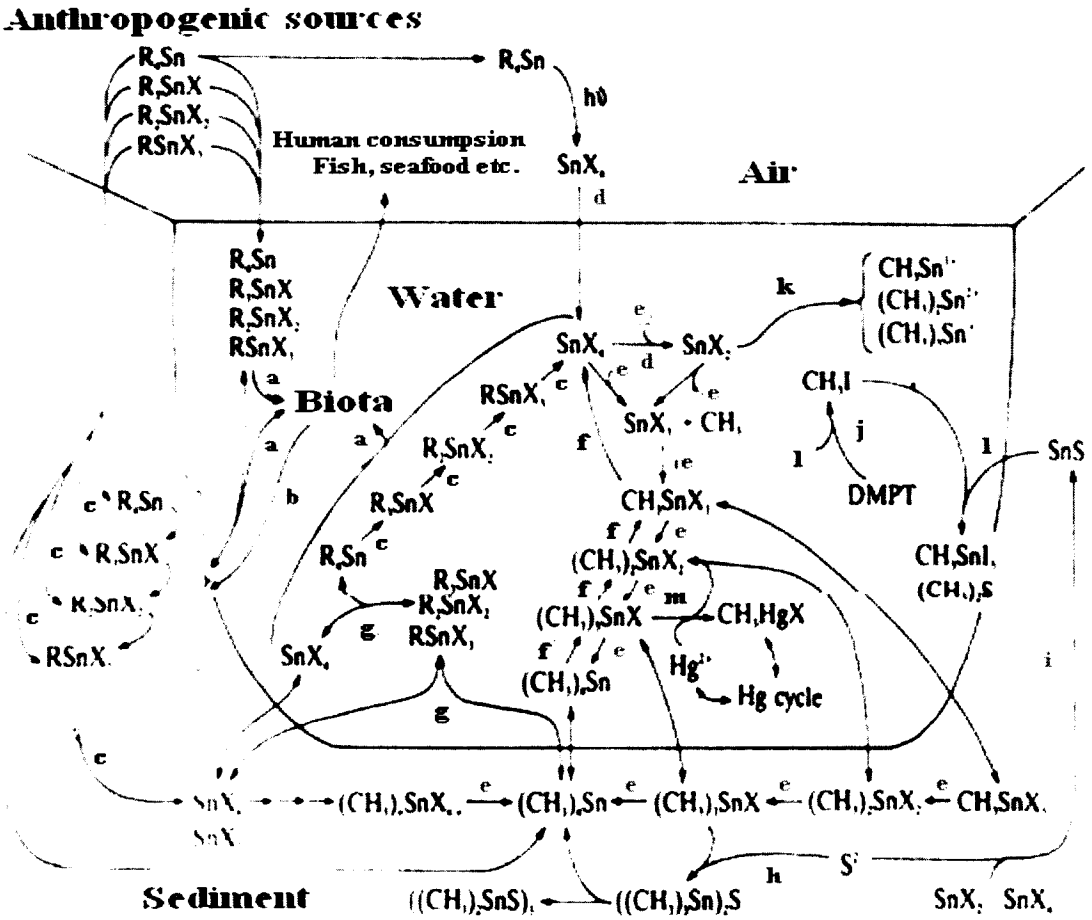
been shown to be mediated by microorganisms, information is still severely limited in relation to mechanism of degradation, tolerance mechanism of microbes and their relative significance and also the role of anionic radicals in degradation process in natural habitats (Cooney, 1988; Gadd, 1993; 2000). 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. 11. 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.1.4). 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: (i) the surface microlayer (ii) the water column and (iii) the sediment (Clark et al., 1988). In aquatic environment tributyltin and other organotins accumulate on the surface microlayer, in sediments, and also on suspended particulates. Binding of tin compounds to sediments varies greatly with the type and nature of the sediments and tin species and influenced by salinity, pH, and amount of particulate matter (Cooney, 1988). The bio-availability of organotins to microorganisms is a key determinant for its uptake, bioaccumulation and toxicity, which depends on the chemical speciation of organotin in aquatic ecosystem (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 > 89 days (Wuertz et al., 1991). Half life of TBT from a clean water site (0.03 µg/L of TBT) was 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 monobutyltin. Complete mineralization of TBT measured by the formation of $^{14}CO_2$, preceded slowly with a half-life of 50-75 days. 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 bioavailable (Evans, 1999). Interaction of microorganisms with organotins is significantly influenced by environmental conditions. In aquatic ecosystem, both pH and salinity can determine organotin speciation / bioavailability and hence, 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 response of the organisms which includes changes in intracellular compatible solute and membrane composition (Cooney et al., 1989). These environmental factors may also alter selectively the resistance of microorganisms in polluted aquatic ecosystems (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 surface water is about a week, whereas in sediments it takes 2.5

years (Atireklap et al., 1997). This clearly indicates that sorption of TBT in the silty sediments strongly reduced the bioavailability of this organotin 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).



- This scheme was constructed from a number of sources including Thayer (1984), Blunden and Chapman (1988 a), Brinckman and Olson (1988) and reproduced from Gadd (1993), with permission.

Fig.1.4 A model for the biogeochemical cycling of organotins. Tributyltin compounds are included in the nomenclature R_3SnX. 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; 9d) 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 reaction of dimethyl propiothetin (DMPT) with aqueous iodide; (k) CH_3I methylation of SnX2; and (m) transmethylation reactions between organotins and mercury.

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 radiation, 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 centimeters 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 (Fig. 1.5).

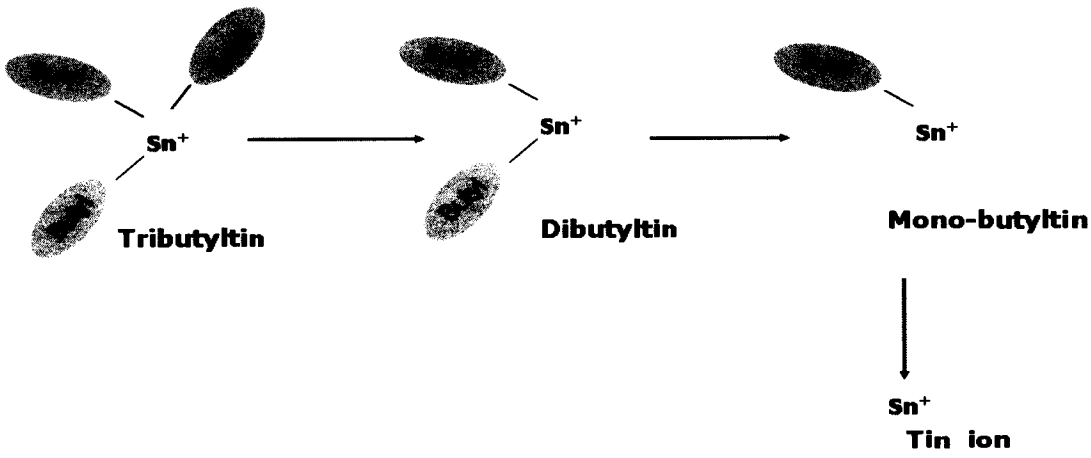


Fig. 1.5 Schematic diagram showing degradation of TBTCl.

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 butyl methyltin 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) $Bu_3Sn^+ + Me^- \rightarrow Bu_3MeSn$

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 TBTCl 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.

Samuel et al., (1998) suggested that single stranded DNA and dibenzyltin dichloride (phenanthroline) complex indeed hydrolyse the Sn-Cl bond in appreciable amounts at physiological pH i.e. 6.0. 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 Chesapeake 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.

Another study at Brest Naval harbor 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 of sea water i.e., ~ 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 vary in freshwater and marine environments and the level of Na^+ or Cl^- ions is considered in toxicity studies (White et al., 1999).

Influence of media constituents on apparent organotin toxicity to microorganisms has been previously reviewed (Cooney and Wuertz, 1989). Jonas et al., (1984) have reported that the media composition can alter the physico-chemical equilibrium of the metal species compared to natural water. 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).

1. 11. 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, Alcaligenes 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 few *Pseudomonas* sp. have 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).At concentrations of 1 µg/L or less, TBT can be toxic to many marine organisms, including algae. Some types of microalgae can tolerate a TBT

concentration of 25 µg/L and degrade TBT to dibutyltin (DBT), butyltin (MBT) and inorganic tin. It is interesting to note that only one pathway in microalgae for TBTCl degradation has been reported so far (Lee et al., 1989). β-hydroxylation is predominant during the degradation of TBT, however other types of hydroxylation are also possible (Fig. 1.6).

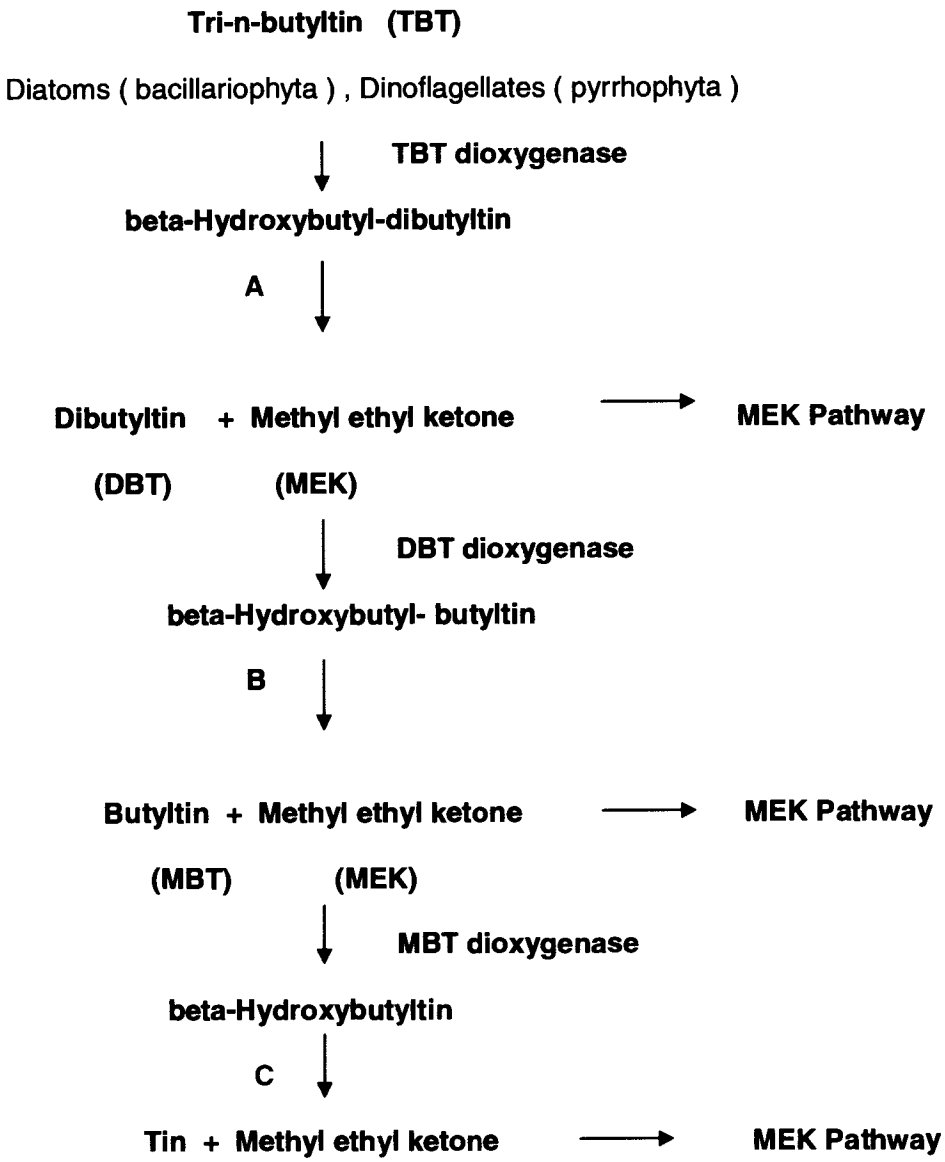


Fig. 1.6 Tri-n-butyltin Degradation Pathway Map

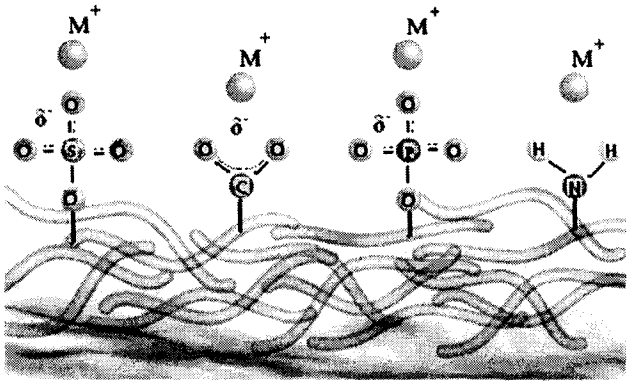
1.14. Biochemical basis of tributyltin resistance in bacteria

Microorganisms have various inherent mechanisms to withstand stresses viz. oxidative, heavy metal and organometal, xenobiotic, osmotic (salinity) and physical (temperature and pH). Microbial cells show enhanced production/ secretion of various biomolecules viz. exopolysaccharides and metallothionein like proteins in response to stress.

1.14.1. Microbial 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 conditions and they greatly extend the range of available polymers because of their unique properties (Ashtaputre et al., 1995b). Bacteria have devised complex regulatory mechanisms 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 hydrocarbon polluted sea water. This fact suggests that EPS produced by *Rhodococcus rhodochrous* S-2 could be useful for the bioremediation of spilled oil in marine environments, and especially for the bioremediation of polyaromatic hydrocarbons that remain in the environment even after a traditional chemical 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 (Fig. 1.7). 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 metabolize and reproduce more efficiently. Microbial exopolysaccharides 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.



(Source : Braissant et al., 2007)

Fig. 1.7 Diagram showing the functional groups commonly associated with exopolymeric substances (EPS), and their possible interactions with metals (M+).

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 secrete exopolysaccharide which acquire resistance to the antibiotic, bacitracin by stopping synthesis of their exopolysaccharide (Pollock et al., 1994). Most of the EPS produced by bacteria which show

metal binding are from the capsule or slime layers. The majority of these exopolymers are composed of polysaccharides, 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). So far there is no report on TBTCl induced exopolysaccharide production in bacteria.

1.14.2. Synthesis of microbial proteins in response to toxic compounds

It is well understood that heavy metal induced specific polypeptides play a very important role in metal ion homeostasis in cyanobacteria (Olafson et al., 1979). Some bacterial strains are also known to synthesize cystein rich low molecular weight polypeptides which play an important role in biosorption of these metals and ultimately resulting in immobilization of toxic metals thereby, protecting their vital metabolic processes catalysed by enzymes (Gadd, 2000; Higham et al., 1984). The periplasmic space is involved in various biochemical pathways including nutrient acquisition, synthesis of peptidoglycan, electron transport, and alteration of substances toxic to the cell. In *E.coli* and *S. typhimurium*, periplasmic proteins are involved in transport and chemotaxis (Miller et al., 1983). Certain heavy metal tolerant bacteria such as *Pseudomonas putida* and *Vibrio alginolyticus* exhibit metal induced synthesis of low molecular weight, cysteine rich polypeptides (methallothioneins) 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, 1995 ; 1998).

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 TBTCl resistant bacteria, *Vibrio* M1 isolated from natural marine environment showed

interesting properties when grown with TBTCl. It was found that in the mid log 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 TBTCl, although the function of the polypeptides are not known definitely. Preliminarily studies have shown that both polypeptides were detected in 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).

1. 15. Molecular basis of organotin resistance in bacteria

Molecular 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 TBTCl 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. 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 16S rRNA 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 encoding genes on chromosomal genome (Fukagawa and Suzuki, 1993).

One of the most interesting reports on molecular mechanism defining bacterial resistance to TBTCl is on isolated from a TBT- polluted harbor in France. In *Pseudomonas stutzeri* strain 5MP1, TBT resistance was found to be associated with the *tbtABM* operon, which is homologous to the resistance – nodulation – cell division (RND) efflux pump family (Jude et al., 2004). TbtABM operon exhibited the greatest homology with the TtgDEF and SrpABC operons, involved in aromatic compound resistance in *Pseudomonas putida*. TbtABM operon also conferred multidrug resistance (MDR) to nalidixic acid, chloramphenicol, and sulfamethoxazole. This is the first report of a MDR (drug efflux pump) found in *Pseudomonas stutzeri* (Fig. 1.8).

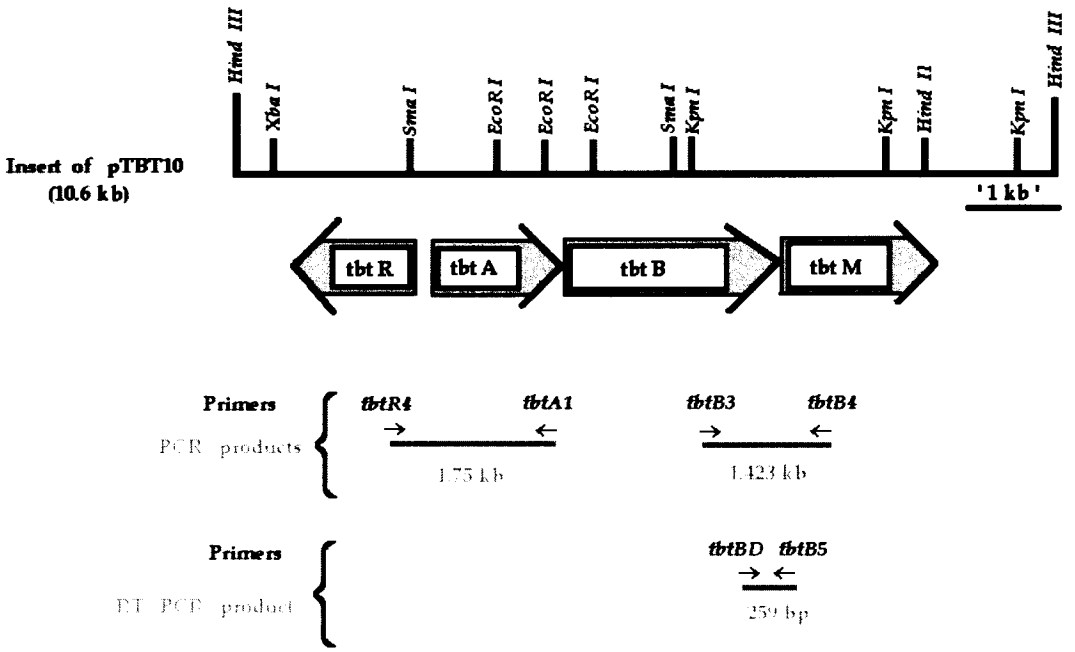
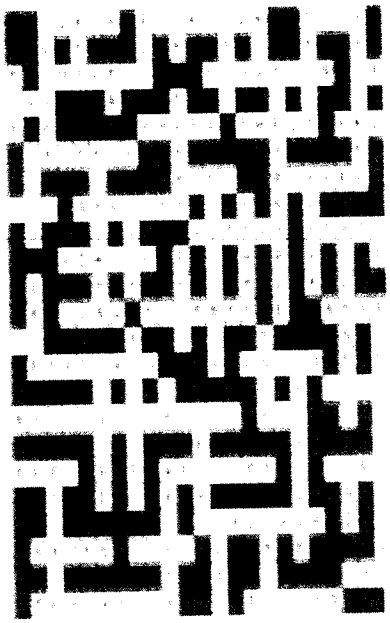
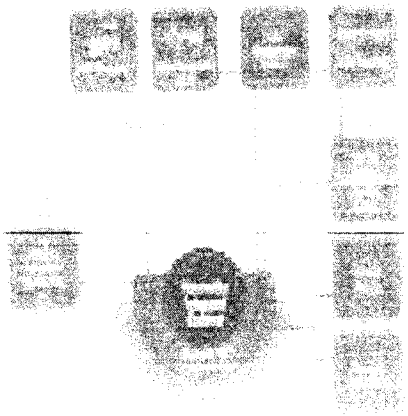
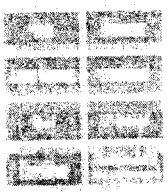


Fig. 1.8 Partial restriction map of the Hind III insert carrying the tbt ABM operon of *Pseudomonas stutzeri*.





AIMS & OBJECTIVES



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 TBTCl resistant bacterial strains such as *Escherichia coli, Pseudomonas fluorescens, P. aeruginosa, Proteus mirabilis, Serratia marcescens* as Gram negative and *Staphylococcus 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).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 marine sediment bacteria) in biodegradation process as they get naturally enriched in organotin contaminated marine environment (Fukagawa et al. 1994).

The Environmental Protection Agency (EPA), U.S.A. issued a – 'Ambient water quality criteria document for tributyltin (TBT)' on August 7, 1997 which states that the permissible **chronic level of TBT in aquatic environment should not exceed 0.001 ug/l or 1ng/l.**

EPA has recently updated its Environmental Risk Characterization (ERC) data for TBT, considering the following attributes:

- TBT is an **immunosuppressing agent** and an **endocrine disruptor**.
- TBT **biomagnifies through the food chain** and has been found in tissues of marine mammals.
- TBT **causes adverse reproductive and developmental effects** in aquatic organisms at very low concentrations.
- TBT **degrades much more slowly in sediment** and persists in sediments at concentrations which cause adverse biological effects.

It has been reported that the total butyltins from the Dona Paula Bay, West coast of India in the surface waters was 21 to 89 ng/l, biofilm samples 10 to 822 ng/g and tissues of marine organisms 58 and 825 ng/g dry wt (Bhosle et al.,2004) which is almost **20 to 800 times more than the chronic criterion issued by the EPA**.

One of the main industries in Goa is the Shipping industry and aquatic organisms (fish, shrimps, Shell fish, oysters etc.) are the most common seafoods consumed by the people in this state, hence the chances of contamination and biomagnification of TBT is high in this region and other coastal cities in India.

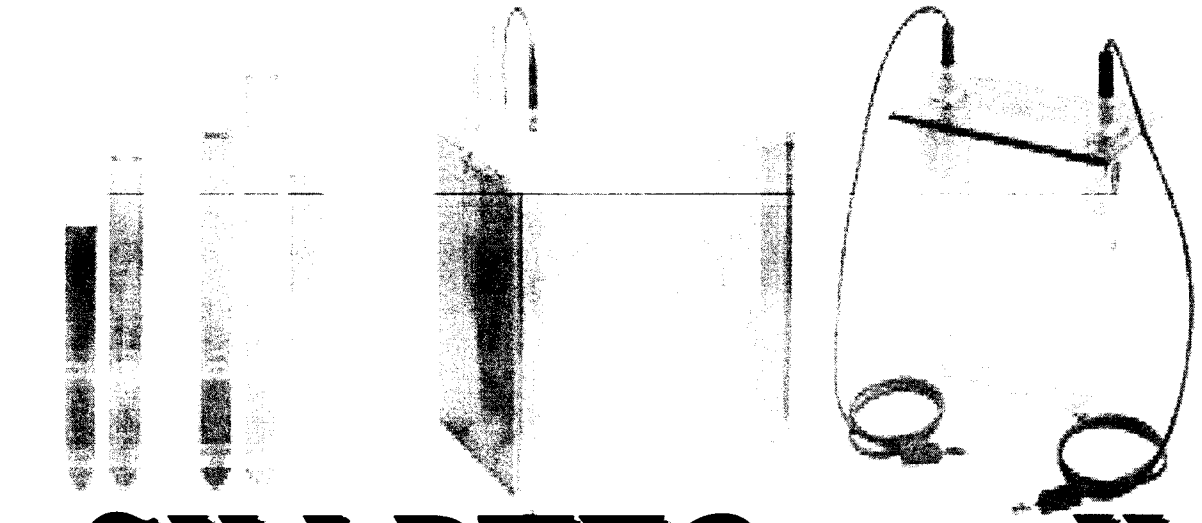
The Goa Shipyard Ltd., (GSL) is a prominent site for TBTCl contamination in the west coast of India, due to regular ship building activity and other services. In a previous work (Krishnamurthy et al., unpublished data); the sediment samples from GSL were screened for TBTCl tolerant bacteria. Five potential bacterial strains that could tolerate

very high concentrations of TBTCl were isolated. One of these isolates was chosen for further characterization.

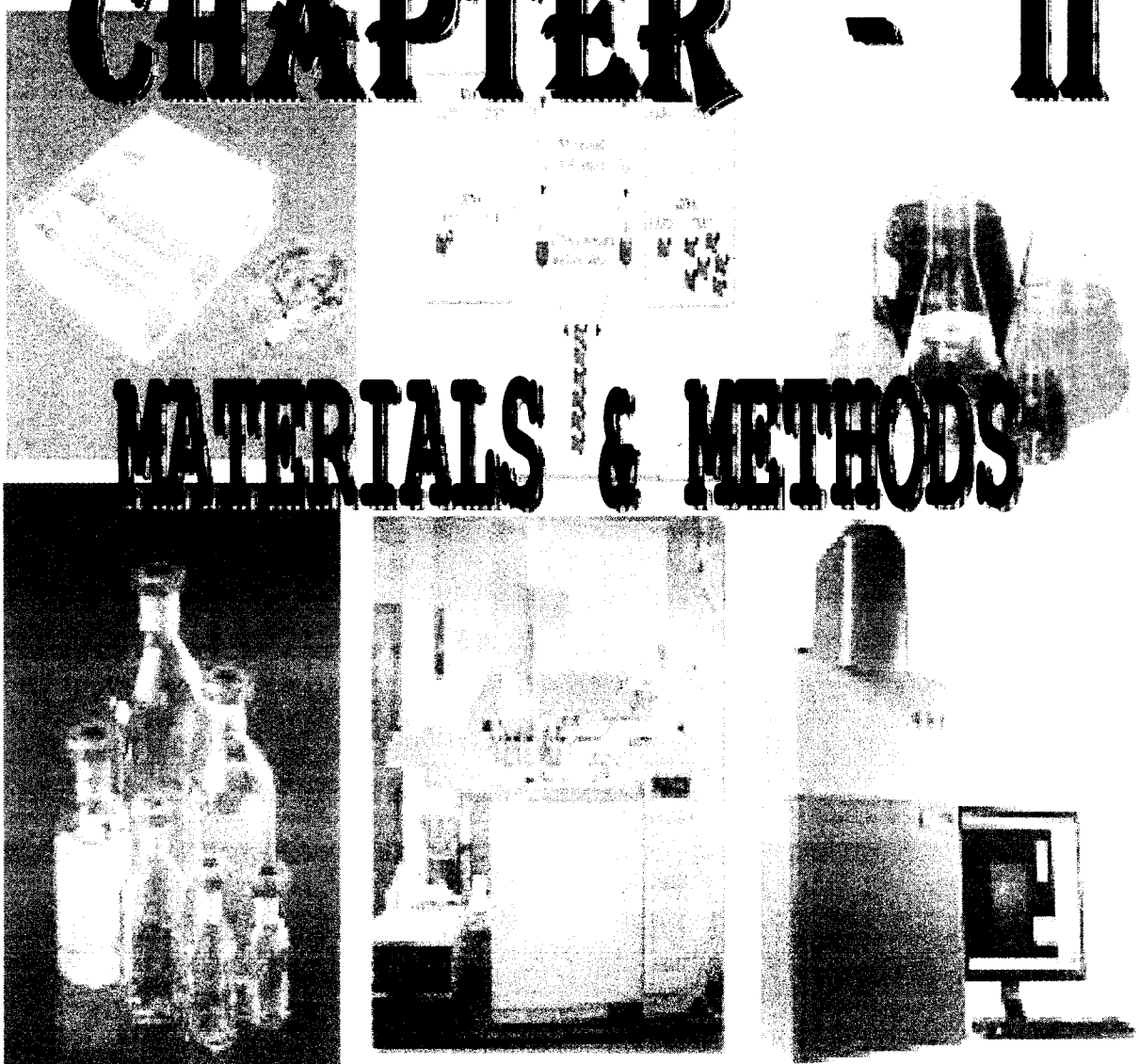
The studies on TBT tolerant bacteria from these contaminated ship building sites from west coast of India would enable us to understand the mechanisms of degradation and employ natural as well as genetically modified bacterial strains in the task of organotin (TBTCl) bioremediation.

The objectives of the present study

- Morphological, biochemical and molecular (16S rDNA) characterization of the TBTCl resistant bacterial isolate.
- Physiological and Biochemical characterization of the isolate with respect to growth behavior, exopolysaccharide (EPS) production, TBTCl tolerance limit and protein profile under TBTCl stress.
- Molecular and Genetic characterization with respect to plasmid profile, localization of TBTCl resistance genes on plasmid or chromosomal genome (plasmid curing and transformation experiments).
- Molecular cloning of TBTCl resistance genes from plasmid / genomic DNA in *E.coli* host using a suitable cloning vector.
- Screening of TBTCl resistant clones from plasmid / genomic library made in *E.coli*.
- Characterization of TBTCl resistance clones with reference to insert size and growth in the presence of TBTCl.



CHAPTER - II



MATERIALS & METHODS

2.1. Identification of the isolate by morphological, biochemical and molecular characterization

Morphological (colony morphology and gram character) and biochemical characteristics of the isolate were determined following the methods by Cruickshank et al. (1972) (Appendix B and C) and *Bergey's Manual of Systematic Bacteriology* (Krieg, and Holt 1984). Identification of the isolate was further confirmed by 16S rDNA sequencing followed by BLAST search (Altschul et al., 1990). Partial 16S rDNA amplicon (~ 1 Kbps) of the isolate was PCR amplified using a single set of oligonucleotide primers to the universally conserved sequences at the 5' and 3' termini of the eubacterial gene encoding 16S rRNA. The following eubacterial primers were used for PCR amplification :

forward (17 mer) [f 341]: CCT ACG GGA GGC AGC AG

reverse (20 mer) [r 1387]: GCC CGG GAA CGT ATT CAC CG

The isolate was maintained on Mineral Salts Medium (MSM) supplemented with 2 mM TBTCl (Appendix A.1 and F.1).

2.2. TBTCl tolerance limit and growth behaviour of the bacterial isolate

The bacterial isolate, grown overnight (18 hrs) in MSM supplemented with 0.1% glucose was used as inoculum for all growth studies carried out. The tolerance limits of the isolate were determined in MSM using different concentrations of TBTCl (1 – 5 mM). The culture flasks were maintained at 28 °C and 180 rpm on a rotary shaker and growth was monitored after 24 hrs in terms of absorbance at 600 nm and as total proteins following Lowry's method (Lowry et al., 1951). Similarly, the TBTCl tolerance limit in ZMB supplemented with different concentrations of TBTCl was also determined. Cells grown in ZMB only served as a control (Appendix A.4).

The growth behaviour of the isolate in response to TBTCl was studied by inoculating an overnight grown culture in MSM as well as ZMB supplemented with optimal concentration of TBTCl (2 mM) separately. The flasks were incubated at 28°C,

180 rpm upto 72 hrs and growth was monitored as absorbance at 600 nm periodically with an interval of 4 hrs.

2.3. Determination of environmental optimas for growth (pH, salinity and temperature)

(i) pH : In order to determine pH optima of the bacterial strain, overnight grown culture was inoculated to a final concentration of 5% in MSM containing 2mM TBTCl. These flasks were separately adjusted to three different pH values i.e. 4.0, 7.0 and 9.0 before inoculation and were incubated on an incubator-shaker for 72 hrs at 28 °C. Growth was monitored periodically as absorbance at 600nm and the result was recorded as a graph of growth v/s time at different pH separately.

As a control the isolate was grown in the presence of glucose (0.1%) in MSM at pH 4, 7 and 9 separately. The flasks were incubated at 28°C, 180 rpm upto 72 hrs. The growth was monitored as absorbance at 600 nm periodically with an interval of 4 hrs.

(ii) Salinity (% NaCl) : The overnight grown isolate was inoculated in MSM supplemented with 2 mM TBTCl with increasing concentration of NaCl viz. 0.5%, 1.0%, 1.5%, 2.0%, 2.5% and 3.0% (w/v) separately and incubated for 24 hrs at 28 °C. Growth was determined periodically as absorbance at 600nm. Optimal concentration of NaCl was determined from the bar diagram considering growth v/s salinity. Cells grown in MSM with glucose (0.1%) supplemented with NaCl concentrations as above were taken as the control.

(iii) **Temperature:** Culture flasks containing MSM supplemented with 2mM TBTCl were incubated 48 hrs at three different temperatures i.e. 28 °C, 37 °C and 42 °C separately. Growth was determined in terms of absorbance at 600nm and also as total protein content / ml using Lowry's method (Lowry et al., 1951) (Appendix D.1).

2.4. Comparison of growth in media supplemented with TBTCl, ethanol, butanol, and glucose as sole carbon sources

Optimal levels of four different carbon sources, viz. glucose, ethanol, butanol and TBTCl was determined by culturing the isolate in different concentrations of glucose (0.05%, 0.1%, 0.2%, 0.3% & 0.4 % w/v), ethanol (0.01%, 0.07% & 0.14%), butanol (0.10 %, 0.30%, 0.50%, 0.70%, 0.90% & 1%) and TBTCl (1 mM, 2 mM, 3 mM & 4 mM v/v) in MSM. The growth was monitored as absorbance at 600 nm as well as total protein content after 24 hrs of incubation at 28°C.

Optimal concentrations of glucose (0.2% w/v), ethanol (0.14%), butanol (0.50%) and TBTCl (2mM), were used separately as supplements in MSM and growth of the isolate was monitored as absorbance at 600 nm upto 72 hrs to study its growth behaviour.

2.5. Exopolysaccharide (EPS) production

2.5.1. Determination of optimal concentration of TBTCl for EPS production

Overnight grown cells were inoculated (5%) in flasks containing MSM with different concentrations of TBTCl (1 mM, 2 mM, 3 mM, 4 mM & 5 mM respectively). The flasks were maintained at 180 rpm at 28 °C for 48 hrs. The growth was measured as absorbance at 600 nm and the exopolysaccharide produced was estimated as total carbohydrates (Dubois et al, 1956) (Appendix D.2).

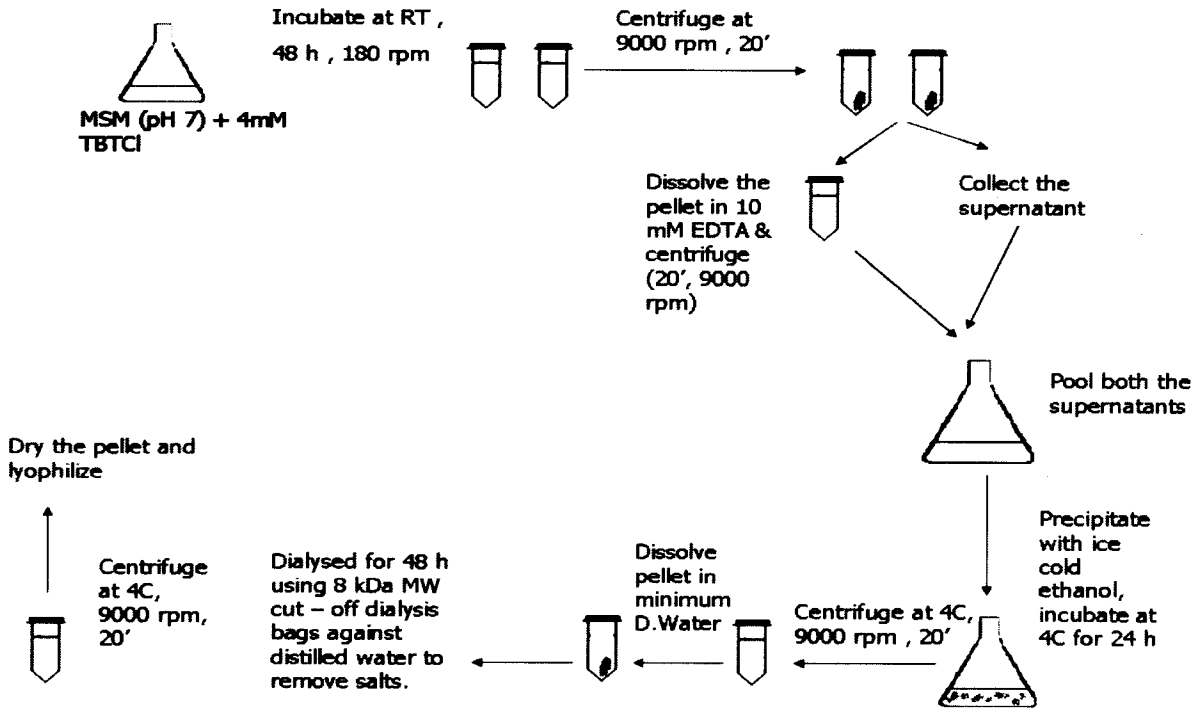
2.5.2. Growth pattern and EPS production in the presence of TBTCl as the sole carbon source.

In order to determine the ideal growth phase for the production of highest amount of EPS, overnight grown cells were inoculated in flasks containing MSM with optimal TBTCl (4mM) for highest production of EPS. The flask was incubated at room temperature for 72 hrs at 180 rpm. 5 ml of the sample was withdrawn and growth was measured as absorbance at 600 nm and the EPS was estimated by phenol-sulphuric acid method periodically.

2.5.3. Bulk production of EPS in the presence of TBTCl as the sole carbon source.

Pure culture of *Alcaligenes* sp. was grown in 2 L flasks containing 1 L of MSM with 4mM of TBTCl. The pH of the medium was adjusted to 7. The culture was grown at 28 °C for 48 hrs. At the end of the incubation period, the cells were harvested by centrifugation at 10,000 rpm for 30 mins and the supernatant was collected. The cell pellet was treated with 10mM EDTA to extract cell - bound EPS. The EDTA – extracted EPS was pooled with the supernatant EPS solution and filtered through 0.22 µm filters concentrated at 35 °C to less than 50 ml using a rotary evaporator. The concentrated EPS was then precipitated using ice – cold absolute ethanol while continuously stirring and kept at 4 °C overnight. The supernatant was decanted; the precipitate was redissolved in minimum volume of distilled water and dialysed for 48 hrs using 8 kDa MW cut – off dialysis bags against distilled water to remove salts. During dialysis, the water was changed every 10 hrs. The high molecular weight EPS retained in the dialysis bag was then reprecipitated, lyophilised and stored at -20 °C until further analysis (Fig. 2.1).

Fig. 2.1 Schematic digram showing bulk production of EPS.



2.5.4. Wet weight/Dry weight

The wet weight and dry weight of the EPS produced was weighed for both the samples.

2.5.5. Bulk characterization of EPS

The EPS of *Alcaligenes* sp. was analysed for its bulk chemical characteristics like total carbohydrates (TCHO), proteins, uronic acid (UA) and sulphates.

Carbohydrates (TCHO): TCHO concentrations were estimated following the phenol – sulphuric acid method (Dubois et al 1956) with a slight modification. A subsample of lyophilised EPS was weight and dissolved in appropriate volume of deionized water. If the sample were in the form of clear solution EPS sample was directly used for TCHO estimation. 1ml of EPS solution was mixed with 1ml solution of cold 5 % phenol and 5 ml of concentrated sulphuric acid was added to develop colour. The intensity of the colour was measured spectrophotometrically at 490 nm. Glucose was used as the standard for calibration of method.

Proteins: the protein content of the EPS was estimated following Smith et al., (1993). A known amount of EPS was weighed, and dissolved in appropriate volume of distilled water and used directly for protein estimation. In order to analyse the protein content of the EPS, 100µl of the EPS sample was transferred into clean test tubes and 2ml of bicinchoninic acid (BCA) reagent was added to it. The samples were then incubated at 37 °C for 30min to complete the reaction and develop the colour. The intensity of the colour formed was used as standard for the calibration of method.

Uronic acid : UA content in the EPS was measured by following the modified meta-hydroxyl biphenyl method of Filisetti – cozzi and Nicholas (1993). The modified method remains the same as the old meta hydroxyl biphenyl method (Blumenkrantz and Asboe 1973) except for the pre-treatment with sulphamic acid. A known amount of EPS was dissolved in deioniozed water and 0.4ml was taken in a clean test tube. To this 40 ul of 4 M sulphamic acid was added and mixed thoroughly. After mixing properly, 2.4 ml of

sulphuric acid – tetra borate solution was added and vortex mixed and the colour developed was measured at 525nm. Galacturonic acid was used as the standard for calibration of method.

Sulphate estimation : Sulphate in the EPS were estimated following $BaCl_2$ – gelatine method (Dodgson and Price 1963). A known amount of EPS was dissolved in deioniozed water and 1 -2 ml of the sample was transferred into glass ampoule and dried under the flow of nitrogen gas. To this ampoule, 1ml of 1N HCl was added and the sample was flushed with Nitrogen. There after, the ampoule was sealed and the sample was hydrolysed at 105 C for 17 h. After hydrolysis the sample was cooled and the content was transferred to test tube containing 3.8 ml of 3% trichloroacetic acid (TCA) solution. To this mixture, 1 ml of $BaCl_2$ – Gelatin reagent was added and mixed thoroughly. The solution was kept at room temperature for 15 -20 min and the turbidity formed was measured at 360nm. Potassium sulphate dissolved in 1N HCl was used as a standard for calibration.

In order to estimate blanks in all the above estimations, deionized water was used in place of samples and analysed.

2.5.6. Fourier Transformed Infrared (FTIR) analysis of EPS

The major structural groups of the purified EPS were detected using Fourier transformed infrared (FTIR) (Abu et al., 1991). Pellets for infrared analysis were obtained by grinding a mixture of 2 mg polysaccharide with 200 mg dry KBr, followed by pressing the mixture into a 16-mm diameter mold. The Fourier transform-infrared (FT-IR) spectra were recorded on a SHIMADZU- FTIR 8201PC instrument (Shimadzu, Japan) in the 4000–400 cm-1 region and spectra traced with a Hewlett Packard plotter.

2.5.7. Emulsifying activity of the exopolysaccharide

Lyophilized EPS (0.5 mg) was dissolved in 0.5 ml deionized water by heating at 100 °C for about 15-20 min and allowed to cool to room temperature. The volume was then

made to 2ml using phosphate buffered saline (PBS). The sample mixtures were vigorously vortexed for 1 min after addition of various hydrophobic substrates: diesel and crude oil supplied by the Indian Oil Crop. Ltd; and xylene, toluene (by Qualigens Chemical Comp, Mumbai, India.) and TBTCl (Merck, Germany). Samples were incubated at 30°C with reciprocal shaking speed of 180 rpm. The absorbance at 540 nm was read immediately before and after vortexing (A_0). The fall in absorbance was recorded after incubation at room temperature for 30 and 60 min (A_t). a control was run simultaneously with 2 ml PBS and 0.5 ml hexadecane. The emulsification activity was expressed as the percentage retention of emulsion during incubation for time *t*: A_t / A_0 x 100.

2.5.8. Analytical gas chromatography of EPS

Alditol acetate derivatives of the monosaccharide sugars released by hydrolysis from lyophilized EPS were analysed by a capillary gas chromatography (Perkin – Elmer model 8310; Wellesley, M.A, USA) equipped with a fused silica capillary column coated with CP Sil-88. A flame ionization detector was used to separate the aditol acetate mixture, and oxygen-free dry helium was used as carrier gas at a flow rate of 25-30 ml min^{-1}. The resulting methyl glycosides were converted to their trimethylsilyl derivatives. The gas chromatogram oven temperature was initially programmed at 70 °C and then rapidly raised to 150 °C after the sample was injected. The final analysis temperature was set at 230 °C for 40 min using inositol (1ml min^{-1}) as the internal standard.

2.6. Protein profile of the isolate under TBTCl stress

2.6.1. Whole cell protein profile

Whole cell lysates were prepared by a method adapted from Hitchock and Brown (1983). Briefly, a colony was grown in MSM with 2mM TBTCl for 24 h at 37°C; cells were harvested by centrifugation (3000 rpm x 5 min) at room temperature, washed at least three times with, and resuspend in, 10 ml of PBS (Appendix B.2.i) Cells from 1.5 ml of

the washed culture were harvested in a microfuge tube, resuspended in 100 µl of single strength SDS-PAGE sample lysis buffer (Appendix E.1) and heated at 100°C for 10 min. The lysed samples were then centrifuged (5000 rpm x 10 min) at room temperature (RT), and 10 µl of the filtrates were loaded on a SDS-12% PAGE gel and electrophoresed using Tris-Glycine-SDS buffer system (Appendix E.1) developed by Laemmli (1970). Gels were silver stained by the method by Sambrook et al.(1989).

2.6.2. Periplasmic protein profile

Periplasmic proteins were released by osmotic shock treatment based on the method of Nossal and Heppel (1966) with modifications. Briefly, bacteria were grown in 25 ml of MSM at 37°C for 24 h with shaking; cells were harvested by centrifugation (8000 rpm x 30 min x 4°C) and washed two times with PBS (pH 7.2). The washed cell pellet was resuspended in 0.5 ml of fractionation buffer (Appendix B.2) and incubated on ice for 10 min. Cells were then harvested (5000 rpm x 10 min x 4°C); the pellet was re-suspended in 0.5 ml ice-cold 5 mM $MgSO_4$ with shaking at room temperature for 10 min, and the supernatant containing periplasmic proteins was collected by centrifugation (5000 rpm x 10 min x 4°C). Both the supernatant (after extraction with the fractionation buffer) and the periplasmic fractions from each of the isolates were mixed with equal volumes of double strength SDS-PAGE lysis buffer, boiled (100°C, 5 min) and analyzed on 12% SDS-PAGE (Appendix E.1).

2.6.3. Growth versus protein expression

The isolate was inoculated in sterile MSM broth supplemented with 2mM TBTCl. The flask was incubated at 28°C. Samples were drawn at 6 hr, 12 hr, 18 hr and 24 hr of interval and the total periplasmic proteins were isolated. The samples were prepared as above and run on 12% SDS-PAGE gel.

2.6.4. Preparation of SDS-PAGE gels (Laemmli et al.,1970)

The glass plates after washing and drying were wiped with acetone and clamped together with spacers in place. The assembly was sealed using molten 1% agar. All the reagents (Appendix E.1) used for separating gel were taken in a clean flask and mixed well. APS and TEMED were added, 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. Isobutanol was added above the resolving gel to get a uniform surface. The isobutanol was drained out and the surface of the gel was washed with distilled water to remove any traces of unpolymerized mix or isobutanol. Similarly, stacking gel was cast over the resolving gel. A comb was inserted into the stacking gel and allowed to set. 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.

2.6.5. Staining of the gel :

Commassie Blue staining :

The gel was placed in staining solution for 6-8 hrs. It was then destained in solution I for 1 h and in solution II (Appendix E.1) until clear bands appeared. Gel was washed with deionised water and stored in the same.

2.7. Molecular and genetic characterization of the isolate

2.7.1. Plasmid profile

Extraction of plasmid DNA was carried out following 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 microfuge tube and was centrifuged at 8,000 rpm for 5 mins at 4°C. The supernatant was discarded leaving the

bacterial pellet as dry as possible. The pellet was suspended in 100 µL of ice-cold glucose tris - EDTA buffer, freshly prepared SDS buffer (200 µL) was subsequently added and the contents were mixed by inverting the tube gently. The microfuge tube was incubated on ice for 10 mins followed by addition of 150 µL of Potassium acetate and the contents were gently mixed by inversion. The tubes were centrifuged at 11,000 rpm for 5 mins at 4°C. The supernatant was transferred to a fresh microfuge tube and the plasmid DNA was precipitated with double volume of the ice-cold ethanol. The contents were mixed gently and allowed to stand for 1 h on ice. The tube was centrifuged at 12,000 rpm for 5 mins at 4°C. The supernatant was decanted and the tube was inverted on a paper towel, to drain all the ethanol. The pellet was dissolved in 70% (v/v) chilled ethanol and centrifuged at 12,000 rpm for 5 mins at 4°C. The supernatant was discarded and the pellet was air dried and then dissolved in 20 µL of TE buffer containing DNase free RNase (20 µg/mL) (Appendix E.2.3). Plasmid DNA was stored at -20°C.

2.7.2. Agarose Gel Electrophoresis of plasmid DNA (Sambrook et al)

2.7.2.a. Preparation of agarose gel : Agarose gel 0.8% (w/v) was prepared in 1 x -TAE buffer (pH 8.0) (Appendix E.2). To the molten agarose (50 mL), 1 µL of Ethidium bromide (10 mg/ mL) was added to get final concentration of approximately 4 µg/ mL, and allowed to set at room temperature (Appendix E.2). The casting tray containing agarose gel was placed in the horizontal electrophoresis chamber filled with 1 x TAE buffer and the combs were removed.

2.7.2.b. Loading and electrophoresis of DNA sample:

DNA sample (10 µL), mixed with 2 µL of loading dye was loaded in the well of agarose gel using pipetteman. Appropriate molecular weight markers were loaded in parallel with samples (GENEI) was used (Sambrook et al. 1989; Ausubel et al. 1992).

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 70 V and the electrophoresis was carried out at constant voltage for 2 hrs.

2.7.2.c. Visualization of DNA

After electrophoresis, the gel was observed on a UV photodyne transilluminator and the photograph of the ethidium bromide stained bands in the gel was captured using a Gel documentation system (Vilber - Lourmat, France).

2.8. Localization of TBTCl resistance genes on plasmid or chromosomal genome(plasmid curing)

2.8.1. Selection of ideal curing agent.

Three curing agents acridine orange, novobiocin and sodium dodecyl sulphate were used separately to cure the plasmid. An overnight grown culture was inoculated in flasks containing different concentrations of novobiocin (20 – 200 µg/ml), SDS (5 – 20 %) and acridine orange (20 – 200 µg / ml) (Appendix F.3.). The flasks were incubated for 48 hrs at room temperature at 180 rpm. The culture flasks containing acridine orange were covered with aluminium foil. Growth was measured in terms of absorbance at 600 nm. 50 µl of the sample from each flask showing sublethal (near killing) level of the curing agent was spread plated on Zobell Marine Agar (ZMA) plates and incubated at room temperature for 24 hrs.

2.8. 2. Screening and confirmation of cured cells

The colonies obtained after treating with the three curing agents were screened for the presence of plasmids by alkaline lysis method. The plasmid cured colonies were maintained on separately on MSM agar plates containing 0.2% glucose.

2.8. 3. Confirmation of genomic DNA mediated TBTCl tolerance

In order to confirm if the TBTCl tolerance of the isolate was genomic DNA mediated, overnight grown uncured cells and acridine orange cured cells were inoculated separately in flasks containing:

(i) MSM with 2 mM TBTCl

(ii) MSM with 0.2 % glucose

(iii) ZMB with 2 mM TBTCl, and

(iv) Only ZMB

The flasks were kept at 28 °C at 180 rpm for 60 hrs and growth was monitored as absorbance at 600 nm periodically.

2.9. Molecular cloning of TBTCl resistance genes from the isolate

Preparation of genomic DNA library of the isolate by Shotgun cloning

2.9.1. Bulk preparation of genomic DNA

Overnight grown cells were inoculated in a 100ml flask containing 50 ml of ZMB and incubated at 28 °C for 18 hrs at 180 rpm. The genomic DNA of the isolate was isolated by PCI method (Sambrook et al., 1989). Concentration of genomic DNA isolated was determined spectrophotometrically at 260 nm (Wilson and Walker, 1990).

2.9.2. Restriction enzyme digestion of genomic DNA

1 ul genomic DNA (0.25 µg / µl) was digested using restriction enzyme Hind III (Sambrook et al., 1989). All the buffers, enzymes and DNA samples were kept on ice in a ice bucket. The reaction mixture was prepared as follows:

	Test	Control
Enzyme	Hind III (1 µl)	Nil
DNA (0.25 µg / µl)	1 µl	1 µl
Reaction buffer (10 x)	1 µl	1 µl
Sterile Milli Q water	7 µl	8 µl

Total volume of reaction mix	10 µl	10 µl

2.9.3. Preparation of Vector DNA

2.9.3.a. Restriction enzyme digestion of the vector DNA

pUC18 plasmid (Bangalore Genei, India) was used as the cloning vector to prepare genomic DNA library of the isolate in *E.coli* HB101. 2 µl of the pUC18 plasmid DNA (approx. 0.25 µg/ µl) was digested with restriction enzyme Hind III as described above. The activity of Hind III was tested by electrophoresing 5 µl of digested pUC 18 DNA.

2.9.3.b. Dephoshorylation of vector DNA

The Hind III digested pUC18 DNA was dephosphorylated using Calf intestinal Alkaline Phoshatase (CIP) (Bangalore Genei, India). The reaction mixture was prepared by combining the following in a 1.5 ml sterile eppendorff tube :

Hind III digested pUC 18 DNA in TE buffer (20 pmol)	-	9 µl
CIP (10 units / µl)	-	2 µl
10 x Alkaline phosphatase buffer	-	5 µl
Sterile Milli Q water	-	34 µl
Total volume	-	50 µl

The microfuge tube containing the reaction mix was incubated at 37 °C for 30 mins. Phenol: Chloroform (1:1) extraction of the DNA sample was done twice using the same volume of the phenol: chloroform. The sample was treated with chloroform (100 µl) and then with 2.5 µl of 3M of ice cold Sodium acetate (final concentration 150mM). The contents were precipitated with chilled ethanol (2.5 x volumes) and incubated at -20 °C

for 30 mins – 1hr. The pellet (dephosphorylated vector DNA) was dissolved in sterile TE buffer (10ul).

2.9.4. Ligation of vector DNA with insert DNA

Dephoshorylated vector DNA and DNA inserts (generated by partial digestion of genomic DNA of the isolate with Hind III) were ligated using T4 DNA ligase (Bangalore Genei, India). The ratio of vector DNA: insert DNA taken was 1:3 in terms of DNA concentration. The ligation mix consisted of the following components:

vector DNA	- 2 µl (1 µg)
insert DNA	- 6 µl (20 µg)
10 x ligation buffer	- 5 µl
10mM Ribo – ATP (pH 7.5)	- 0.5 µl
4 U/ µl T4 DNA ligase	- 0.5 µl

The total volume of ligation mix was made upto 50 µl with sterile Milli Q water.

2.9.5. Preparation of competent cells

E.coli HB101 cells were grown overnight. 2ml of overnight grown cells was inoculated into flask containing LB medium. The cells were grown at 37 °C till the population density reached 6 x 10^8 cells / ml (A_{650} = ~ 0.6). The flask was chilled on ice for 15 min with swirling and then centrifuged at 6000 rpm for 15 min at 4 °C. The cell pellet was washed with 20 ml 0.01 M NaCl (prechilled to 4 °C). The cells were centrifuged at 6000 rpm again for 5 min. The supernatant was discarded. The cell pellet was resuspended in 20 ml of ice cold 0.03 M $CaCl_2$ and incubated on ice for 20 min. The cell pellet was collected after centrifugation at 6000 rpm for 5 min and resuspended in 3 ml of 0.03 M $CaCl_2$ (Appendix F.5).

2.9.6. Transformation experiment (Hanahanan et al., 1993)

The competent cells were thawed on ice and gently mixed by tapping. 100 µl of this was transferred into a pre chilled 15 ml Falcon 2059 polypropylene tube. 1.7 µl of freshly

diluted (1:10) β – mercaptoethanol was added to the 100 µl of competent cells, giving a 25mM final concentration. The tube was swirled gently and kept on ice for 10 min, swirling gently every 2 min. 1-2 µl of recombinant DNA (50ng) was added to the cells and swirled gently. As a control, 1 µl of pUC 18 test insert was added to another 100 µl aliquot of cells and swirled gently. The tubes were incubated on ice for 30 min and heat pulsed in a 42 °C water bath for 45 sec. After the heat shock the tubes were incubated on ice for 2 min. 0.9 ml of preheated (42 °C) SOC medium was added and incubated at 37 °C for 1 h shaking at 225 rpm (Appendix A.6).

2.10. Screening of TBTCl resistant clones

2.10.1. Plating of transformation mix on LB agar plates with Ampicillin

100 µl of the transformation mix was spread plated on LB agar plates containing 50 µg / ml of ampicillin. Plates were incubated at 37 °C for 16 hrs and observed for colonies (transformants). *E.coli* HB101 cells transformed with pUC18 DNA served as the positive control while, the negative control was set by plating *E.coli* HB101 competant cells on LB agar plate with 50 µg / ml ampicillin.

2.10.2. Repilca plating of colonies obtained after initial screening

The transformants obtained were replica plated on:

(i) LB agar plate with 50 µg/ml of ampicillin

(ii) LB agar plate with 50 µg/ml of ampicillin and 5mM TBTCl

The above plates were incubated at 37 °C for 24 hrs except for plates containing 5 mM TBTCl, the incubation time was extended to 72 hrs.

2.11. Characterization of TBTCl resistant clones: (insert size)

The recombinant plasmid DNA of the six clones was isolated by alkaline lysis method. The plasmid DNA from each clone was quantified and used for restriction enzyme digestion with Hind III. The samples were analysed on 0.8% agarose gel. Supermix

DNA ladder and 100 bps DNA ladder were used to determine the DNA insert size. The size of the DNA insert was determined statistically using Vilber – Lourmat software.

2.12. Characterization of TBTCl resistant clones: (growth in presence of TBTCl)

2.12.1. Determination of optimal concentration of TBTCl for growth

In order to determine the TBTCl tolerance limit, overnight grown recombinant clones were inoculated in:

(i) LB broth containing different concentration of TBTCl (0.5 mM, 1 mM, 1.5 mM and 2 mM)

(ii) MSM containing different concentration of TBTCl (0.5 mM, 1 mM, 1.5 mM and 2 mM).

The culture flasks were incubated for 48 hrs at 37 °C at 180 rpm and growth was monitored as absorbance at 600 nm.

2.12.2. Study of growth pattern of recombinant clones in LB and MSM

Overnight grown cultures of the six clones were inoculated in flasks containing only LB broth (control) and in LB broth containing the optimal TBTCl concentration for the respective clones separately. The flasks were incubated at 37 °C at 180 rpm for 72 hrs and growth was monitored periodically as absorbance at 600 nm. Similarly, the growth pattern of the six clones was studied in MSM with the optimal concentration of TBTCl for the respective clones to find out if any of these clones utilize TBTCl as sole carbon source. *E.coli* HB101 inoculated in MSM and LB with TBTCl separately served as a control in this study.

3.1. Morphological, biochemical & molecular identification of the isolate.

As per the Bergey's Manual of Systematic Bacteriology (Kreig and Holt, 1984), the bacterial isolate appeared to be a motile Gram negative rod, positive for oxidase, catalase, citrate, esculin and malonate. However it was negative for sugars fermentation, methyl red test, Vogues - Proskauer test, starch and gelatin hydrolysis (Table 3.1). This isolate was further identified as *Alcaligenes* sp. by 16S rDNA sequencing and NCBI-BLAST homology search (GenBank Accession No - EU401443).

3.2. TBTCl tolerance limit and growth behaviour of *Alcaligenes* sp.

The bacterial isolate was primarily screened on the basis of TBTCl utilization as sole carbon source. Most of the bacterial isolates which were screened previously initially grew on MSM agar supplemented with 2 mM TBTCl, lost their viability on the same medium after repeated subculturing, because organotin compounds inhibit or kill the aquatic microorganisms (Pettibone and Cooney, 1986). Interestingly only few isolates could survive at higher concentration of TBTCl i.e. 2 mM.

Although the marine sediment isolate, *Alcaligenes* sp. could tolerate up to 4 mM TBTCl in MSM, but the optimal concentration for its growth was found to be 2 mM (Fig. 3.1). This concentration has been reported to be highly toxic for other non target organisms (Dubey et al., 2003). It is interesting to mention that an initial lag of 4 - 5 hours was observed when the isolate was grown in MSM with 2mM TBTCl, which could be attributed to the time taken by the isolate to acclimatise to this biocide (Fig. 3.2). A similar response was observed in *Aeromonas* sp. which could tolerate upto 3mM TBTCl (Cruz et al., 2007).

Interestingly, the isolate showed better tolerance and growth in ZMB supplemented with 4mM of TBTCl as compared to its growth in MSM with 4 mM TBTCl (Fig. 3.3). When the isolate was grown in ZMB with 2mM TBTCl no lag phase was observed and it is interesting to mention that the stationary phase was also prolonged as

compared to the cells grown in MSM with TBTCl (Fig. 3.4). Tolerance of the isolate is enhanced in nutrient rich medium (i.e., ZMB) due to availability of yeast extract present in the medium as a preferred carbon source. It has been reported that *E. coli* shows a reduction in lag phase when grown in complex medium compared to any minimal medium like MSM (Singh et al., 1989). The reduction of lag phase observed in complex medium indicated reduction of TBTCl toxicity. This reduction of toxicity could be attributed to complexation of TBT with organic compounds present in the complex growth medium such as tryptone, yeast extract, peptic digest etc. Similar studies conducted earlier have also revealed reduced toxicity of TBTCl in nutrient rich medium (Blair et al., 1982).

It was found that in *Alcaligenes* sp. toxicity of TBTCl was more pronounced in MSM broth as compared to nutrient rich media tested (Fig. 3.3). In case of *E. coli*, toxicity of TBTCl was increased atleast three fold in minimal medium than in complex medium (Singh, 1989).

Growth of *Alcaligenes* sp. in MSM with TBTCl (2 mM) clearly indicates that it utilizes TBTCl as the sole carbon source for its growth and multiplication, since there is no other carbon source in MSM. This medium was therefore selected for further characterization of TBTCl degrading bacterial isolate, *Alcaligenes* sp.

3.3. Optimal temperature for growth

Growth of the isolate determined in terms of total protein content at variable incubation temperatures (28 C, 37 C and 42 C) revealed that 28 °C was optimal as indicated by maximum protein content (i.e., 453 µg / ml) (Fig. 3.5). Therefore, it was evident from this experiment that the isolate grew and utilized 2mM TBTCl better at ambient temperature 28 °C whereas at 42 °C, growth as well as TBTCl utilization declined significantly. *Pseudomonas chlororaphis* also showed its optimum growth and triphenyltin degradation activity at 28 °C (Inoue et al., 2000). On the contrary, Fukagawa et al., (1994) have

reported that 25 °C is the optimum temperature for growth of *Vibrio* sp. in presence of TBTCl and other bacteria respectively. Temperatures lower than 28 °C (i.e. 25 °C), prolonged the incubation period for appearance of colonies on sea agar plates containing TBTCl (Callow and Willingham, 1996).

3.4. Optimal pH for growth

The bioavailability of organotin depends on the pH and the content of organic matter (Fent, 2003). The ideal pH for growth of the bacterial isolate without TBTCl stress was found to be 9 as it's a known alkaliphile (Fig. 3.6). It showed better growth in MSM supplemented with 2 mM TBTCl at pH 7 than at pH 9 and a marked decrease in growth was noticed at pH 4 due to increased availability of the tributyltin cation, $[Bu_3Sn(H_2O)_2]^+$ in the medium. Therefore, pH 7 was observed to be the optimal pH in MSM with TBTCl as sole carbon source (Fig. 3.6). In case of *Rhodospirillum rubrum,* triphenyltin inhibits the hydrolysis of chromophore membrane bound pyrophosphatase in a pH dependent manner, being maximal at pH 9 -10. Similar result was observed in case of chromophore bound H^+ ATPase (Celis et al., 1998). Another factor that affects the toxicity of TBTCl is it's solubility in water. It is interesting to note that pH of the aquatic environment causes selective enrichment of TBT resistant microorganisms in TBT contaminated sites (Fukagawa et al., 1994; White et al., 1999).

3.5. Optimal salinity for growth

When the isolate was grown in MSM supplemented with 2mM TBTCl containing different concentrations of NaCl ranging from 0.5 to 3 % it showed a decline in growth as the concentration of NaCl increased. While, in the presence of glucose (0.1%) the optimal salinity for growth was 2.5 % (Fig. 3.7). Since, TBTCl is very hydrophobic, its solubility greatly depends on pH and salinity of the aquatic environment (Alzieu, 2000).The aqueous solubility of a organotin compound such as TBTCl decreases with increased salinity (Inaba et al.,1995). This clearly suggests that TBTCl toxicity is reduced at higher

salinity levels. As the availability of TBTCl is high at low concentration of NaCl, it becomes toxic to the cells; the toxicity of TBTCl 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).

3.6. Effect of selected carbon sources: glucose, TBTCl, ethanol and butanol on growth of *Alcaligenes* sp.

The marine sediment isolate *Alcaligenes* sp. responded differently when it was allowed to grow in presence of glucose, ethanol and butanol in MSM separately. The optimal concentrations of the carbon sources glucose, ethanol and butanol were 0.2% (w/v), 0.01% (v/v) and 0.05% (v/v) respectively (Fig. 3.8 - 3.10). It was observed that all three carbon sources i.e., glucose, butanol and TBTCl supported the growth of the isolate in MSM (Fig. 3.11). In order to confirm that the growth of the test isolate was supported entirely by TBTCl, the culture was grown in MSM with ethanol (0.14%, the concentration used to dilute TBTCl) and also in MSM supplemented with 2mM TBTCl and ethanol respectively. Interestingly, growth of the organism was best in 2mM TBTCl with ethanol, because of higher availability of TBTCl to bacterial cells, whereas, there was no growth in medium supplemented with ethanol (Fig. 3.10). Similarly, butanol (0.05 %) also supported growth of the isolate in MSM, which clearly indicates that the isolate utilizes butanol as a carbon source (Fig. 3.11).

Based on these observations, it can be hypothesized that since this isolate can utilize butanol as a carbon source it could be degrading TBTCl to dibutyltin chloride later to monobutyltin chloride and finally releasing tin ions, while butyl group is released in each degradation step.

3.7. TBTCl induced exopolysaccharide production.

Alcaligenes sp. produced maximum EPS (85.9 µg/ml) when exposed to 4 mM TBTCl after 48hrs at an incubation of 28°C. Interestingly, the amount of EPS produced increased gradually with increase in concentration of TBTCl i.e., 1- 4 mM (Fig. 3.12). Therefore, it can be hypothesized that this *Alcaligenes* sp. shows TBTCl induced enhanced production of EPS for possible sequestration of TBTCl, rendering the cells more tolerant to it.

As lipophillic nature of TBT ensures its association to the cell wall components (Gadd, 2000), it is quite possible that attachment of TBT molecule to the cell surface enhances the EPS production in bacteria. It has been reported that cell to cell attachment increases EPS production in *Pseudomonas aeruginosa, Pseudomonas putida* and *Pseudomonas fluorescens* (Read and Costerton, 1987; Danese, et al., 2000). Exopolymers itself possess high affinities for many suspended compounds present in seawater. It forms a micro-environment around the microbial cell, which allows it to metabolise and reproduce efficiently under controlled conditions and even in presence of heavy metals and toxins (Decho, 1990; Ashtaputre and Shah, 1995a). *Sphingomonas galtibidus* and *Vibrio* sp. are reported to produce gellan like polymers (Sarradin et al., 1995). 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 exopolymer, which has high metal binding activity (Kachlany et al., 2001). EPS production by *Pseudomonas aeruginosa* has been well characterized (Ashtaputre and Shah, 1995b).This study strengthens our understanding about exopolysaccharide mediated TBTCl resistance mechanism in this well known proteo bacterium *Alcaligenes* sp.

3.8. Growth pattern and EPS production in the presence of TBTCl

The study of growth Vs. EPS production in *Alcaligenes* sp interestingly revealed that in the presence of 4mM TBTCl the maximum amount of EPS was produced at 48 hrs (after late log phase, i.e., 36 hrs) although the optimal concentration for growth is 2 mM (Fig. 3.13). Most microbial EPSs are produced both in the exponential as well as stationary growth phase (Uhlinger and White, 1983), with an exception of a non-marine pseudomonad which produces EPS only in the stationary growth phase (Williams and Wimpenny, 1977).

3.9. Bulk production of EPS

Most of the EPS-producing marine bacteria, isolated from various sites are Gram-negative rods belonging to the genus *Vibrio, Flavobacterium, Pseudomonas,* and *Alteromonas* are known to produce acidic polysaccharides (Geesey et al., 1992). The EPS production in batch culture was highest during the late log phase of growth. When the culture was centrifuged at 15 000 rpm, for 30 min, the supernatant from the isolate was viscous and formed stringy precipitates with cold ethanol (95%) and to enhance the precipitation, the samples were stored at 4°C for 24 hrs. The addition of two volumes of ice-cold ethanol showed a better precipitation and recovery of the biopolymer. The precipitates recovered by centrifugation at 15 000 rpm for 20 min were vacuum dried to obtain a crude biopolymer or were directly dissolved in Milli Q water. Various methods including high speed cold centrifugation (Decho, 1990), mild alkali, ethylene-diamine tetra acetic acid (EDTA) and NaCl (Bhosle et al., 1995) have been reported for extraction of EPS from microbial cultures. However, we found fairly a effective extraction of exopolysaccharide from the isolate using cold ethanol precipitation.

3.10. Wet weight and Dry weight of EPS

The wet weight of dialysed EPS was 2 gms whereas after lyophilization it was 1.635 gms (Fig. 3.14).

3.11. Chemical characterization of TBTCl induced EPS

Chemical analysis of the EPS produced by Alcaligenes sp. showed gross variations in its composition with reference to its constituents. This analysis clearly revealed that this exopolysaccharide was acidic in nature and composed of neutral sugars (total carbohydrates), proteins, uronic acids and sulphates (Table 3.2). Various macromolecules, such as polysaccharides, proteins, nucleic acids, and lipids, form the architectural matrix in the intracellular space of microbial biofilms and unattached aggregates in the marine environment (Wingender et al., 1999). Polysaccharides are the most abundant component, generally representing 40 % to 95 % of the extracellular polymeric substances (Flemming and Wingender, 2001). Abundant microbial polysaccharides present in dissolved organic carbon, particulate material, or biofilms are of major significance in the marine environment.

The FTIR spectrum of the purified EPS of *Alcaligenes* sp. revealed characteristic functional groups, such as broad stretching hydroxyl group at 3581.81cm^1 and a weak C-H stretching peak of methyl group at 2926.01 cm^1 (Table 3.3) (Fig. 3.15). Further, an asymmetrical stretching peak was noticed at 1045.42 cm^1 and 1089.88 cm^1, which corresponds to carboxyl groups and a peak at 1558 cm^1 could be assigned to amide II (Helm and Naumann, 1995). A broad stretching peak of C-O-C and C-O at 1000–1200 cm^1 corresponds to the presence of carbohydrates (Bremer and Geesey, 1991). Specifically, the peaks at 1000 – 1125 cm^1 range ascertain the presence of uronic acid and o-acetyl ester linkage bonds. The FTIR spectra of the polymer confirmed the presence of carboxyl groups, which may serve as binding sites for divalent cations. A comparison of variable functional groups of TBTCl induced EPS of *Alcaligenes* sp. with the functional groups of other microbial exopolymers confirmed it to be more complex. The presence of acidic sugars in the EPS may be important, considering the heavy

metal-binding properties of this polymer. The EPS produced by *Alcaligenes* sp. is highly surface active, which can be attributed to high uronic acid content in the polymer.

Exopolysaccharides are organic macromolecules that are formed by polymerization of similar or identical building blocks, which may be arranged as repeated units within the polymer. Bacterial exopolymers are important in the interaction between bacteria and their environment and are chemically diverse. The major organic fractions of the EPS are carbohydrates, proteins and humic substances (Nielsen and Jahn, 1999). A wide range of chemical structures of homopolymeric or heteropolymeric type, made up of sugar and non-sugar components is possible and varied linkage types, makes the exopolymer an excellent emulsifying agent and attributes to diversity in bacteria (Keene and Lindberg, 1983). The bacterial exopolymers are usually acidic heteropolysaccharides possessing the functional groups viz. hydroxyl, carboxyl and phosphoric acid which exhibit high affinity towards certain metal ions (Mittleman and Geesey, 1985). Microbial exopolysaccharides possess several interesting physico-chemical properties which can be exploited for a range of biotechnological applications in industries (detergents, textiles, adhesives, paper, paint, food, oil recovery, mining and petroleum) (Sutherland, 1996; 1998). A wide range of bacteria from various environmental habitats are known to produce complex and diverse EPS occurring as capsular polysaccharides intensively associated with the cell surface or as slime polysaccharides, loosely associated with the cell (Whitfield, 1988). Bacterial growth is often accompanied by production of EPS, which have relevant ecological and physiological functions. The nutrient status and growth phase of surface associated bacteria may influence the quality and composition of the EPS produced (Decho, 1990).

3.12. GC analysis of the exopolysaccharide

The carbon source used for growth determines both the quality and quantity of exopolysaccharide production. Sugars play an important role in the EPS synthesis as

activated precursors. The gas chromatography analysis of sugar composition of EPS clearly revealed the percentage relative contribution of hexoses : galactose (10.08 %) , glucose (3.6 %) ; deoxyhexoses : rhamnose (0.7 %), fucose (0.15 %) and pentoses : ribose (0.2 %), arabinose (0.3 %), xylose (0.45 %), mannose (1.56 %) respectively (Fig. 3.16). The presence of different sugar moieties suggests that the exopolysaccharide is a heteropolysachharide. The occurrence of nonsugars viz. uronic acids, sulfates and proteins indicates acidic nature of the EPS. The heteropolysachharide-containing multiple sugars have been reported in different Gram-negative bacteria. The EPS of *Pseudomonas fluorescens* strain III, contains glucose, glucosamine, rhamnose, fucose, arabinose and acetate, where as, the plant pathogen *Pseudomonas andropogonis*, produces an acidic exopolymer-containing glucose, glucuronic acid, mannose, rhamnose and galactose .

3.13. Emulsification activity

The exopolysaccharide produced by *Alcaligenes* sp. exhibits 53.76 %, 41.09 %, 38.7 %, 43.19 % and 49. 52 % emulsifying activity after 30 mins and 7.81 %, 10.11 %, 10 %, 14.23 % and 13.48 % after 60 mins in crude oil, diesel, xylene, toluene and TBTCl respectively (Fig 3). The emulsifying activity of this EPS clearly indicates that this biosurfactant has immense importance in solubilizing various hydrophobic pollutants by increasing their bioavailability to microbial cells. The emulsion formation not only assists in cell substrate interaction but also concentrates the minute amount of the substrate in water oil emulsion which scavenges the pollutants from the environment [19]. Interestingly, the surfactant activity increased in the presence of TBTCl, we hypothesis that this can be attributed to enhanced production of EPS (bioemulsifier) which can act on it 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, which could tolerate and grow in such a high concentration of (4 mM) TBTCl. Rosenberg et al (1979) [31] 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. It has been suggested that biosurfactant may prove useful in a broad spectrum of potential applications, which is mostly accomplished by synthetic surfactants [28]. The present biosurfactant would be more effective than synthetic surfactants in increasing bioavailability and degradation of the emulsifying hydrophobic compounds.

3.14. Protein profile of *Alcaligenes* sp. under TBTCl stress

Heavy metal induced specific polypeptides play a very important role in metal ion homeostasis in cyanobacteria (Olafson et al., 1979). Some bacterial strains are also known to synthesize cysteine rich low molecular weight polypeptides which play an important role in biosorption of these toxic 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 (Gadd, 2000., Higham et al., 1994). The whole cell protein analysis of this *Alcaligenes* sp. exposed to 2mM TBTCl revealed the presence of three polypeptides (43, 63 and 68 kDa) which were induced solely due to TBTCl and three other polypeptides (14.3, 30 and 40 kDa approximately) were constitutive and up-regulated due to organotin biocide (Fig. 3.19). The periplasmic protein profile of the isolate showed the expression of the same proteins as seen in the whole cell fraction. This clearly shows that all the six proteins that are induced or up-regulated belong to the periplasmic fraction of the bacterial cell.

It is very interesting to note that immediately after 12 hrs of exposure of the cells to TBTCl (2mM), a clear induction and up-regulation of specific proteins was observed (Fig. 3.18 & Fig. 3.19). In bacterial cells the periplasmic space is involved in various biochemical pathways including nutrient acquisition, synthesis of peptidoglycan, electron transport, and detoxification of toxic substances. In *E.coli* and *S. typhimurium*,

periplasmic proteins are involved in transport and chemotaxis (Miller et al., 1983). The up-regulation of protein expression has already been reported in TBTCl resistant *Vibrio* sp., which exhibited enhanced synthesis of two polypeptides of approximately 30 and 12 kDa when cells were grown in presence of 125 µM TBTCl (Fukagawa et al., 1992). Transcriptome analysis of a tributyltin-resistant bacterium, *Pseudomonas aeruginosa* exposed to TBTCl (500µM) revealed six genes to be up-regulated and 75 genes were down-regulated. It is very interesting to note that most of the down regulated genes were involved in transcription and translation (Dubey et al., 2006).

Certain heavy metal tolerant bacteria such as *Pseudomonas putida* and *Vibrio alginolyticus* commonly show induced synthesis of low molecular weight, cysteine rich polypeptides which bind with specific metals such as cadmium and copper making them unavailable to the bacterial cells (Higham et al., 1994; Gadd, 1992, 1993., Pazirandeh et al., 1995, 1998). 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 also acts on mitochondria and chloroplast by causing ion exchange through membranes and inhibiting phosphorylation and ATP sythase activity (Ballmoos et al.,2004). Some of the enzymes like glucose dehydrogenase, glucose-6-phosphate dehydrogenase, β-galactosidase, galactohydrolase and alkaline phosphatase are also inhibited by TBT. Interestingly, NADH oxidase activity was stimulated as the concentration of TBT was increased in resistant strains of *Pseudomonas putida* TBT-6 and *Pseudomonas* sp. BP-4 (Tsing et al., 1995). These studies have confirmed that even toxic compounds could affect protein synthesis as it has been reported that a 45 kDa protein produced by *Acinetobacter radioresistens* is highly effective in stabilizing the solubilization of hydrocarbons, including polycyclic aromatic hydrocarbon (Ron et al., 2002).

Table : 3.1 : Morphological and biochemical characteristics of a marine sediment TBTCl tolerant isolate

MORPHOLOGICAL AND BIOCHEMICAL CHARACTERISTICS OF THE ISOLATE	
Colony characteristics	Round, opaque, non - pigmented, butyrous, entire, 2mm-3mm diameter
Gram Character	Gram negative long rods
Motility	motile
Biochemical test	**Result**
ONPG	Positive
Lysine decarboxylase	Positive
Ornithine decarboxylase	Positive
Urease	Negative
Phenylalanine deamination	Negative
Nitrat reduction	Negative
H_2S production	Negative
Citrate utilization	Positive
VP	Negative
Methyl red	Negative
Indole	Negative
Malonate	Positive
Esculin	Positive
Arabinose	Negative
Xylose	Negative
Adonitol	Negative

Rhamnose	Negative
Cellobiose	Negative
Melibiose	Negative
Saccharose	Negative
Raffinose	Negative
Trehalose	Negative
Glucose	Positive
Lactose	Negative
Oxidase	Positive
Catalse	Positive
Amylase	Negative
Gelatin Hydrolysis	Negative
Production of acid and gas during utilization of sugars	Negative
Production of ammonia	Negative
Hugh & Leifson	Oxidative

Table 3.2. : Chemical characterization of EPS induced by TBTCL in *Alcaligenes* sp.

Component	µg/gm of lyophilized EPS	Methodology
Total Carbohydrates	0.032	Dubois et al ,1956
Proteins	0.021	Smith et al,1993
Uronic Acid	0.013	Filisetti – cozzi & Nicholas,1993
Sulphates	0.002	Dodgson & Price,1963

Table 3.3. : FTIR of EPS produced by *Alcaligenes* sp.

Peak cm^{-1}	Possible group	Reference
1045.42	A broad stretching of C-O-C, C-O : corresponds to the presence of carbohydrates	Helm and Naumann, 1995
1089.78	A broad stretching of C-O-C, C-O : corresponds to the presence of carbohydrates	Helm and Naumann, 1995
1145.72	Presence of uronic acid, o-acetyl ester linkage bonds	Bremer and Geesey 1991
1558.48	amide II	Helm and Naumann, 1995
2926.01	A weak C-H stretching peak of methyl group	Bremer and Geesey 1991
3581.81	Broad stratching hydroxyl group	Bremer and Geesey 1991

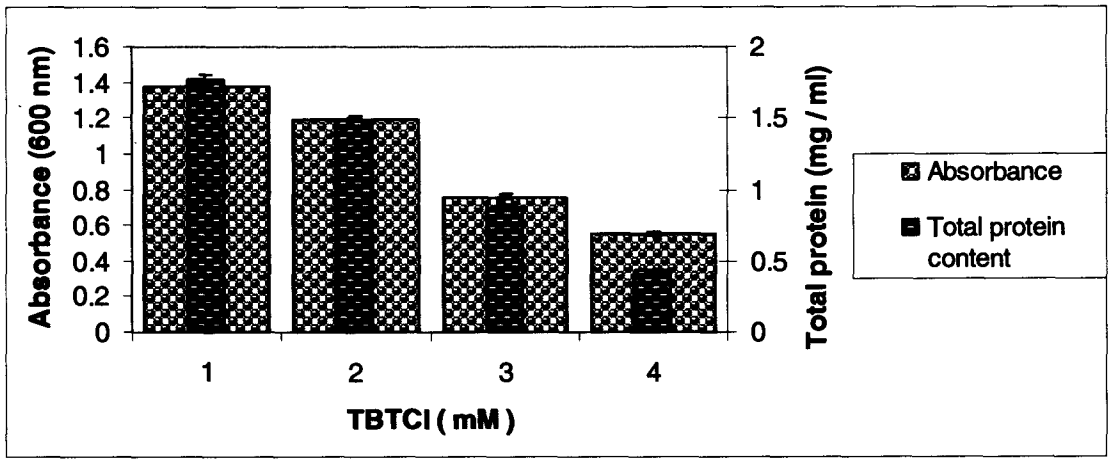


Fig. 3.1 TBTCl tolerance limit of *Alcaligenes* sp. in MSM

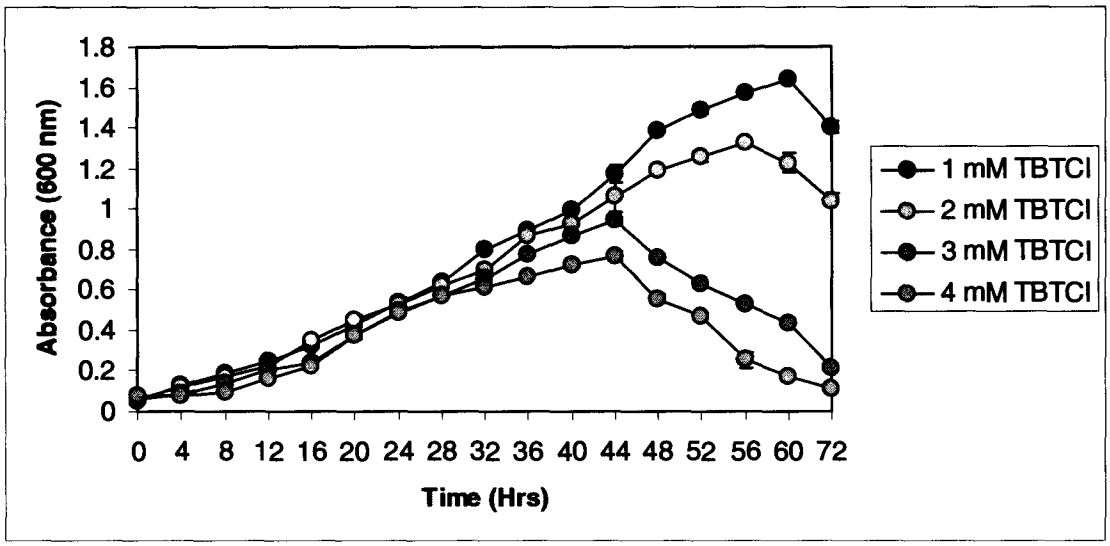


Fig. 3.2 Growth behaviour of *Alcaligenes* sp. in MSM with different concentration of TBTCl

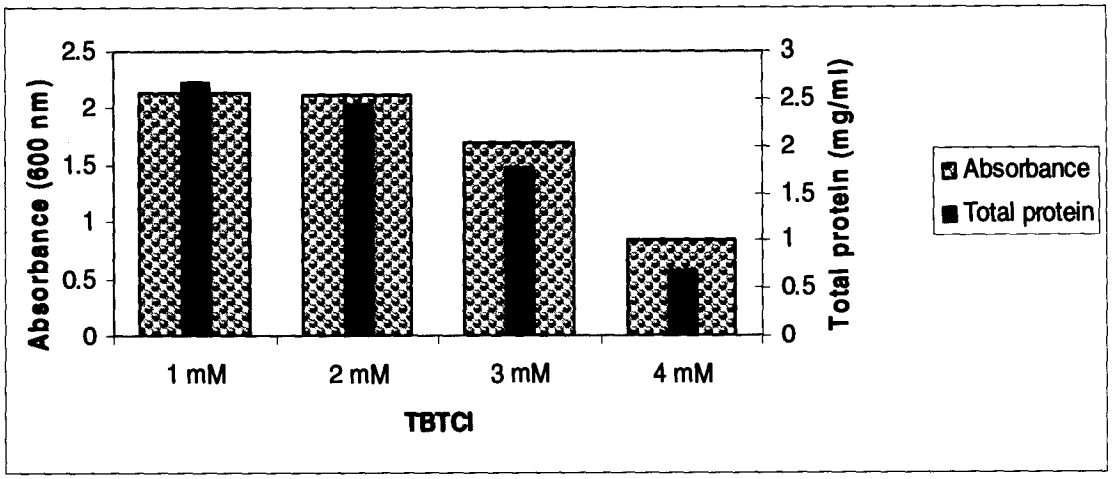


Fig. 3.3 TBTCl tolerance limit of *Alcaligenes* sp. in ZMB

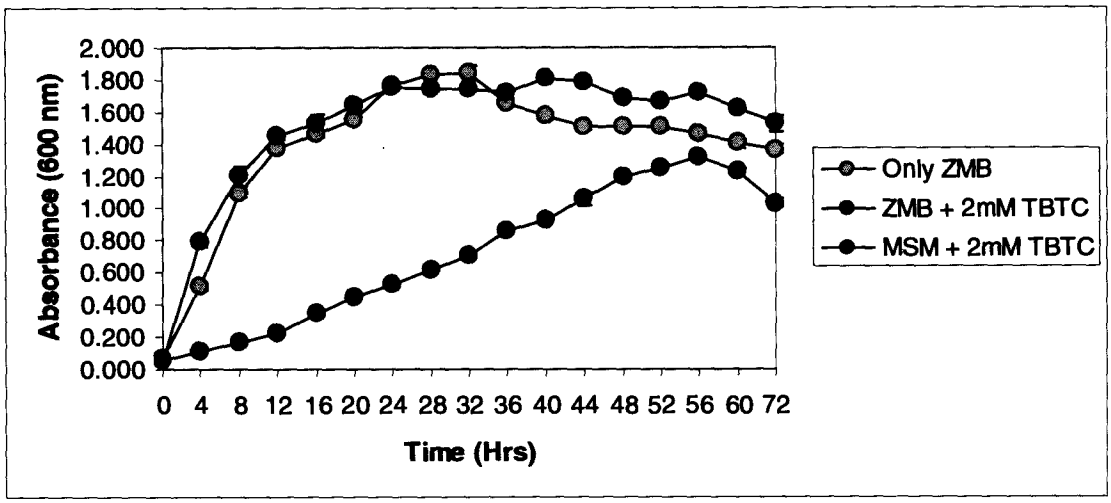


Fig. 3.4 Comparative growth of *Alcaligenes* sp. in ZMB with and without 2mM TBTCl and in MSM with 2mM TBTCl.

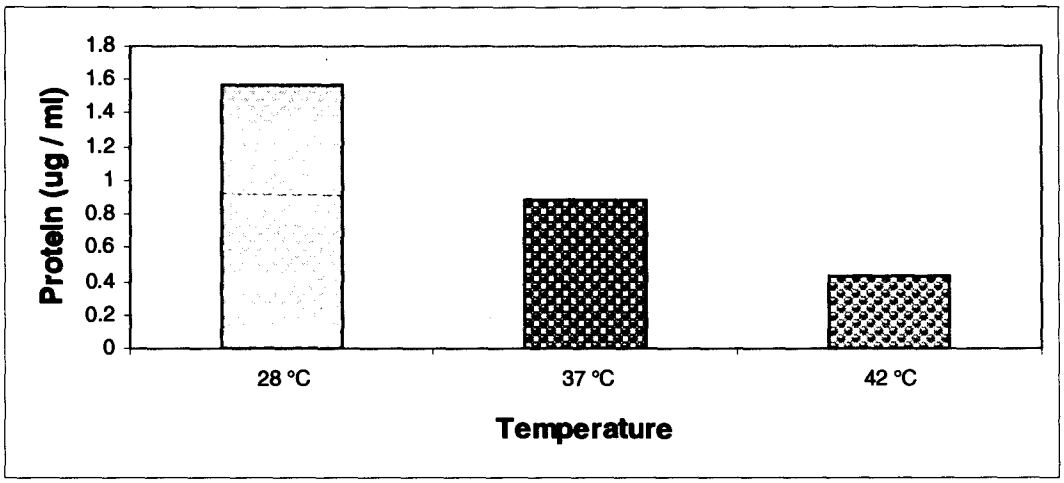


Fig. 3.5 Growth of *Alcaligenes* sp. in MSM with 2mM TBTCl at different temperatures.

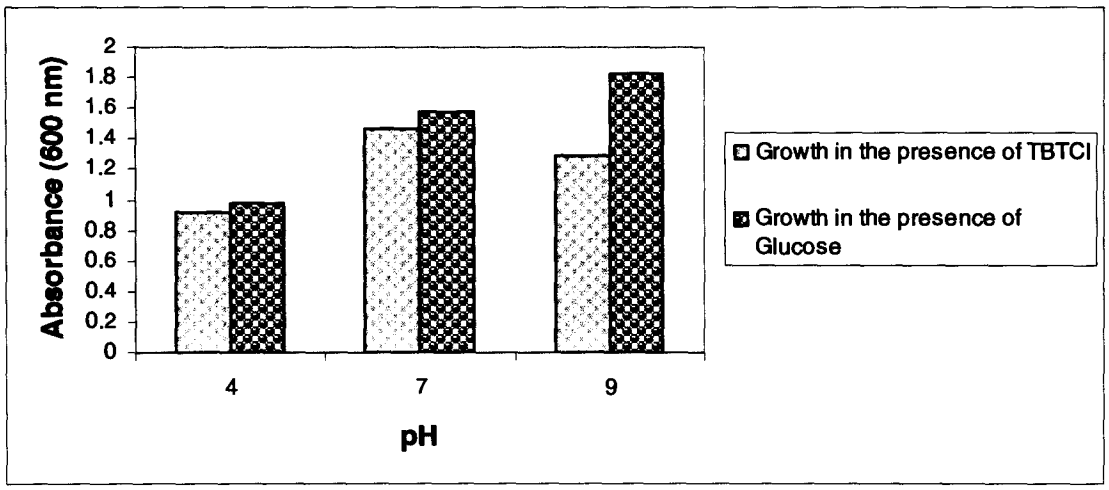


Fig. 3.6 Growth of *Alcaligenes* sp. in MSM supplemented with 0.1% Glucose at different pH.

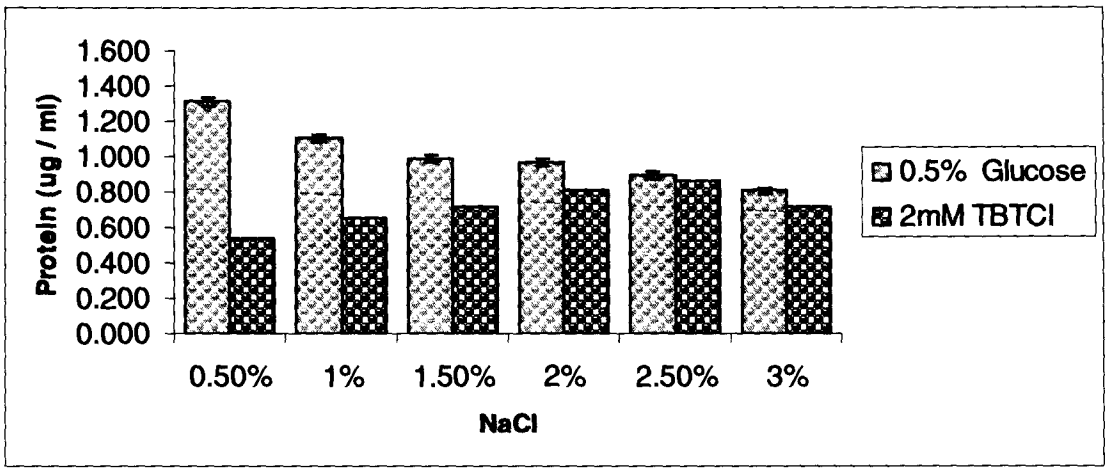


Fig. 3.7 Growth of *Alcaligenes* sp. in MSM supplimented with 2mM TBTCl and 0.1 % Glucose at different NaCl concentration.

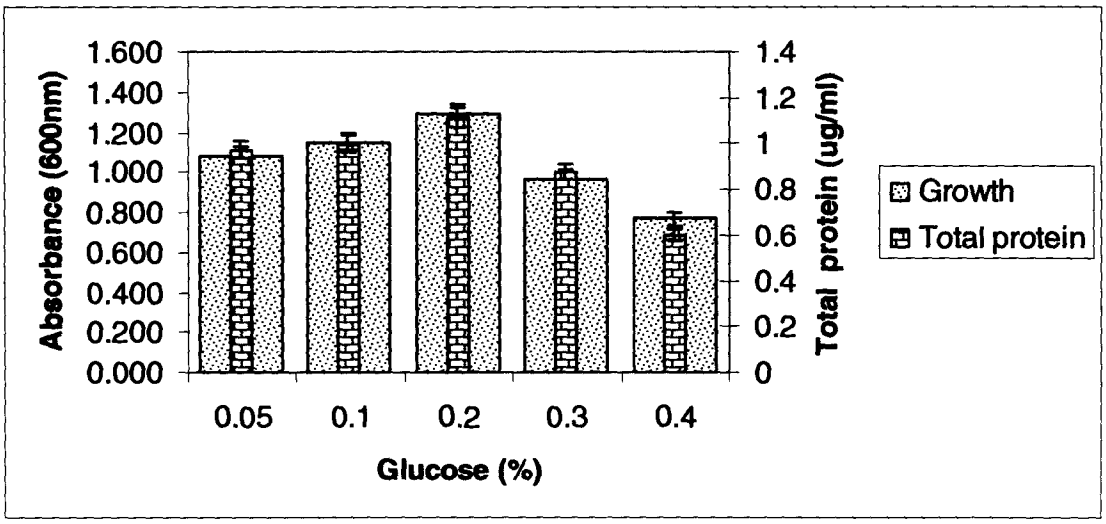


Fig. 3.8 Optimal concentration of glucose for growth of *Alcaligenes* sp. in MSM.

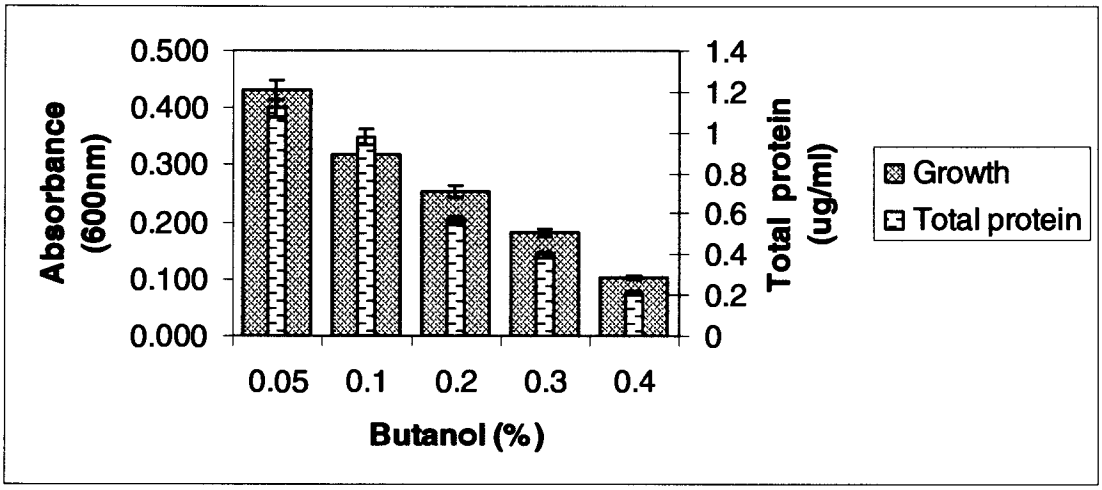


Fig. 3.9 Optimal concentration of butanol for growth of *Alcaligenes* sp. in MSM.

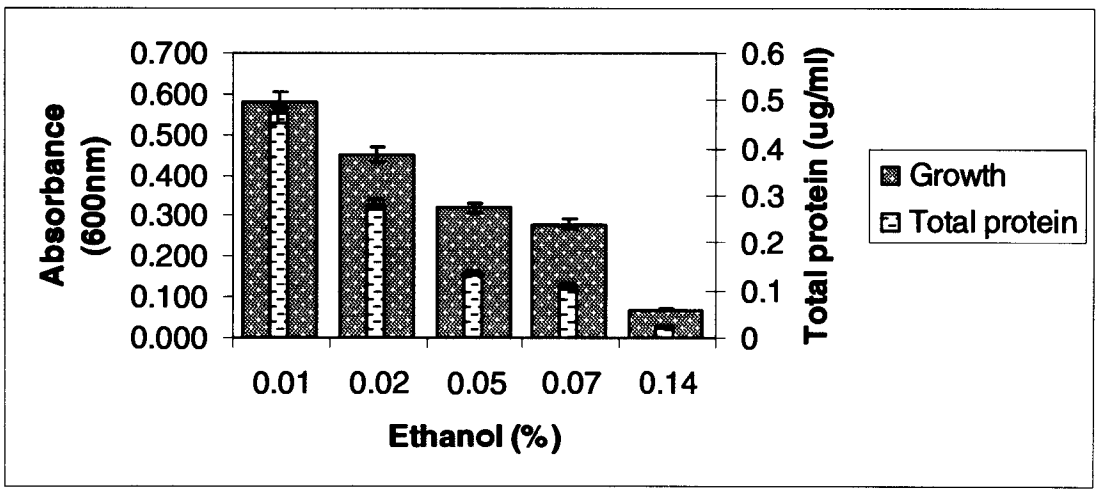


Fig. 3.10 Optimal concentration of ethanol for growth of *Alcaligenes* sp. in MSM.

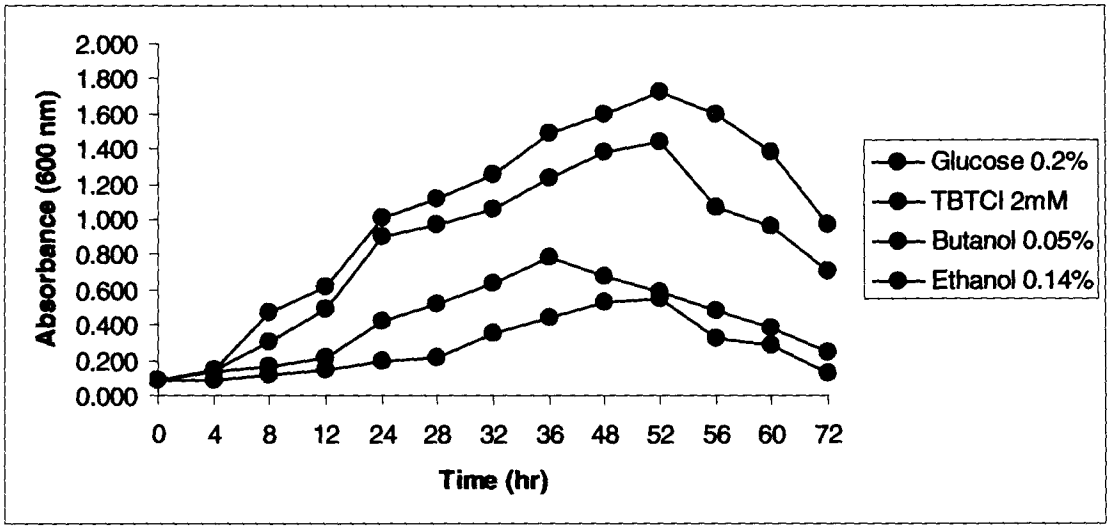


Fig. 3.11 Comparative growth study of *Alcaligenes* sp. in MSM supplemented with optimal concentration of four different carbon sources.

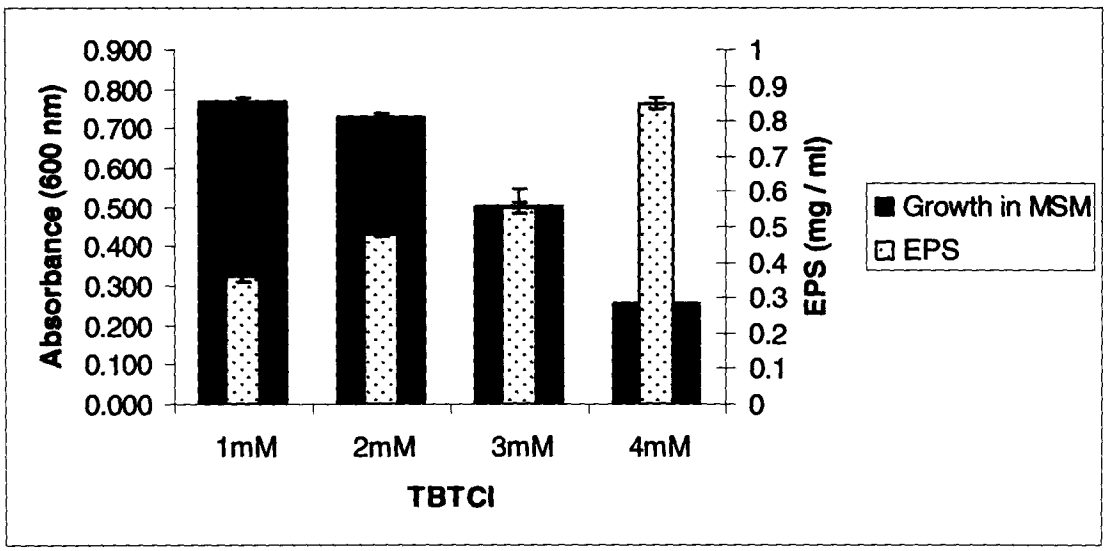


Fig. 3.12 Optimal concentration of TBTCl for EPS production.

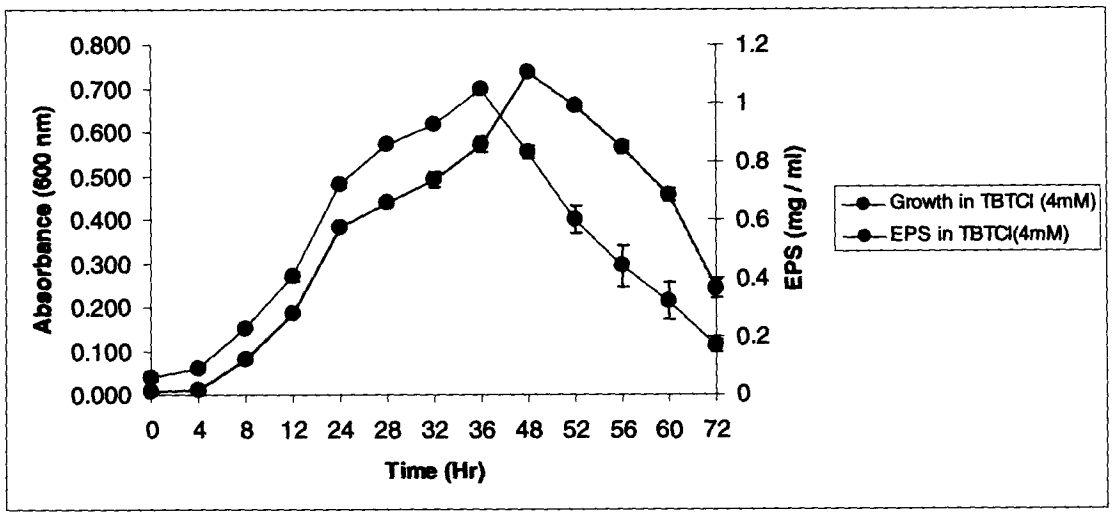


Fig. 3.13 Growth phase study and EPS production in the presence of TBTCl by *Alcaligenes* sp.

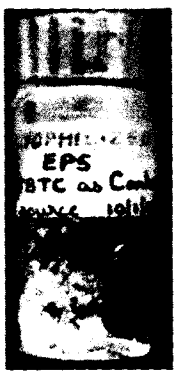


Fig. 3.14 Lyophilized EPS produced by *Alcaligenes* sp. utilizing TBTCl as the sole carbon source.

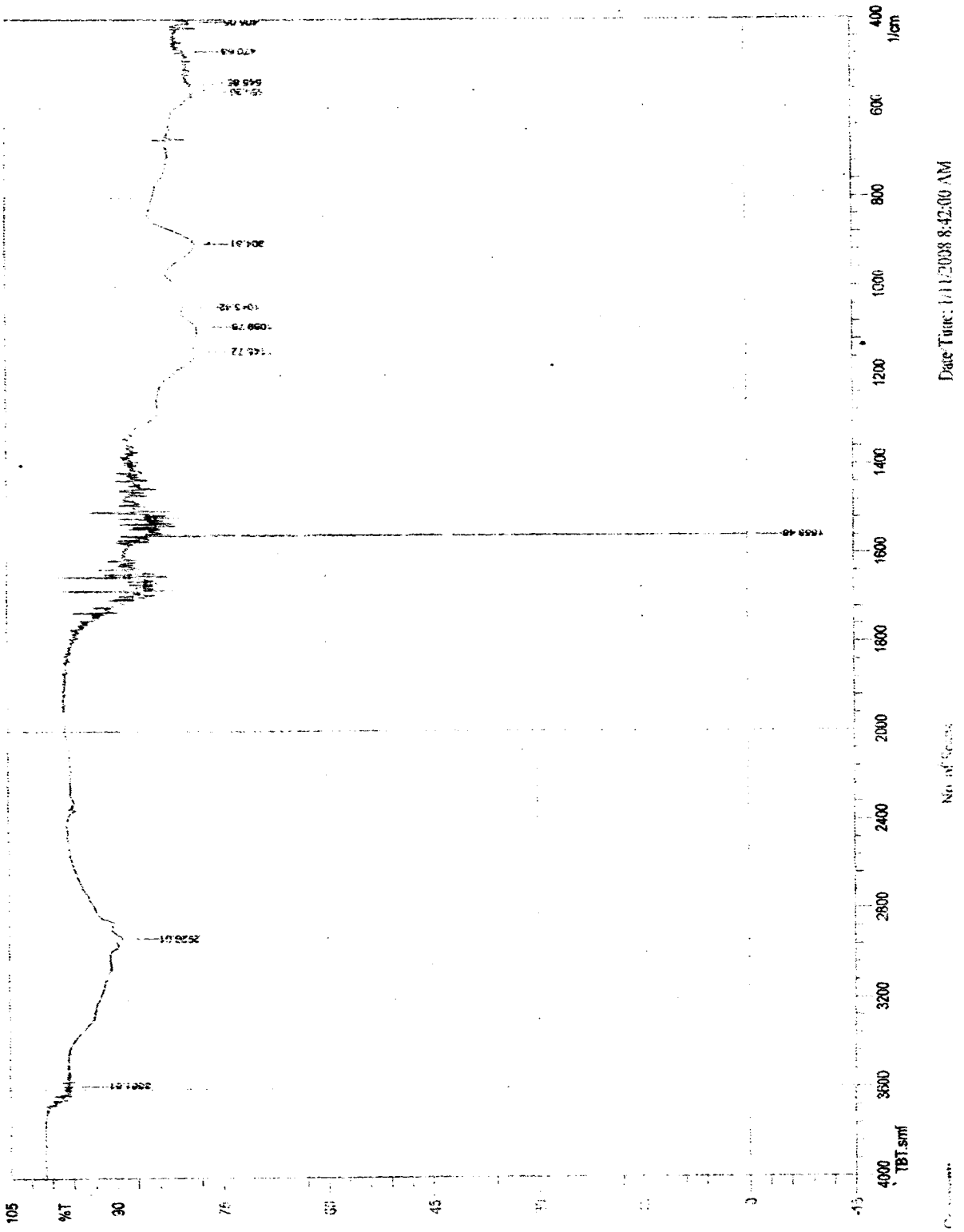


Fig. 3.15 FTIR scan of EPS produced by *Alcaligenes* sp. under TBTCl stress.

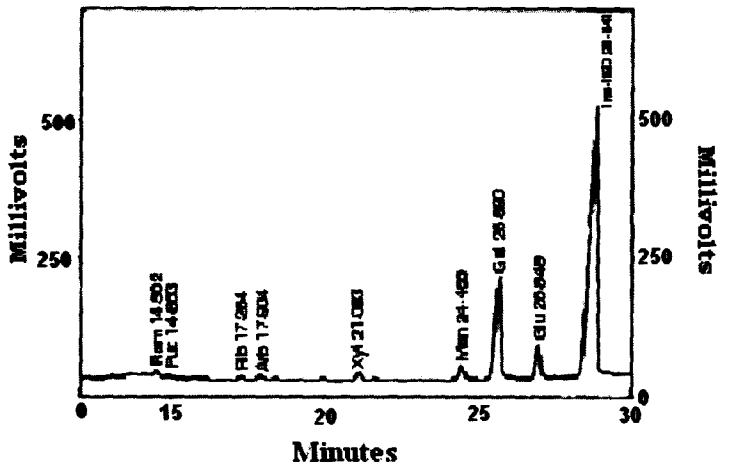


Fig. 3.16 GC chromatograph of exopolysaccharide produced by *Alcaligenes* sp. utilizing TBTCl as sole carbon source.

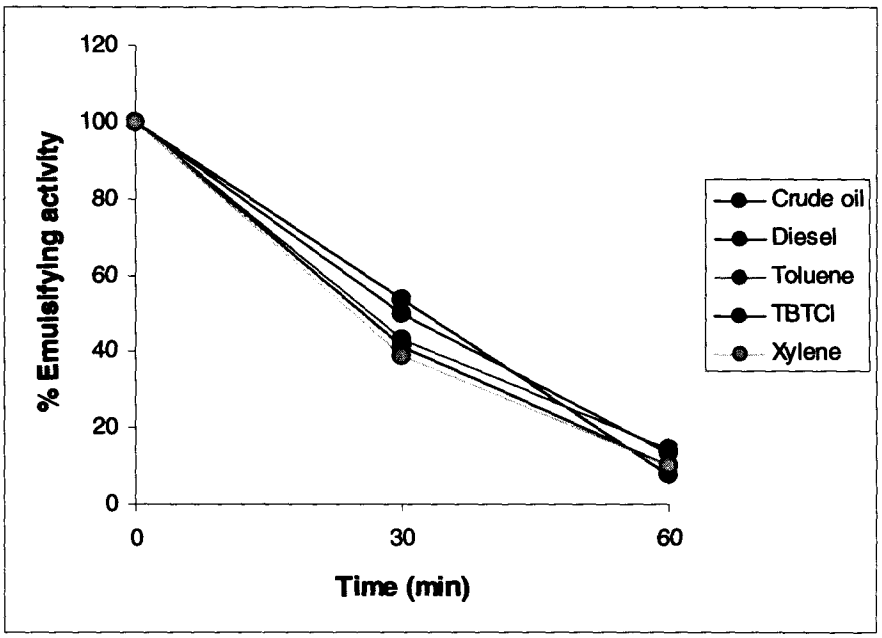


Fig. 3.17 Emulsifying activity of the exopolysaccharide produced by *Alcaligenes* sp.

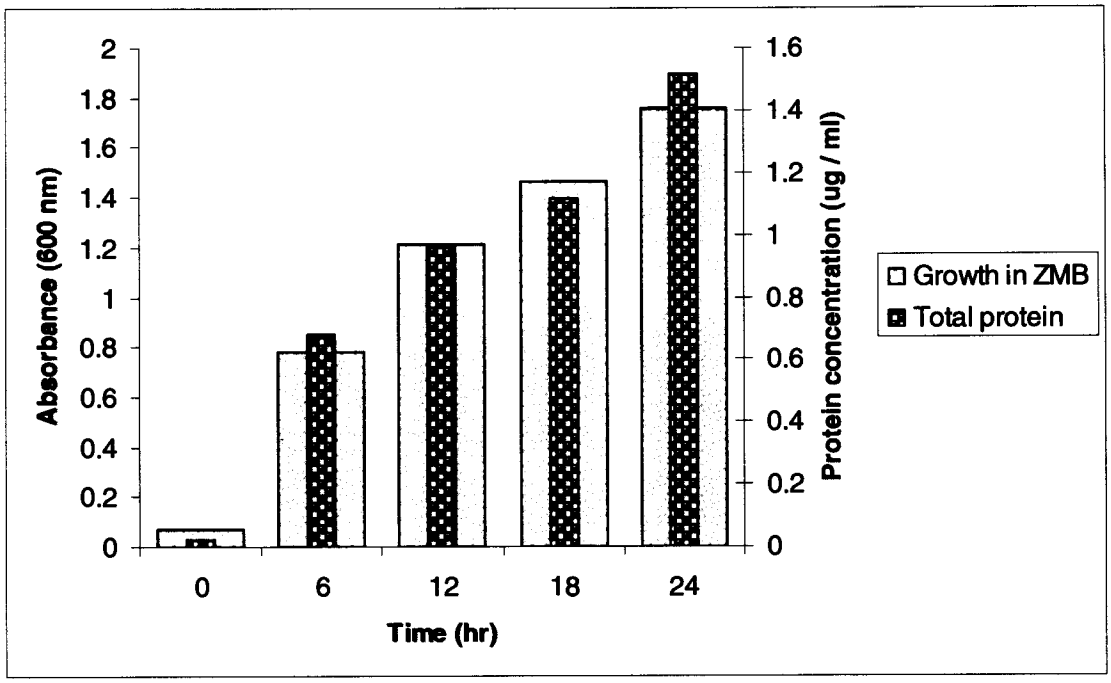
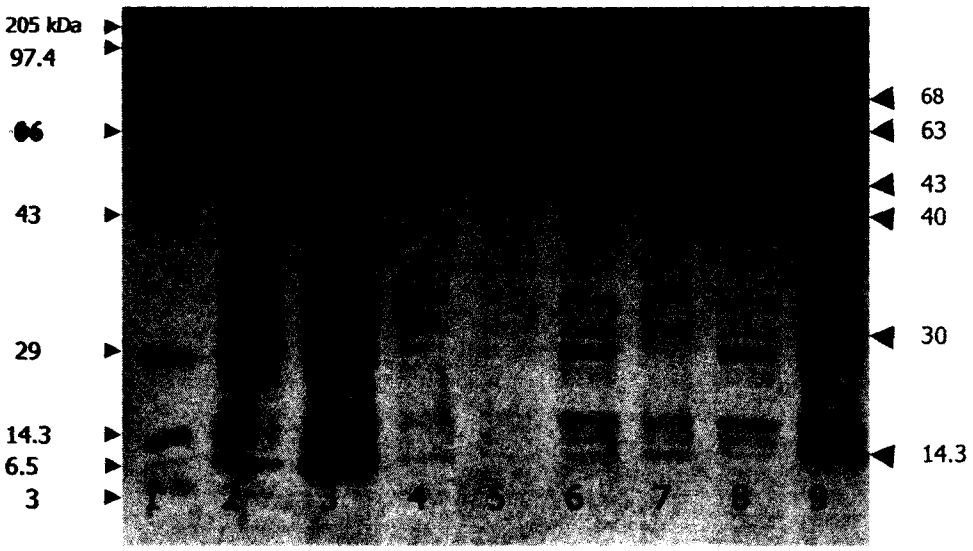


Fig. 3.18 Growth versus total cell protein content of *Alcaligenes* sp exposed to TBTCl (2mM).



Lane 1 : Protein molecular weight ladder

Lane 2 : Whole cell protein (control)

Lane 3 : Whole cell protein (cells grown with 2mM TBTCl)

Lane 4 : Periplasmic proteins (control : 6 hr old cells)

Lane 5 : Periplasmic proteins (test : 6 hr old cells)

Lane 6 : Periplasmic proteins (control : 12 hr old cells)

Lane 7 : Periplasmic proteins (test : 12 hr old cells)

Lane 8 : Periplasmic proteins (control : 18 hr old cells)

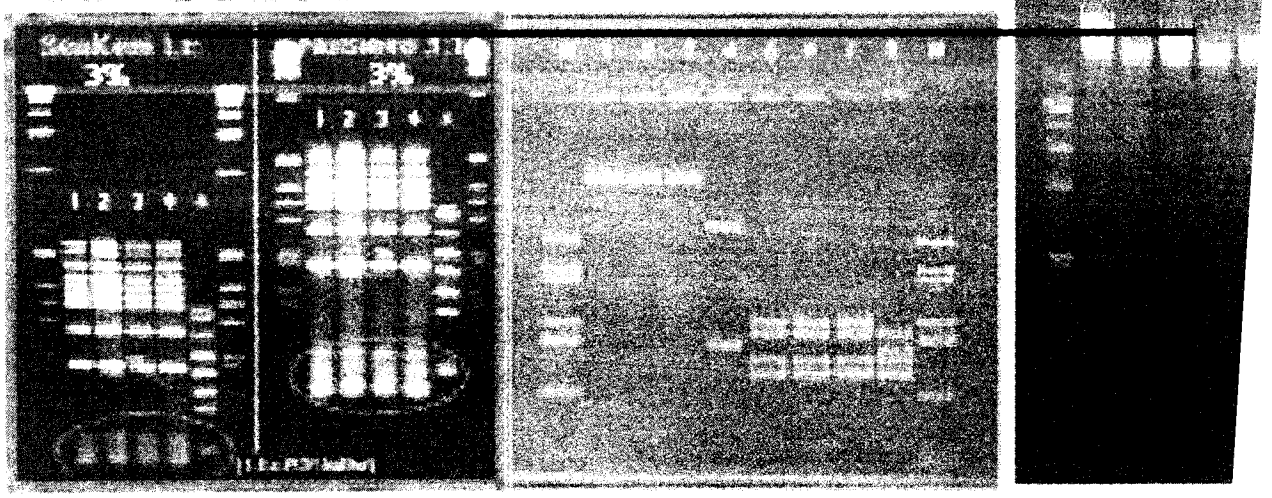
Lane 9 : Periplasmic proteins (test : 18 hr old cells)

Fig. 3.19 Growth versus protein expression by *Alcaligenes* sp. under TBTCl stress.

CHAPTER - IV

RESULTS & DISCUSSION

MOLECULAR AND GENETIC CHARACTERIZATION OF A TBTCL RESISTANT BACTERIUM



4.1. Plasmid profile

The plasmid DNA from Alcaligenes sp. was isolated by alkaline lysis method and agarose gel electrophoresis revealed the presence of a small supercoiled plasmid (Fig. 4.1).

4.2. Confirmation of localization of TBTCl resistance genes on plasmid or chromosomal genome

Although some heavy metals are essential trace elements for metabolism in organisms majority of them are toxic to all forms of life, including microbes, as they bind with –SH groups of enzymes. Microorganisms are exposed to toxic metals, organometals and biocides due to their release in the environment as a result of natural and anthropogenic activities. Microbes possess several mechanisms of tolerance by efflux, sequestration, and metal ion reduction to use them as terminal electron acceptors in anaerobic respiration. The involvement of bacterial plasmids in the detoxification of heavy metals has already been reviewed (Silver and Phung, 1996). There are several reports on the degradation of toxic organic compounds by bacteria which includes fenitrothion degradation by *Burkholderia* sp. strain NF100 involving two plasmids (Hayatsu et al., 2000) and PCB metabolism mediated by plasmid in *Alcaligenes eutrophus* H850 (Bedard et al., 1987). Other reports include conjugative, degradative plasmid in xenobiotic-degrading *Sphingomonas* strains (Basta et al., 2004) and denitrifying plasmid in *Alcaligenes eutrophus* (Romermann and Friedrich, 1985). *Alcaligenes* sp. is known to possess large plasmids harbouring genes which confer resistance to toxic heavy metals, antibiotics and other organic biocides including polyaromatic hydrocarbons (Nies et al., 1995).

Most mechanisms studied earlier involve the efflux of metal ions outside the cell, and genes for this general type of mechanism have been found on both chromosomes and plasmids.

4.2. a. Selection of the ideal curing agent.

In order to confirm plasmid mediated TBTCl resistance and degradation, attempt was made to eliminate the plasmid from cells of *Alcaligenes* sp. cells by treatment with curing agents: acridine orange, novobiocin and sodium dodecyl sulphate (SDS). It is very interesting to note that lethal concentrations of novobiocin (i.e., 170 µg / ml) and SDS (20 %) could not eliminate the plasmid and only acridine orange (140 µg / ml) was found to be an effective curing agent (Fig. 4.2.a, 4.2.b, 4.2.c). Hence, acridine orange was used subsequently in curing experiments. The plasmid DNA from Alcaligenes sp. Was completely cured after 48 hrs of incubation with 140 µg / ml of acridine orange. (Fig. 4.3). It has been reported that acridine orange inhibits the replication of bacterial plasmids by causing mutation in absence of light at the site of semiconservative DNA replication (Webb and Hass, 1984; Trevors, 1986).

Bacterial plasmids can be eliminated from bacterial species grown as pure or mixed bacterial cultures in the presence of sub-inhibitory concentrations of non-mutagenic heterocyclic compounds such as novobiocin, sodium dodecyl sulphate etc (Gabriella et al., 2006).

4.2. b. Genetic confirmation of TBTCl resistance

The presence of plasmids in TBTCl resistant bacteria without their involvement in its resistance has already been reported (Wuertz et al., 1991; Cruz et al., 2007). *Alcaligenes* sp. is known to possess large plasmids harbouring genes which confer resistance to toxic heavy metals, antibiotics and other organic biocides including polyaromatic hydrocarbons (Nies et al., 1995). In the present study we have observed that TBTCl resistance in *Alcaligenes* sp. isolated from the marine sediment of Goa Shipyard Ltd. was genomic DNA mediated which was confirmed by the growth of acridine orange cured cells in MSM with 2 mM TBTCl (Fig 4. 4). The uncured and plasmid cured cells both showed similar pattern of growth in MSM supplemented with 2mM TBTCl. If the TBTCl resistance encoding genes

were located on the plasmid of *Alcaligenes* sp. loss of plasmid DNA would have made the plasmid cured cells sensitive to TBTCl. Therefore, the growth of plasmid cured cells in MSM with TBTCl clearly confirms that these cells are able to utilize TBTCl as carbon source even with the loss of plasmid DNA and the genes governing TBTCl resistance and utilization are located on the genomic or chromosomal DNA of this isolate.

4.3. Molecular cloning of TBTCl resistance genes from genomic DNA of *Alcaligenes* sp.

Preparation of genomic DNA library by Shotgun cloning

Genomic DNA from Alcaligenes sp. was purified and checked by agarose gel electrophoresis (Fig. 4.5). The Hind III digested pUC 18 cloning vector showed the presence of a single band corresponding to approximately 2.7 Kbps (Fig. 4. 6 and 4.7). This was subsquently dephosphorylated and ligated with the insert using T4 DNA ligase (Fig. 4.8). This mixture containing the vector with the insert was used further for transformation.

4.4. Transformation experiment

Transformation experiment using *E.coli* HB101 (host) and ligation mix revealed appearance of several ampicillin resistant colonies after 16 hrs of incubation the LB agar plates supplemented with 50µg/ ml ampicillin. The positive control i.e., pUC18 vector transformed into *E.coli* HB101 competant cells showed mat growth because, the plasmid pUC 18 harbours genes for ampicillin resistance. While no growth was seen on the plate containing only *E.coli* HB101 competant cells since, these cells are sensitive to ampicillin (Fig. 4. 9).

4.5. Replica plating of transformants

Although the colonies replica plated from the master plate on LB agar plates grew within 16 hrs of incubation at 37 °C, the plates containing LB with 5 mM TBTCl showed six prominent colonies only after 72 hrs of incubation (Fig. 4. 10). This could be attributed to the fact that *E.coli* HB101 cells are highly sensitive to TBTCl, while the positive transformants may possess genes for TBTCl resistance. The TBTCl resistant transformants (clones) obviously

took 72 hrs to appear on the plate as they possibly need time to synthesise enzymes for TBTCl resistance / degradation. The six positive clones were refered as TBT 1, TBT 2, TBT 3, TBT 4, TBT 5 and TBT 6 respectively.

4.6. Characterization of TBTCl resistant clones

4.6.1. Insert size

The plasmid DNA from the six positive clones was isolated and analysed on 0.8 % agarose gel (Fig. 4. 11). Molecular characterization of the six positive clones with restriction enzyme Hind III revealed that interestingly, they possess DNA inserts of different sizes. The DNA inserts were found to be 5.6, 5.7, 5.0, 5.2, 3.4 and 1.8 kbps for clones TBT 1, TBT 2, TBT 3, TBT 4, TBT 5 and TBT 6 respectively (Fig. 4. 12, 4.13, 4.14 and 4.15).

These positive clones with the exception of TBT 6 appear to harbour TBTCl resistance / degradation encoding genes. Further characterization (DNA sequencing) of positive clones with longer inserts will reveal exact mechanism of TBTCl resistance in *Alcaligenes* sp.

4.6.2. Growth behaviour in TBTCl

For any gene cloning experiment it is very important to confirm if the clones obtained are truly positive since in most cases host bacteria (*E.coli* JM109) possess inherent mechanisms to survive stress like organotin (TBTCl) (unpublished data).Therefore , a TBTCl host i.e., *E.coli* HB101 would be preferred for cloning experiments and exposure / subculturing of the positive clones in TBTCl containing media would enable to identify them.

Growth studies of the recombinant clones revealed that four TBTCl resistant clones viz. TBT 1, TBT 2, TBT 3 and TBT 4 could grow and utilize upto 1mM TBTCl as the sole carbon source in MSM while, remaining two clones (TBT 5 and TBT 6) did not utilize TBTCl but were resistant to it since they grew well in LB broth supplemented with 2 mM TBTCl. As a sensitive host *E.coli* HB101 could neither grow in MSM with TBTCl (1 mM) nor with 0.14 %

ethanol. This clearly indicates that the recombinant clones are positive clones showing TBTCl resistance (Table 4.1) (Fig. 4. 20, 4.21, 4.22, 4. 23, 4. 24 and 4.25).

Therefore, from these growth studies we can infer that the recombinant clones with inserts larger than 5 Kbps (i.e., TBT 1, TBT 2, TBT 3 and TBT 4) may be harbouring the complete gene cluster (operon) which mediates degradation of TBTCl , whereas, clones with smaller insert size (TBT 5 & TBT 6) may harbour incomplete gene cluster resulting in TBTCl resistance only. Ethanol utilization in *E.coli* sp. is mediated by ethanol dehydrogenase, in the present study it was observed that the host cells (*E.coli* HB101) was unable to survive in the concentration of ethanol used to dilute TBTCl i.e., 0.14%. This clearly indicates that the growth of the recombinant clones in MSM supplemented with 1 mM TBTCl (diluted in ethanol) is solely due to the presence od DNA inserts from *Alcaligenes* sp. which harbours genes for TBTCl utilization.

Molecular 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 TBTCl 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. Therefore, this membrane protein mediated TBT efflux 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 16S rRNA 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 encoding genes on chromosomal genome (Fukagawa and Suzuki, 1993).

One of the most interesting reports on molecular mechanism defining bacterial resistance to TBTCl is on a isolate from a TBT- polluted harbor in France. In *Pseudomonas stutzeri* strain 5MP1, TBT resistance was found to be associated with the *tbtABM* operon, which is homologous to the resistance – nodulation – cell division (RND) efflux pump family (Jude et al., 2004). TbtABM operon exhibited the greatest homology with the TtgDEF and SrpABC operons, involved in aromatic compound resistance in *Pseudomonas putida.* TbtABM operon also conferred multidrug resistance (MDR) to nalidixic acid, chloramphenicol, and sulfamethoxazole. This is the first report of a MDR (drug efflux pump) found in *Pseudomonas stutzeri.* In another report, transcriptome analysis of a tributyltin-resistant bacterium, *Pseudomonas aeruginosa* exposed to TBTCl (500μM) revealed six chromosomal genes to be up-regulated and 75 genes were down-regulated. It is very interesting to note that most of the down regulated genes were involved in transcription and translation (Dubey et al., 2006).

The molecular biological studies conducted (including the present study) to determine the mechanism (s) of tributyltin chloride resistance especially in marine bacteria clearly revealed that most bacteria possess chromosomal genome mediated resistance to this biocide. Hence, we hypothesize from the present study and the previous reports that the mechanism for TBT resistance / degradation is complex in bacteria and involves cluster of genes.

Table 4.1 : Characteristics of TBTCl resistant clones.

Putative clones	Insert size (kbps)	Growth in MSM + 1 mM TBTCl	Growth in LB broth + 2mM TBTCl	Growth in MSM + 0.14 % ethanol
TBT 1	5.6	+ + + + + +	+ + + + + +	No growth
TBT 2	5.7	+ + + + + +	+ + + + + +	No growth
TBT 3	5.0	+ + + + + +	+ + + + + +	No growth
TBT 4	5.2	+ + + + + +	+ + + + + +	No growth
TBT 5	3.4	+ +	+ + + +	No growth
TBT 6	1.8	+	+ + +	No growth
E.coli HB101 (Control)		No growth	No growth	No growth

Key : + + + + + + : very good growth

+ + + + : good growth

+ + + : moderate growth

+ + : poor growth

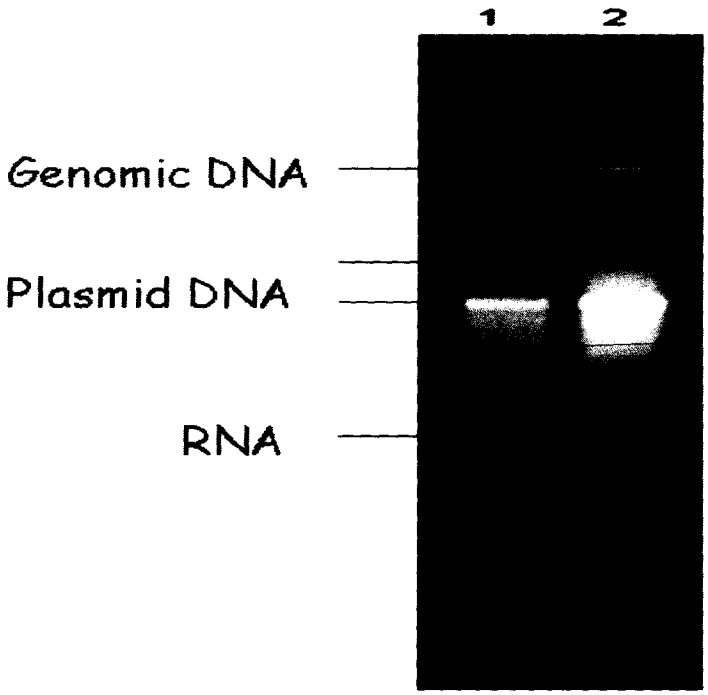


Fig : 4 .1. Plasmid profile of *Alcaligenes* sp.

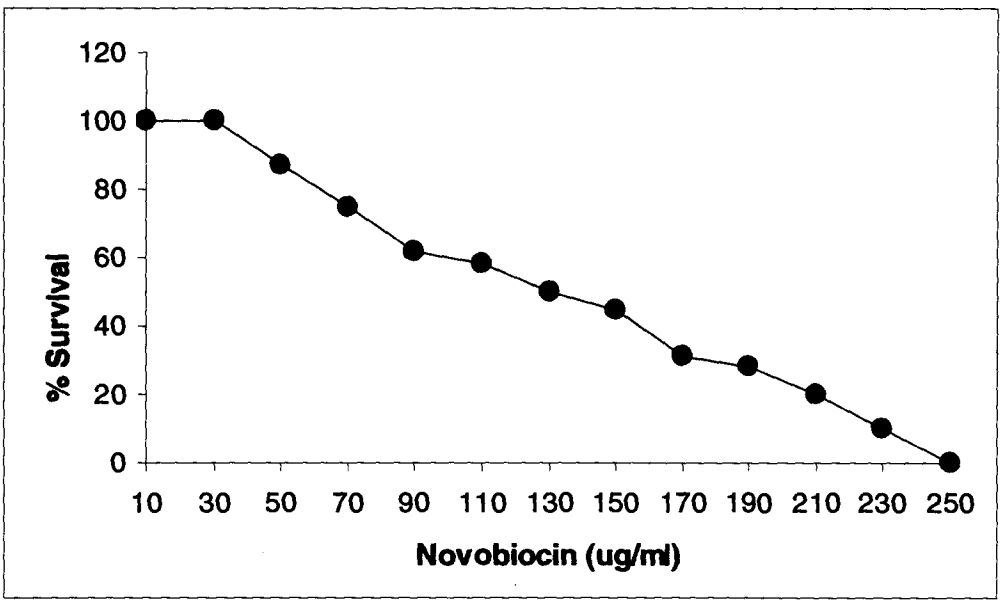


Fig: 4. 2.a. LD_{50} of novobiocin

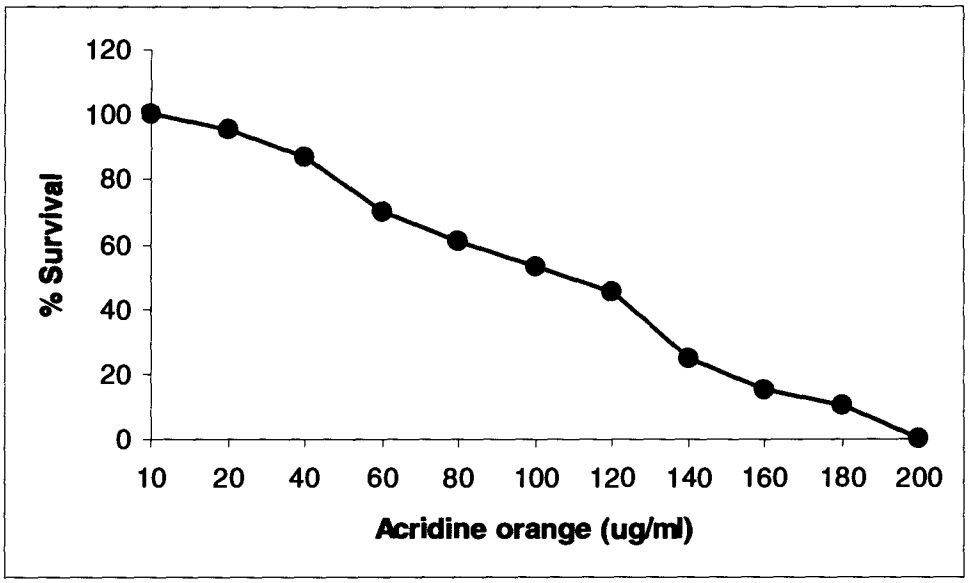


Fig: 4. 2.b. LD_{50} of acridine orange

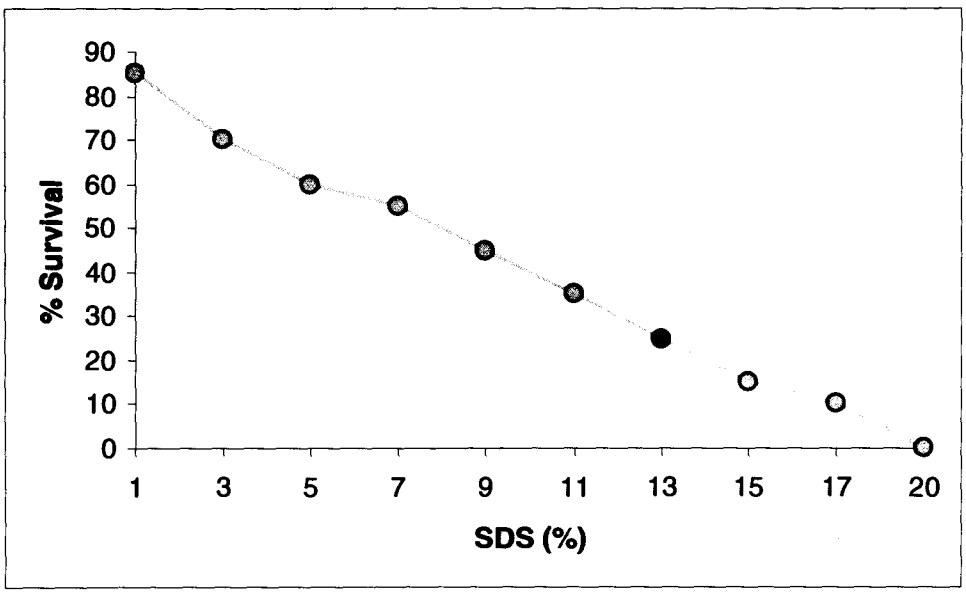
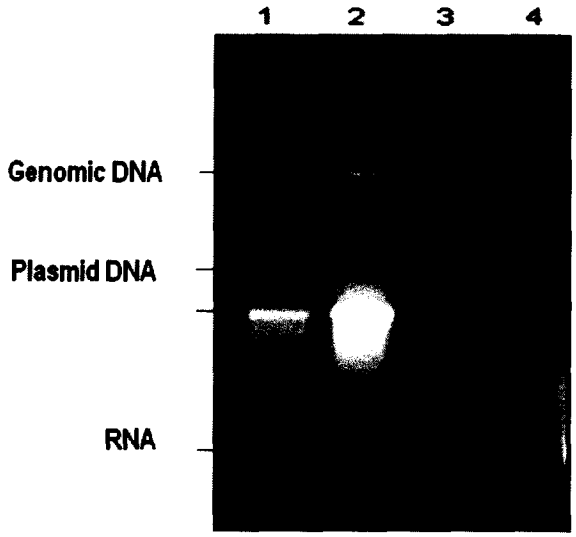


Fig: 4. 2.c. LD_{50} of sodium dodecyl sulphate



Lane 1 & 2 : Uncured cells

Lane 3 & 4 : Cured cells

Fig : 4. 3. Agarose gel electrophoresis of uncured and cured cells of *Alcaligenes* sp.

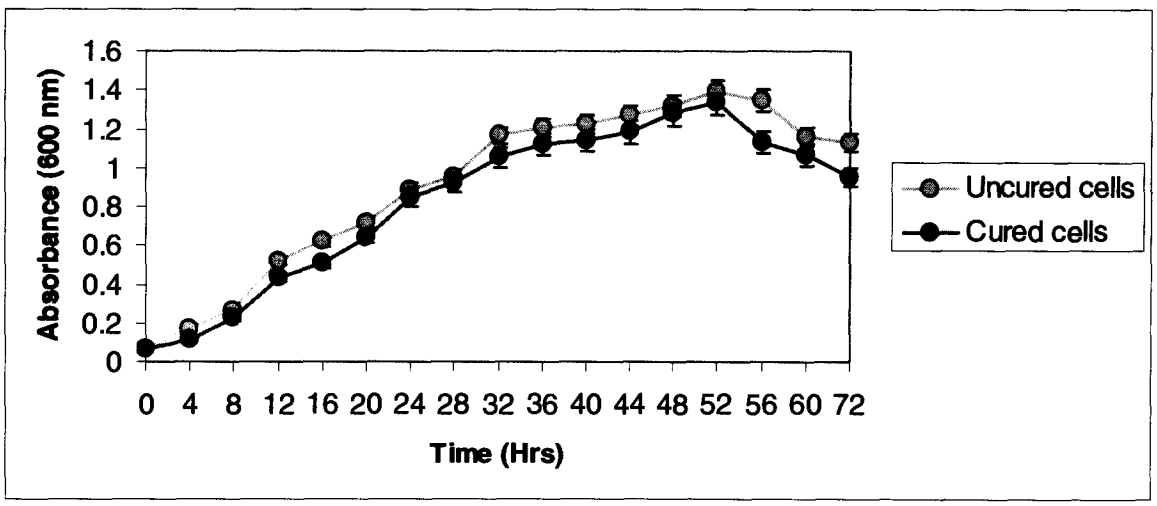


Fig : 4.4. Growth behaviour of uncured and cured cells of *Alcaligenes* sp. in MSM supplemented with 2 mM TBTCl.

Lane 1 : Supermix DNA Marker

Lane 2- 5 : Genomic DNA of *Alcaligenes* sp.

Fig : 4.5. Agarose gel electrophoresis of Genomic DNA isolated from *Alcaligenes* sp.

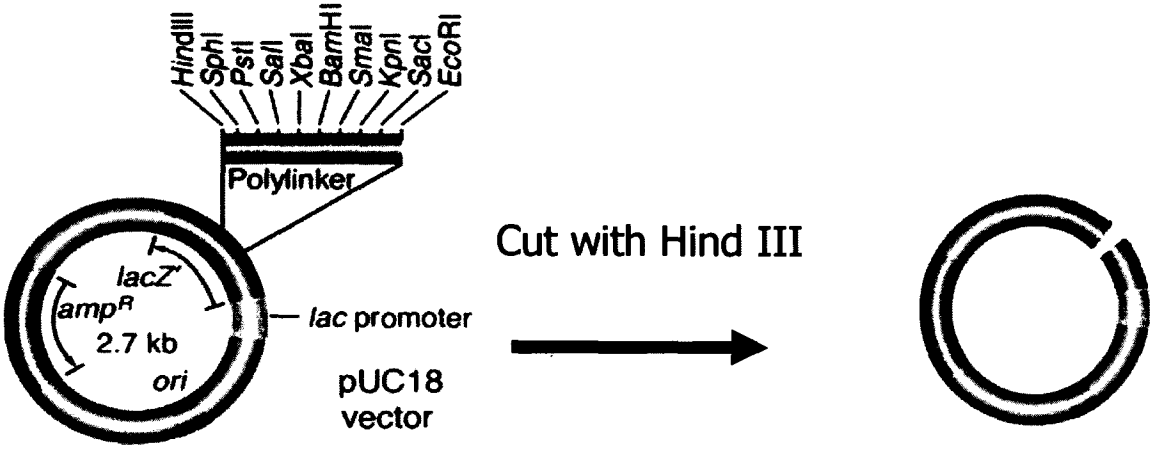
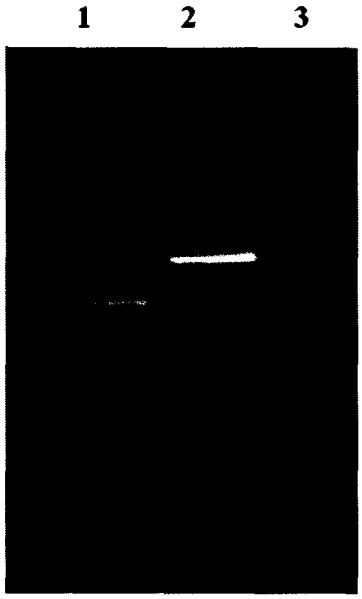


Fig: 4.6. Diagrammatic representation of Restriction Digestion of pUC 18 plasmid with Hind III.



Lane 1 : uncut pUC 18 DNA
Lane 2 : Hind III cut pUC 18 DNA
Lane 3 : Supermix DNA ladder

Fig: 4.7. Agarose gel electrophoresis of pUC 18 plasmid digested with Hind III.

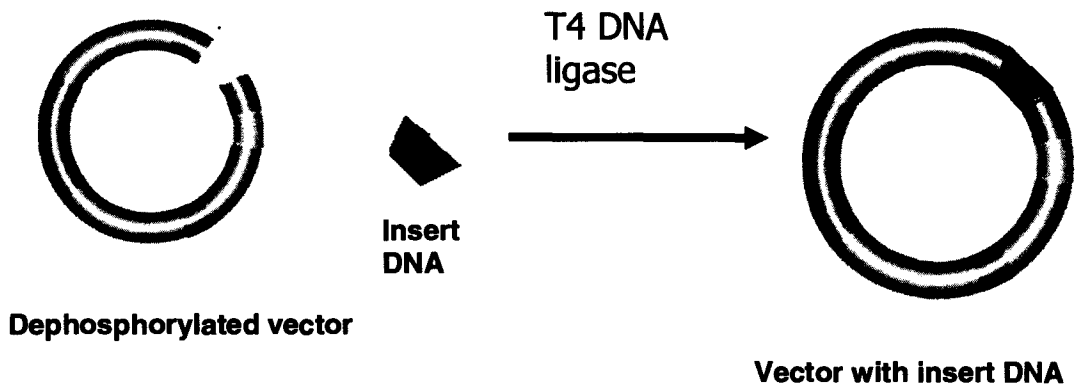
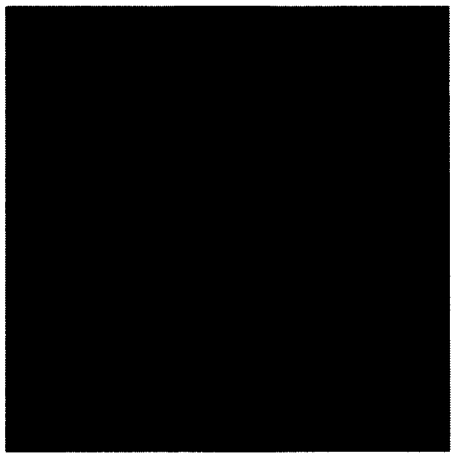
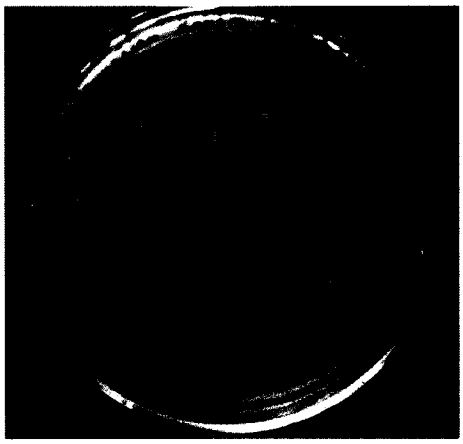


Fig: 4.8. Schematic diagram showing ligation of dephosphorylated pUC 18 with insert DNA (genomic) using T4 DNA Ligase.



Negative Control : *E.coli* HB101 competant cells on LB agar with 50 µg / ml ampicillin

Positive Control : *E.coli* HB101 competant cells with pUC 18 DNA on LB agar with 50 µg / ml ampicillin



Test : *E.coli* HB101 competant cells with recombinant plasmids on LB agar with 50 µg / ml ampicillin

Fig. 4.9. LB agar plates with positive, negative and test samples of transformation experiment after 16 hrs of incubation.

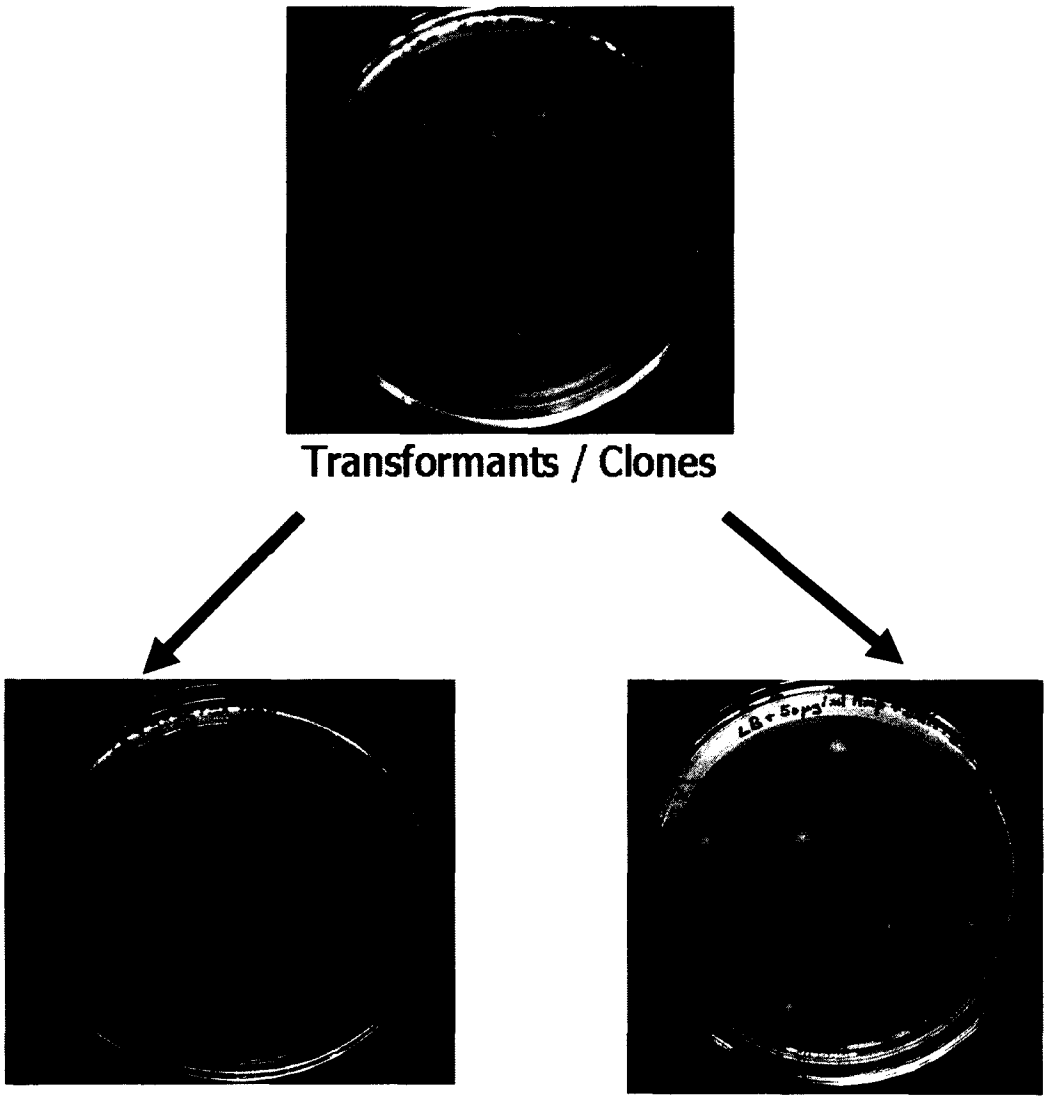
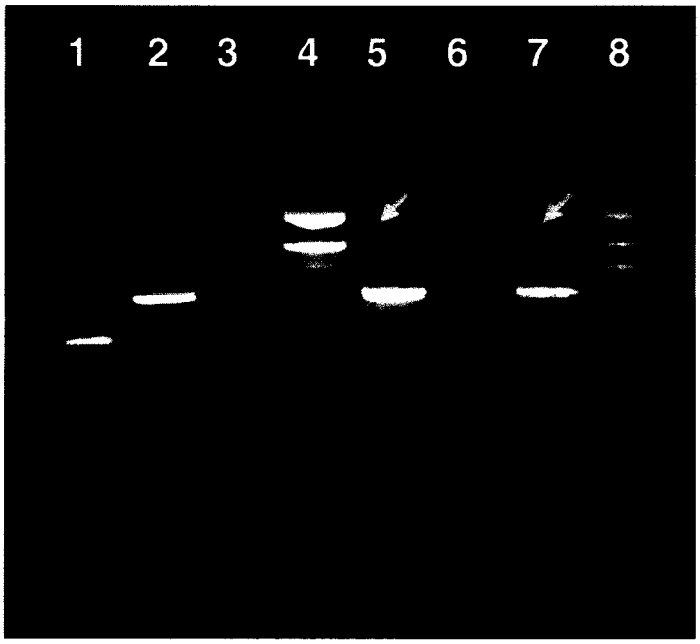


Fig. 4. 10. Replica plating of the transformants from master plate.

Lane 1 : Clone TBT 1
Lane 2 : Clone TBT 2
Lane 3 : pUC 18
Lane 4 : Clone TBT 3
Lane 5 : Clone TBT 4
Lane 6 : Clone TBT 5
Lane 7 : Clone TBT 6

Fig. 4.11. Agarose gel showing uncut positive transformants.



Lane 1 : Uncut pUC 18 DNA

Lane 2 : Hind III digested pUC 18 DNA

Lane 3 and 6 : Supermix DNA Marker

Lane 4 : Uncut clone TBT 1

Lane 5 : Hind III digested clone TBT 1

Lane 7 : Hind III digested clone TBT 2

Lane 8 : Uncut clone TBT 2

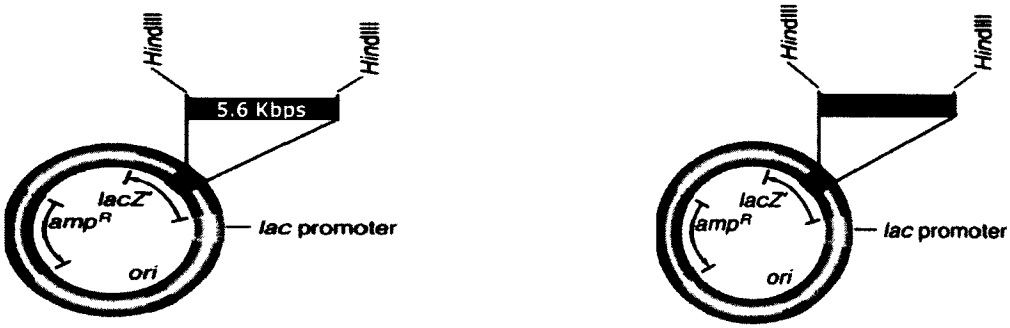
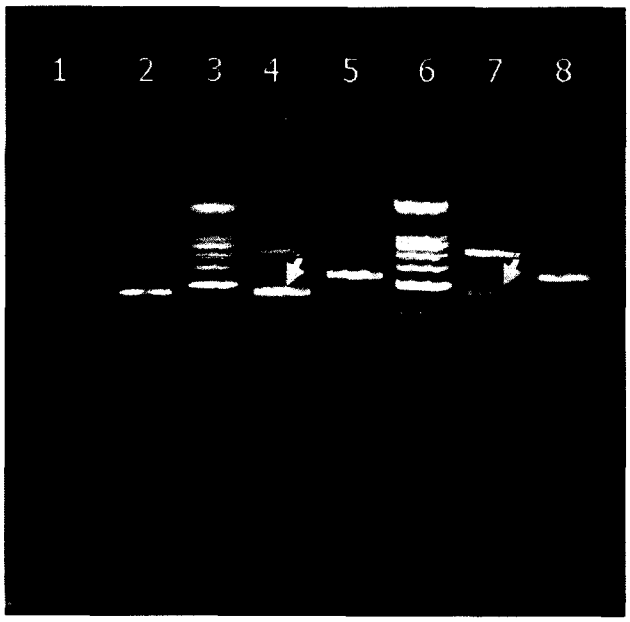


Fig. 4.12. Restriction mapping of recombinant clones TBT 1 and TBT 2 with Hind III and diagrammatic representation to indicate the insert size.



Lane 1 : Uncut pUC 18 DNA

Lane 2 : Hind III digested pUC 18 DNA

Lane 3 and 6 : Supermix DNA Marker

Lane 4 : Uncut clone TBT 3

Lane 5 : Hind III digested clone TBT 3

Lane 7 : Hind III digested clone TBT 4

Lane 8 : Uncut clone TBT 4

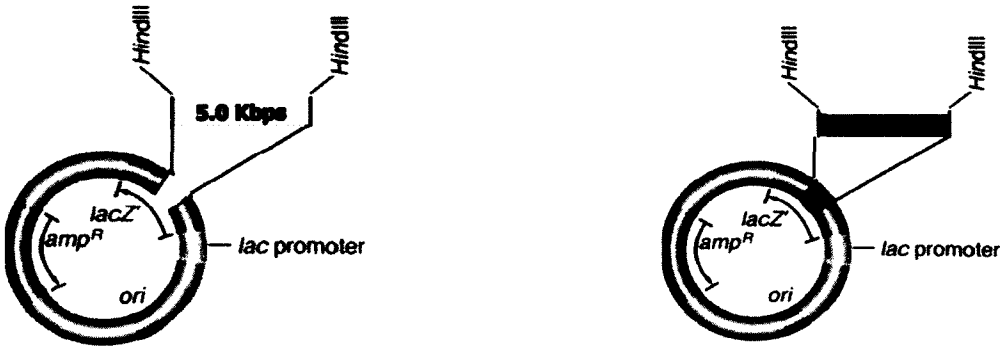
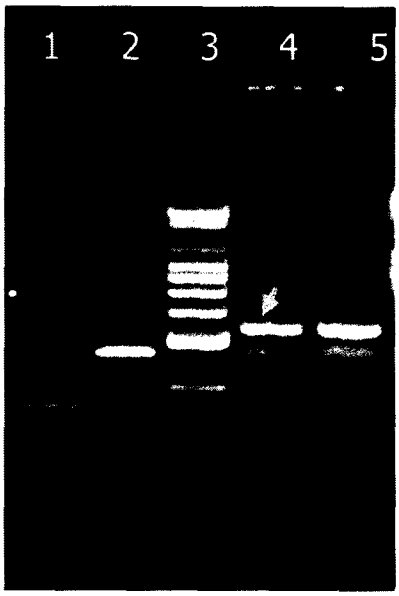


Fig. 4.13. Restriction mapping of recombinant clones TBT 3 and TBT 4 with Hind III and diagrammatic representation to indicate the insert size.



Lane 1 : Uncut pUC 18 DNA

Lane 2 : Hind III digested pUC 18 DNA

Lane 3 : Supermix DNA Marker

Lane 4 : Uncut clone TBT 5

Lane 5 : Hind III digested clone TBT 5

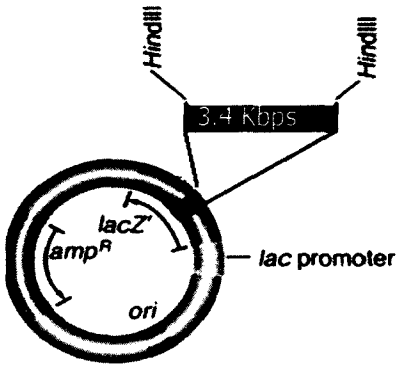
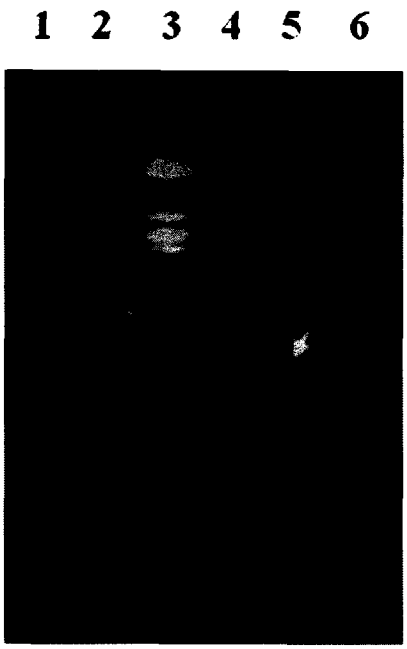


Fig. 4.14. Restriction mapping of recombinant clone TBT 5 with Hind III and diagrammatic representation to indicate the insert size.



Lane 1 : Uncut pUC 18 DNA

Lane 2 : Hind III digested pUC 18 DNA

Lane 3 : Supermix DNA Marker

Lane 4 : Uncut clone TBT 5

Lane 5 : Hind III digested clone TBT 5

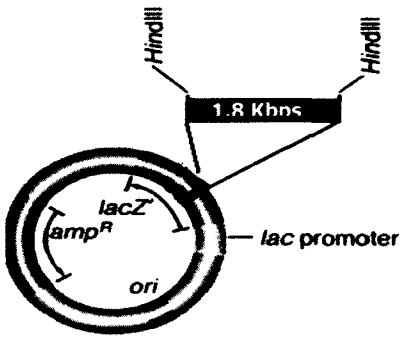


Fig. 4.15. Restriction mapping of recombinant clone TBT 6 with Hind III and diagrammatic representation to indicate the insert size.

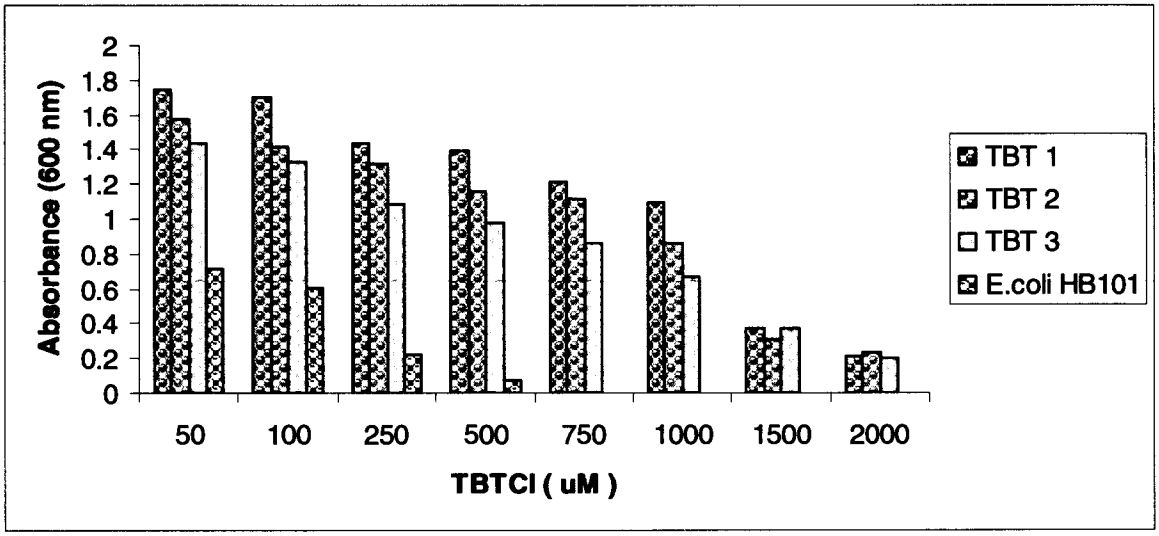


Fig. 4.16 TBTCl tolerance limit of clones TBT 1, TBT 2, TBT 3 and *E.coli* HB101 in MSM

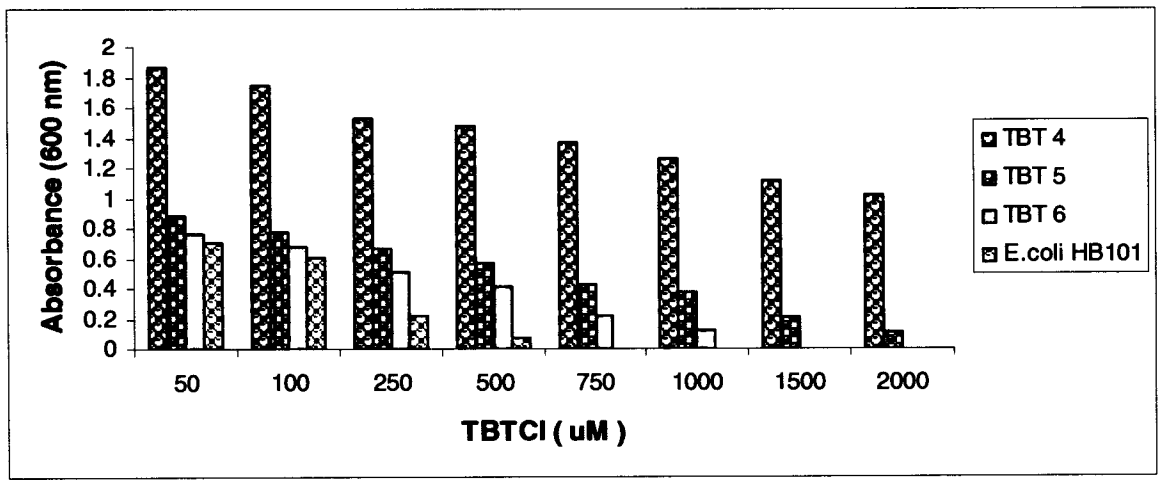


Fig. 4.17 TBTCl tolerance limit of clones TBT 4, TBT 5, TBT 6 and *E.coli* HB101 in MSM

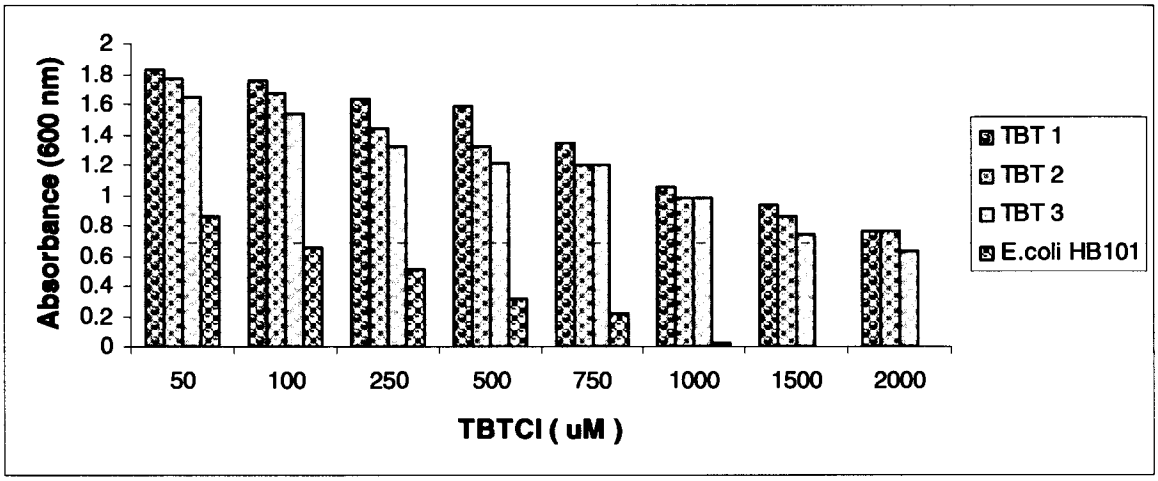


Fig. 4.18 TBTCl tolerance limit of clones TBT 1, TBT 2, TBT 3 and *E.coli* HB101 in LB broth

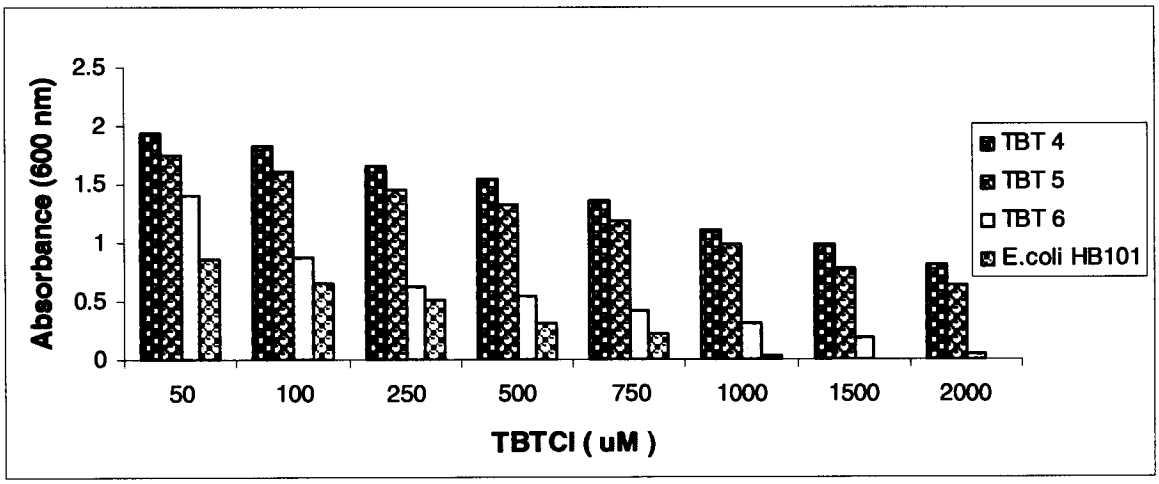


Fig. 4.19 TBTCl tolerance limit of clones TBT 4, TBT 5, TBT 6 and *E.coli* HB101 in LB broth.

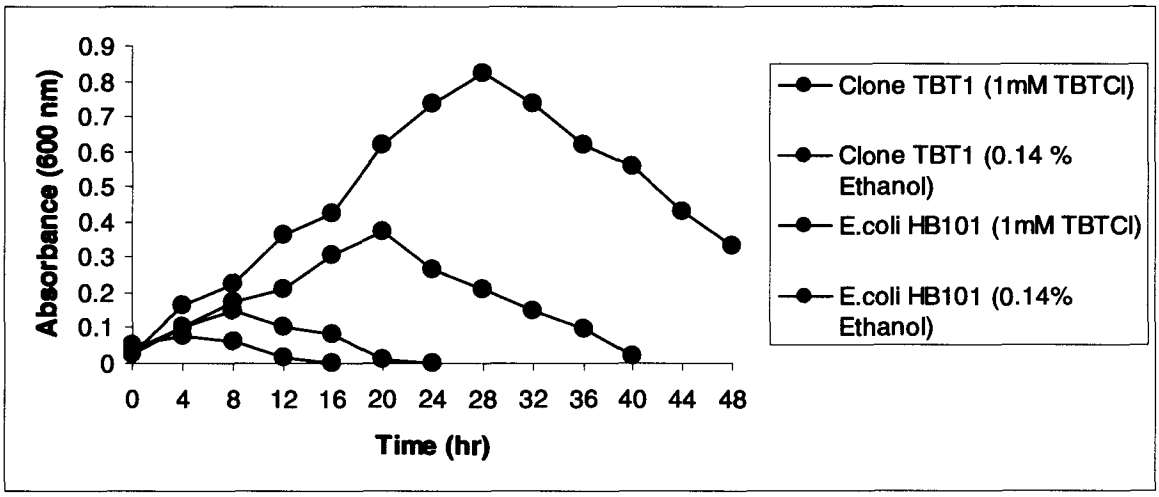


Fig. 4.20 Comparative growth pattern of recombinant clone TBT 1 and *E.coli* HB101 in MSM supplemented with TBTCl and ethanol separately.

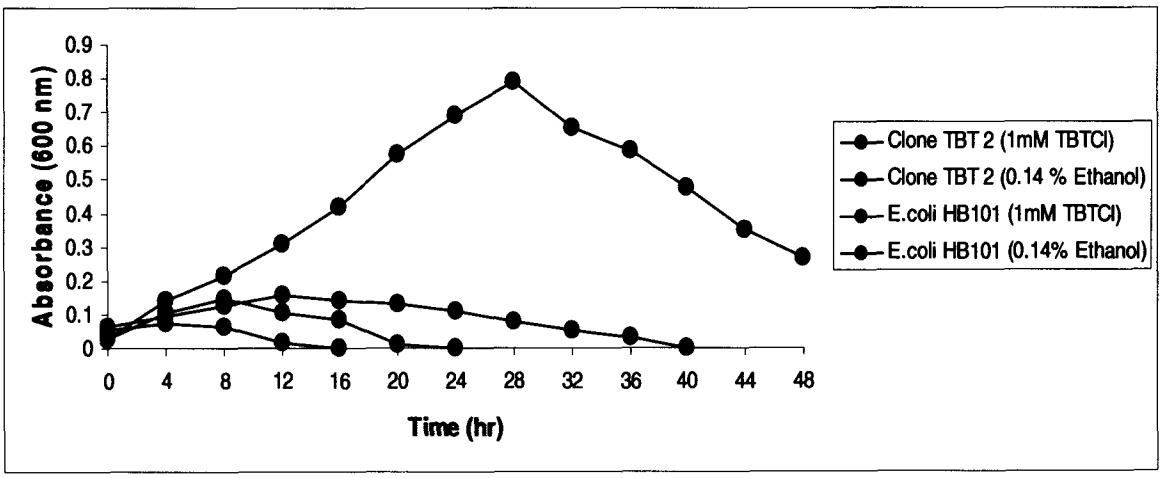


Fig. 4.21 Comparative growth pattern of recombinant clone TBT 2 and *E.coli* HB101 in MSM supplemented with TBTCl and ethanol separately.

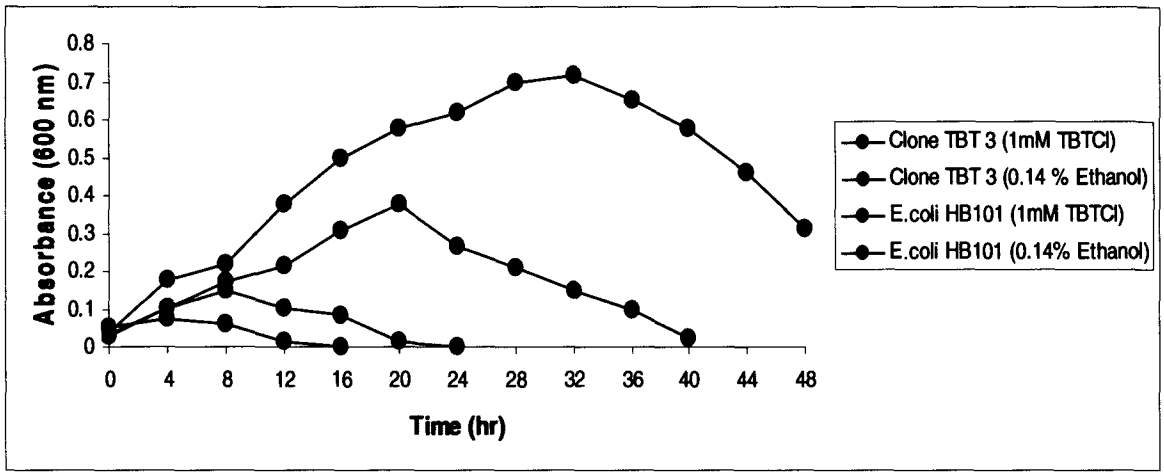


Fig. 4.22 Comparative growth pattern of recombinant clone TBT 3 and *E.coli* HB101 in MSM supplemented with TBTCl and ethanol separately.

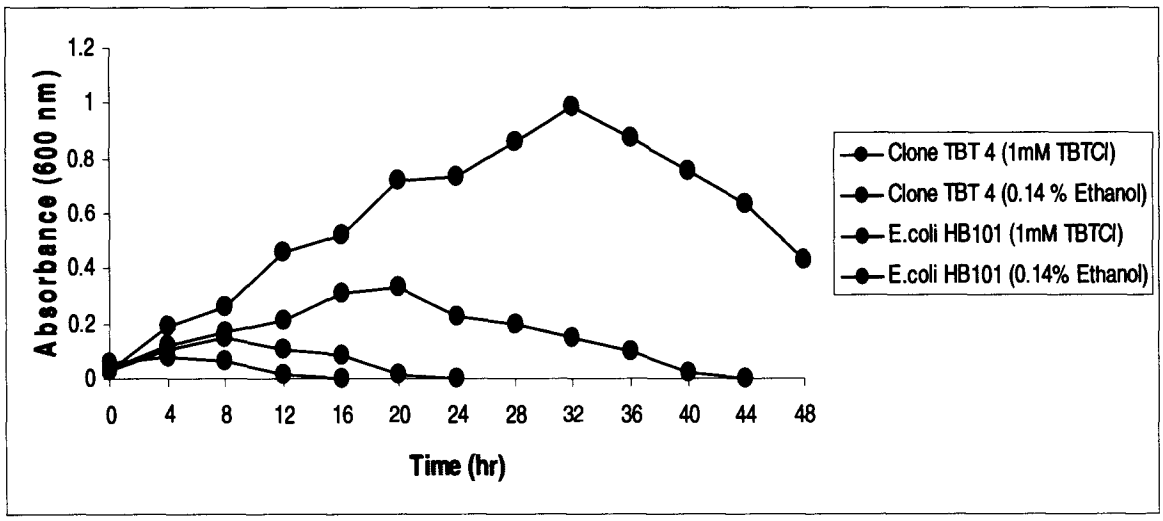


Fig. 4.23 Comparative growth pattern of recombinant clone TBT 4 and *E.coli* HB101 in MSM supplemented with TBTCl and ethanol separately.

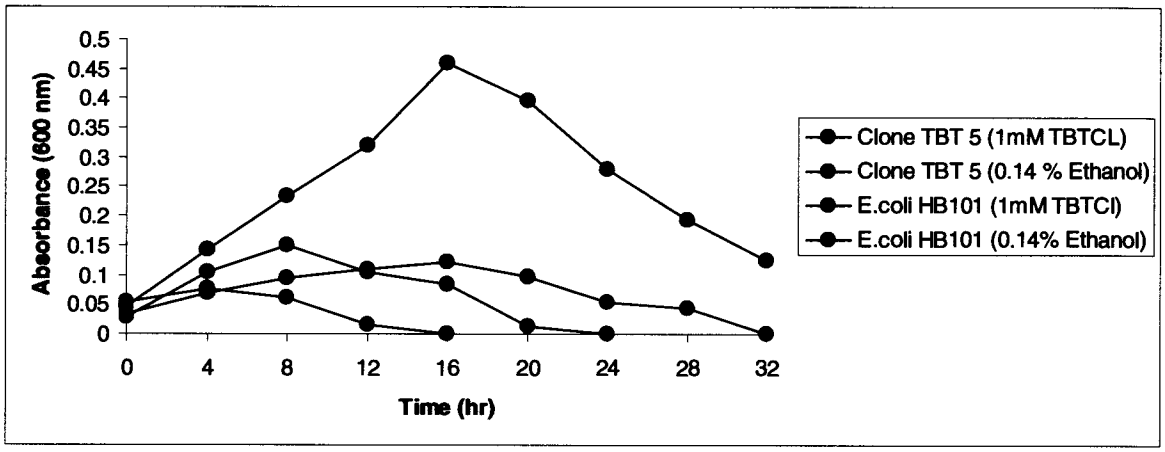


Fig. 4.24 Comparative growth pattern of recombinant clone TBT 5 and *E.coli* HB101 in MSM supplemented with TBTCl and ethanol separately.

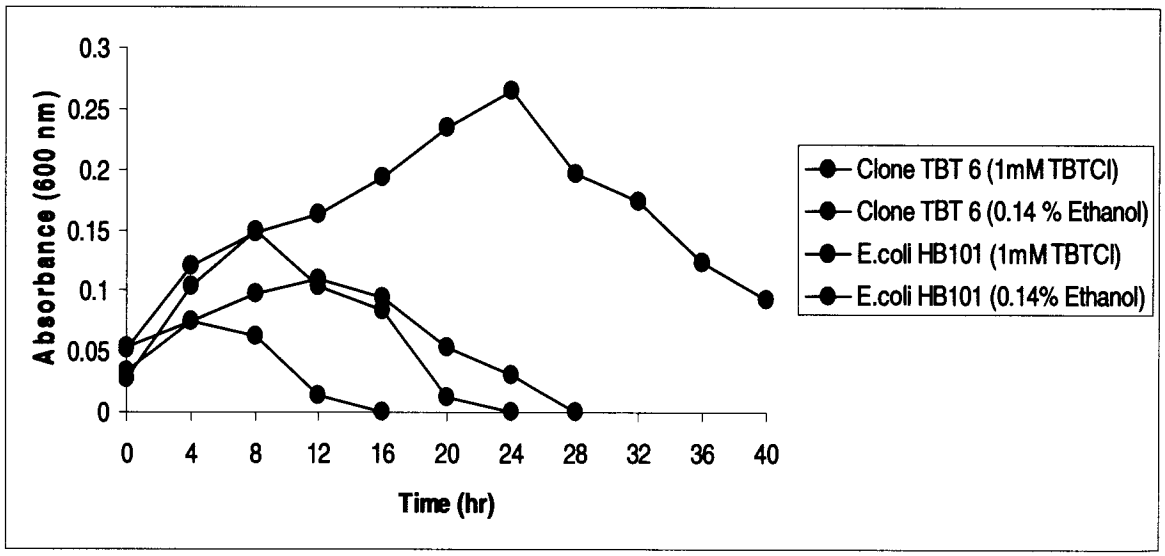
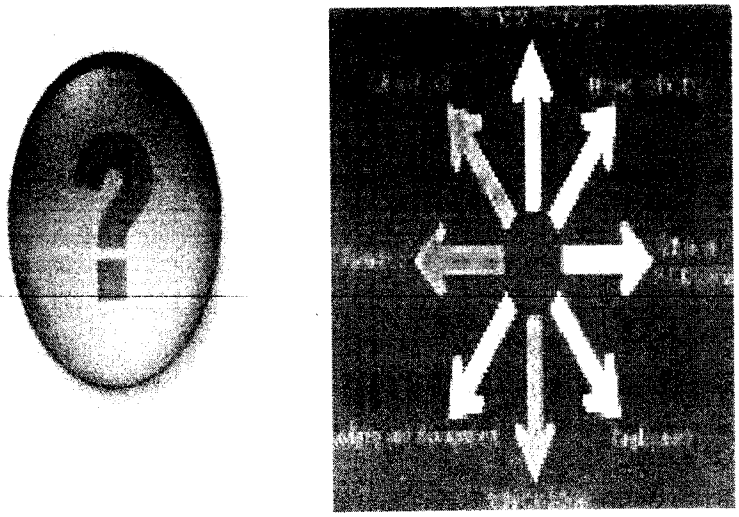
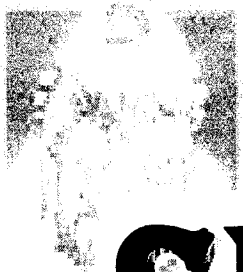


Fig. 4.25 Comparative growth pattern of recombinant clone TBT 6 and *E.coli* HB101 in MSM supplemented with TBTCl and ethanol separately.





CONCLUSIONS

SUMMARY

Salient Features

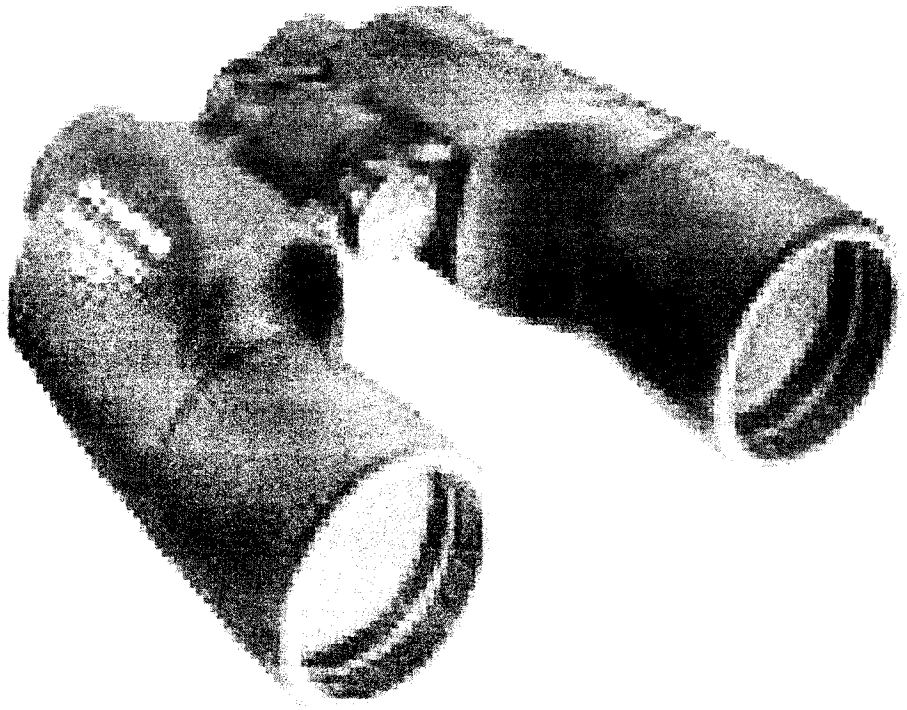
- The marine sediment isolate from Goa Shipyard Ltd., Goa, India was identified as ***Alcaligenes* sp.** which could **tolerate upto 4 mM** TBTCl whereas, the optimal concentration of TBTCl in MSM was found to be 2 mM.
- This is the first report of a bacteria tolerating very high concentration of TBTCl (i.e., 4mM) and **utilization of TBTCl as sole carbon source.**
- The optimal **pH, temperature & salinity (% NaCl)** for growth of the isolate in the presence of TBTCl in MSM was **7, 28 °C & 3 %** respectively.
- The isolate showed enhanced production of exopolysaccharide with increase in TBTCl concentration. The **highest** amount of **EPS was produced at 4mM of TBTCl.** The EPS produced had large amounts of **carbohydrate (26.3 µg/gm), proteins (20.07 µg/gm), uronic acids (11.79 µg/gm) & sulphates (2.12 µg/gm).**
- The protein profile under TBTCl stress showed the presence of 3 **induced proteins (68 kDa, 63 kDa & 43 kDa** respectively) & **up-regulation of 3 constitutive proteins (40 kDa, 30 kDa & 14.3 kDa).** All the 6 proteins were found to be periplasmic proteins. The growth versus protein expression study showed that **the proteins are induced / up-regulated after 12 hr of exposure to TBTCl.**
- This is the first report on induction of 3 periplasmic proteins under TBTCl stress.
- Plasmid curing experiments revealed that the **molecular mechanism** of resistance to TBTCl was **genomic DNA mediated**, since the cured cells could grow in MSM supplemented with 2 mM TBTCl.

- The recombinant TBTCl resistant clones were characterized for their **insert size**. Restriction mapping of recombinant plasmids revealed DNA inserts of **5.6, 5.7, 5.0, 5.2, 3.4 and 1.8 kbps** for **clones TBT 1, TBT 2, TBT 3, TBT 4, TBT 5 and TBT 6 respectively**.

- **All** the positive TBTCl resistance **clones could tolerate upto 1 mM TBTCl** in Nutrient **rich medium** (LB). But only **clones TBT 1, TBT 2, TBT 3 & TBT 4 could tolerate and grow in MSM with 1mM TBTCl indicating their capability to utilize it.** Interestingly, the positive clones TBT 5 & TBT 6 could not utilize TBTCl as sole carbon source possibly due to their small DNA insert.

- This is the **first report** on cloning of TBTCl resistance genes from a marine sediment isolate *Alcaligenes* sp. from West coast of India.

FUTURE PROSPECTS OF THIS STUDY



Future Prospects of the present study

- The bacterium, *Alcaligenes* sp. is a natural isolate that has been isolated from a high tributyltin contaminated site in Goa which can tolerate upto 4 mM TBTCl and utilize upto 2mM of this biocide as the sole source of carbon. This is the *first* report on a marine sediment bacterium that can utilize such high concentrations of TBTCl as sole carbon source. The results of the growth studies reported here clearly indicate that this bacterium exhibits gratuitous mode of metabolism for TBTCl since no other carbon source such as glucose or succinate is required as a primary carbon source. Hence, this isolate is a candidate to study in detail the molecular and biochemical mechanisms of degradation / resistance in sediment bacteria exposed to high concentrations of TBTCl. Using techniques like Flame Photometric Detection / Mass Spectroscopy and Atomic Absorption Spectrophotometry the pathway of degradation of TBTCl by this organism can be determined.

- Interestingly, this bacterium, *Alcaligenes* sp. shows enhanced production of a hetero-exopolysaccharide induced by TBTCl. In the present study we have chemically characterized this EPS. With this basic information it will be very interesting to study the role of this EPS in biofilm formation since the genus *Alcaligenes* is known to play an important role in formation of various natural biofilms. The results of this study will be fruitful to understand the role and importance of biofilms in sequestration of TBTCl also.

- The protein profile of *Alcaligenes* sp. when exposed to TBTCl shows induction of three proteins and up-regulation of three constitutive periplasmic proteins. Further

characterization and identification of these proteins would enable us to find out if these proteins facilitate degradation or utilization of TBTCl.

- In this study six clones containing the genomic DNA fragment of *Alcaligenes* sp. were obtained in *E.coli*. These TBTCl resistant clones will be further characterized to determine the DNA sequence of the gene (s) and in turn the corresponding enzyme (s) to study the mechanism of tolerance exhibited by this isolate.

There are very few reports on the mechanism of tolerance or degradation of TBTCl so far hence, these studies would ultimately enable us to understand the mode or mechanism by which marine bacteria tolerate such biocides and most importantly, to check the potential of these natural as well as genetically engineered bacterial strains for bioremediation of TBTCl contaminated marine and estuarine sites of coastal Goa, India.

APPENDICES

Appendix-A

Media composition:

A.1. Mineral medium (MM) (Double Strength) (Mahtani and Mavinkurve 1979):

a. $FeSO_4$. $7H_2O$ 60 mg, Dissolved in 250ml d/w

b. Stock solutions added as follows:

K_2HPO_4 (12.6%)	50 ml
KH_2PO_4 (18.20%)	10 ml
NH_4NO_3 (10%)	10 ml
MgSO4 (1%)	10 ml
$Na_2MoO_4.2H_2O$ (0.6%)	0.1 ml
$MnSO_4.H_2O$ (0.6%)	0.1 ml
$CaCl_2.2H_2O$ (0.1%)	7.5 ml

Add $CaCl_2$ drop by drop and stir make volume to 500ml with d/w. Store the medium in amber coloured bottle. Sterilize the medium in pressure cooker for 10 minutes. Adjust pH of the medium to 10.5 using 10% Na_2CO_3 solution, sterilized separately.

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)

Commercially available ZMB was used.

For agar plates add 1.5% agar to 100ml of Zobell marine broth and sterilized (autoclaved).

A.5 SOB

deionized H_2O, to 950 ml

tryptone 20 g

yeast extract 5 g

NaCl 0.5 g

Shake until the solutes have dissolved. Add 10 ml of a 250 mM solution of KCl. (This solution is made by dissolving 1.86 g of KCl in 100 ml of deionized H2O.) Adjust the pH of the medium to 7.0 with 5 N NaOH (approx. 0.2 ml). Adjust the volume of the solution to 1 liter with deionized H_2O. Sterilize by autoclaving for 20 minutes at 15 psi. Just before use, add 5 ml of a sterile solution of 2 M $MgCl_2$. (This solution is made by dissolving 19 g of $MgCl_2$ in 90 ml of deionized H_2O. Adjust the volume of the solution to 100 ml with deionized H_2O and sterilize by autoclaving for 20 minutes at 15 psi.

A.6 SOC

deionized H_2O, to 950 ml

tryptone 20 g

yeast extract 5 g

NaCl 0.5 g

SOC medium is identical to SOB medium, except that it contains 20 mM glucose. After the SOB medium has been autoclaved, allow it to cool to 60°C or less. Add 20 ml of a sterile 1 M solution of glucose. (This solution is made by dissolving 18 g of glucose in 90 ml of deionized H_2O. After the sugar has dissolved, adjust the volume of the solution to 100 ml with deionized H_2O and sterilize by passing it through a 0.22-µm filter.)

Appendix-B

Composition of stains and buffers and reagents

B.1. Stains

(i) Gram stain reagents

Crystal violet

Solution A-2g of crystal violet dissolved on 20ml ethanol

Solution B-0.8g ammonium oxalate dissolved in 80ml d/w

Mixed solution A and B and filtered through Whatman paper No. 1

Gram's iodine

Dissolved 1g iodine and 2g potassium iodide in 300 ml d/w. Filtered through Whatman filter paper No. 1 (diameter = 12.5 cm).

Safranine

2.5 g Safranine was dissolved in 10 ml ethanol made the volume to 100ml with d/w and filtered through Whatman filter paper No. 1.

Procedure

Prepared smear of the organism on a slide and heat fixed it. Flood the smear with crystal violet for a min. Washed with tap water and flooded with gram's iodine for a min. Washed with tap water and decolorized with 60% ethanol prepared in d/w. Counter stained with safranine for 45 seconds. Washed with tap water, blot dried with tissue paper and examined under oil immersion.

B.2 Buffers :

(i) Phosphate buffer (0.05M)

Solution A: (0.05M monobasic hydrogen phosphate): 6.0g of NaH_2PO_4 dissolved in 1000ml d/w.

Solution B: (0.05M dibasic hydrogen phosphate): 7.1g of NaH_2PO_4 dissolved in 1000ml d/w.

X ml of A + **Y** ml of B mixed to obtain buffers of the desired pH

X	Y	pH
87.7	12.3	6.0
39.0	61.0	7.0
5.3	94.7	8.0
2	98	9.5

(ii) Tris-HCl buffer (0.05M)

Solution A: (0.05M Tris): 6.0g of Tris dissolved in 1000ml d/w.

Solution B: (0.05M HCl): 4.4ml of 11.35 N HCl added to 1000ml d/w.

70ml of A and 30ml of B mixed to obtain buffer of pH 9.0

(iii) Tris-HCl buffer (0.1M)

Add Xml of 0.2 mol/lit HCl to 50 ml of 0.2 mol/lit Tris-base and make volume upto 100ml.

X ml	pH
43	7.2
24	8.2
6	9.0

Add 6ml of 0.2 mol/lit HCl to 50ml of 0.2 mol/lit Tris and make volume upto 100 ml.

(iv) Carbonate-bicarbonate buffer (0.2 M)

Solution A (0.2 M anhydrous sodium carbonate): 21.2g of anhydrous sodium carbonate dissolved in 1000ml d/w

Solution B (0.2 M sodium bicarbonate): 16.8g of sodium bicarbonate dissolved in 1000ml distilled water. 27.5ml of A + 22.5ml of B, diluted to a total volume of 200ml with d/w to obtain buffer of pH 10.0. Carbonate-bicarbonate buffer (pH10) was sterilized by autoclaving for serial dilution technique.

(v) Fractionation Buffer

Sucrose 20 g

EDTA 1 mM

Tris – HCl 30 mM

Make up the volume to 100 ml with distilled water and set the pH to 8.

Appendix C

C.1. Biochemical media used for identification (Gee *et al* 1980 and Sneath *et al* 1986)

Carbohydrate fermentation

Peptone	5.0g
Beef extract	3.0g
*Sugar	0.5g
D/w	To make 1L
O-Cresol red	0.01g
pH	10.5 adjusted by using 10% Na_2CO_3 solution

* arabinose, glucose, mannitol and xylose

Media with Durhams tube was autoclaved at 15 psi for 20 minutes. Tubes inoculated and incubated at R.T. for 24-48h. Change in color and presence or absence of gas bubble was noted. Uninoculated tubes serve as the control.

Nitrate reduction test

Media

Peptone	5g
Beef extract	3g
KNO_3	1g
d/w	Make volume to 1L
Na_2CO_3	10g
pH	10.5

Nitrate broth is inoculated and incubated at R.T. for 24-48h. After incubation, 5 drops of sulfanilic acid and 5 drops of α-naphthylamine were added. Red coloration indicated a positive test while in a negative test, red coloration is observed after addition of 5mg of zinc. Uninoculated tubes served as the control.

Citrate utilization test

Simmons citrate agar

Ammonium dihydrogen phosphate	1g
Diammonium phosphate	1g
Sodium chloride	5g
Magnesium Sulphate	2g
Sodium carbonate	10g
Agar	20g
d/w	Make volume to 1L
pH	10.5

Inoculate Simmon's citrate agar slants by means of stab inoculation and incubate for 24-48h at R.T. Observe the slants for presence or absence of growth. Citrate utilizers were indicated by the presence of growth on the slant.

Catalase test

Three or four drops of 3% (v/v) hydrogen peroxide were mixed with a loopful of culture in a plate. Evolution of gas bubbles caused by liberation of free oxygen was indicative of catalase positive organisms.

Oxidase test

A filter paper strip was soaked in Tetramethylparaphenylenediamine (TMPD) dye. A loopful of fresh bacterial culture was smeared on the moist filter paper. Production of a deep purple colour in 5-10 seconds indicated a positive oxidase test.

Gelatin liquefaction

Nutrient Gelatin

Peptone	5g
Beef extract	3g
Gelatin	120g
d/w	To make 1L
Sodium Carbonate	10g
pH	10.5

Inoculate tubes and incubate at R.T. for 24-48 h, the tubes were refrigerated for 30 min and the medium were observed. Liquid medium after refrigeration showed a positive test.

Starch hydrolysis

Starch agar medium

Peptone	5g
Beef extract	3g
Soluble starch	2g
Agar	20g
D/w	Make volume to 1L
Sodium Carbonate	10g
pH	10.5

Inoculate starch agar plates by spot inoculation. Incubate the plates at R.T. for 24-48 h, then flood the plates with Gram's iodine for 1 min and pour off the excess stain. Clear zone surrounding the colony indicated a positive test.

Casein Hydrolysis

Inoculate milk agar plates and incubate at R.T. for 24-48 h, then examine the plates for the presence or absence of a clear area around the colony. A clear area around the bacterial colony indicates a positive proteolytic activity.

Hugh and Leifson's test

Peptone	2g
NaCl	5g
K_2HPO_4	0.3g
Glucose	10g
O-cresol red	0.01g
Sodium Carbonate	10g
D/w	Make volume to 1L
pH	10.5

Heat in boiling water bath and cool immediately. Inoculate young culture in the medium and dispense into two tubes. The medium of one tube was overlayered with sterile liquid paraffin. Growth and color change of the indicator dye was noted in the two tubes. Strict aerobes grow only in aerobic conditions. Facultative anaerobes grow in both aerobic and anaerobic conditions. The anaerobic organisms grow only in anaerobic conditions.

C.2 Reagents for biochemical tests

Reagents for nitrate reduction

Solution A (Sulfanilic acid)

Sulfanilic acid	8g
Acetic acid (5N)	One part of glacial acetic acid added to 2.5 parts of d/w
D/w	to make 1L

Solution B (α-naphthylamine)

α-naphthylamine	5g
Acetic acid (5N)	1L

C.3. Solutions for determination of Buffering Capacity (Krulwich *et al* 1985 , Guffanti and Hou 1987 , Zychlinsky and Martin 1983) :

(a) **200mM KCl:** Dissolved 14.912g of KCl in 1000mld/w. (Mol. wt. of KCl = 74.56)

(b) **0.05MKOH**: Dissolved 2.811g of KOH in 1000mld/w. (Mol. wt. of KOH = 56.11)

(c) **10% Triton X-100**: Dissolved 10ml of Triton X-100 in 90ml of 200mM KCl and mixed well before use.

Appendix-D

Chemical Estimations and standard graphs

D.1 Folin Lowry's method for Proteins (Lowry et al 1953)

Reagent A: 2% Na_2CO_3 in 0.1 N NaOH

Reagent B: 0.5% $CuSO_4$ in 1% potassium sodium tartrate.

Reagent C: Alkaline Copper solution-Mixed 50ml of A and 1ml of B prior to use.

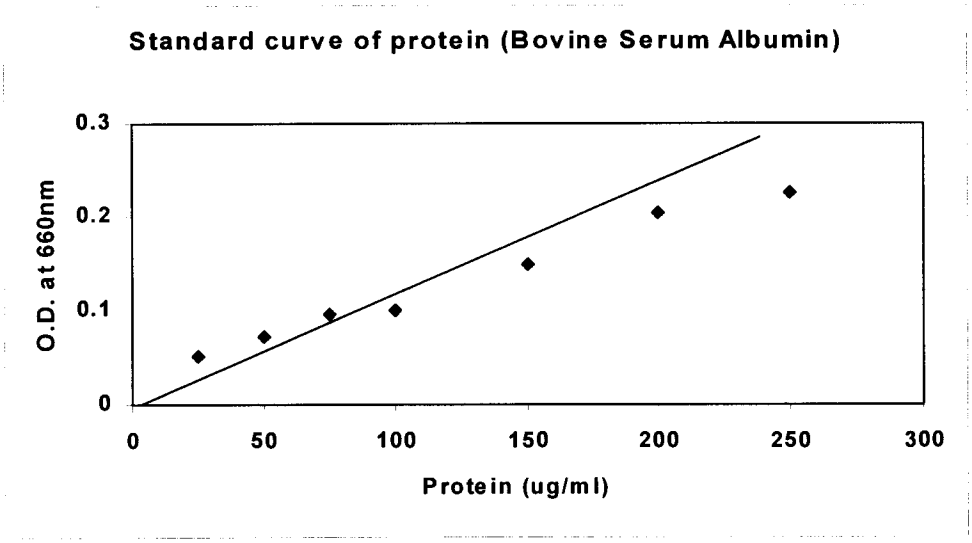
Reagent D: Folin and Ciocalteau's phenol reagent

Commercially available reagent diluted with equal volume of d/w on the day of use. This reagent is a solution of sodium tungstate and sodium molybdate in phosphoric and hydrochloric acids.

Standard bovine serum albumin solution: 0.1mg of BSA dissolved in one ml of d/w

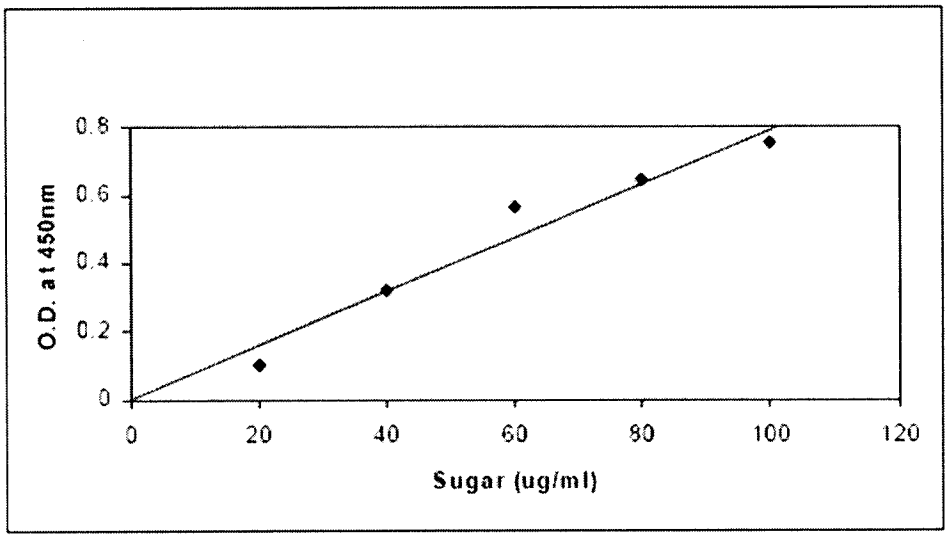
Procedure: To 1ml of the sample, 5 ml of copper sulphate solution was added and kept at room temperature in the dark for 10 minutes. 0.5 ml of Folin and Ciocalteau's phenol reagent was then added and kept in the dark for 20 minutes. Absorbance was measured at 750 nm against reagent blank and the concentration of the samples detemined from standard graph and Factor F calculated using Bovine serum albumin as the standard (0-100 μgms/ml).

Standard graph of protein :



D.2. Standard graph for sugar (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 10 min. The absorbance was measured at 490 nm. Standard curve was plotted using glucose (0-100 mg/mL) as standard.



Appendix-E

E.1. Stock Solution for PAGE and SDS – PAGE:

Acrylamide-bis-acrylamide solution [Monomer solution]: 29% acrylamide and 1% (w/v) N,N methylene *bis* acrylamide was dissolved in warm d/w. Checked pH to be 7.0 and stored in dark bottles at 4°C and used within 30 days.

Resolving gel buffer [1.5 M Tris, pH 8.8]: Prepared by dissolving 18.615g Tris, in 70 ml d/w water and added 100 µls of 10% SDS in d/w. The pH of the solution was adjusted to 8.8 using concentrated HCl and the volume was build up to 100ml with d/w. The solution was stored at 4°C.

Stacking gel buffer [1.0 M Tris pH 6.8]: Prepared by dissolving 12.11g Tris, in 70 ml d/w water and added 50 µls of 10% SDS in d/w. The pH of the solution was adjusted to 6.8 using concentrated HCl and the volume was build up to 100ml with d/w. The solution was stored at 4°C.

Ammonium per sulfate (APS, 10% w/v): Prepared by dissolving 0.1g of APS in 1.0 ml d/w. The solution was prepared afresh each time.

Electrophoresis buffer:

Composition of 1X buffer is as follows:

Tris	3.0g
Glycine	14.4g
SDS(10%)	10ml
D/w	to make 1000ml
pH	8.4

Sample buffer:

Composition of 4X buffer is as follows:

TriS- HCl (1 M pH 6.8)	0.04ml
Glycine	0.04g
SDS	0.004g
β-Mercaptoethanol	0.004ml
d/w	to make 10ml

Tracking dye:

50% sucrose	10 ml
Bromophenol blue	10mg

Staining Solution:

Coomassie Brilliant Blue G - 250 solution was prepared by dissolving 0.25g Coomassie Brilliant Blue G - 250 in 100ml of 25% methanol, 10% glacial acetic acid and 65% d/w.

Destaining Solution I

Methanol	40ml
Acetic acid	10ml
d/w	50ml

Destaining Solution II

Methanol	5ml
Acetic acid	7ml
d/w	88ml

Preparation of gel monomer

The composition of the resolving and stacking gels is as follows:

Solution	Resolving gel (10%) (ml)	Stacking gel (5%) (ml)
Monomer	2.5	0.33
1.5M Tris, pH 8.8	1.875	-
1.0M Tris, pH 6.8	-	0.625
10% SDS	0.075	0.025
10% APS	0.0375	0.025
D/w	0.003	1.525
TEMED	0.005	0.005

(a) **Preparation of sample:** 100 µls of cell pellet (containing 100 mg of protein) was mixed with 10µls of 25% SDS and boiled for 2 minutes at 100°C. 50µls of sample buffer was then added and boiled for 5 minutes at 100°C. After cooling, 20 µls of

bromothymol blue was added and 50µls of the samples were loaded in the gel with SDS PAGE molecular weight markers (sigma- St.Louis, MO USA).

(b) **Procedure:** The PAGE and SDS - PAGE were carried out in a Bangalore Genei apparatus. After a pre-run for 10 minutes, 30µls of the samples containing 50µgms of proteins along with the standard molecular weight markers were loaded in the gel. The electrophoresis was carried out at a constant voltage of 80 V for stacking gel and 120 V for resolving gel till tracking dye (Bromothymol blue) reached the bottom of the gel. At the end of the run, the gel was stained by Coomassie blue.

(c) Staining and destaining procedure:

i) **Coomassie blue staining**: The gel was stained in Coomassie Brilliant Blue G-250 solution Staining was carried out overnight; followed by destaining under mild shaking using de staining solution I for 3-4 hours and destaining solution II for several hours till the protein bands became clearly visible with no background colour. The gels were dried and preserved between cellophane sheets .

ii) **Silver staining of SDS-PAGE gels** (Sparnins and Chapman., 1976)

(i) Fixative solution

Ethanol	25 ml
Acetic acid	10 ml
D/W	100 ml
Dithiothreitol (5mg/ml)	
Dithiothreitol	0.5 mg
Double Distilled Water	100 ml

(ii) Silver nitrate solution (0.1%)

$AgNO_3$	0.1 gm
Double Distilled Water	100 ml

(iii) 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.

E.2 Solutions for DNA extraction and agarose gel electrophoresis :

(1) 0.5M Tris-HCl (pH 8)

Tris-HCl (mol. wt)-157.6

Dissolve 7.88gms of Tris-HCl in 50ml d/w and adjust pH to 8.0 using NaOH. Make up the volume to 100ml using d/w

(2) 0.1M Tris-HCl (pH 8)

Dissolve 1.576gms in 50ml d/w and adjust pH to 8.0 using NaOH. Make up the volume to 100ml using d/w

(3) TE buffer

10ml 0.1M Tris-HCl (pH 8) was diluted with 90ml of distilled water and 0.0372gms of EDTA was added and pH was adjusted to 8.0.

(4) **70% ethanol :** 70ml of ethyl alcohol + 30ml d/w

(5) Lysozyme (15 mg/ml)

15mg lysozyme + 1ml Tris-HCl (pH 8) vortex and store at 20°C

(6) TAE buffer (5X)

12.1 gms Tris-base

2.85ml glacial acetic acid - dissolve in 1000ml d/w

5ml of 0.5M EDTA. Adjust the pH to 8. Make the volume to 500ml using d/w.

Appendix- F

F.1 Tributyltin chloride (TBTCl)(C12H27ClSn)(F.W-325.49g/mol),obtained from MERCK ,Germany.

Preparation of 1M stock of TBTCl

Absolute ethanol (72.2 ml) + TBTCl (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.

F.2 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.

F.3 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).

F.4 Ampicillin

100 µg was weighed and dissolved in 1ml of double distilled water and filter sterilized. The solution was kept at 4°C.

F.5 Novobiocin

100 µg was weighed and dissolved in 1ml of double distilled water and filter sterilized. The solution was kept at 4°C.

F.5 $CaCl_2$ for preparation of competent cells

Reagent Amount	per liter	Final concentration
1 M potassium acetate (pH 7.5)	10 ml	10 mM
Magnesium Chloride	8.91 g	45 mM
Calcium chloride	1.47 g	10 mM
Potassium chloride	7.46 g	10 mM

H_2O to 1 liter

Sterilize the solution by filtration through a disposable prerinsed Nalgene filter (0.45-µm pore size).Dispense the solution into 40-ml aliquots and store the aliquots at 4°C.

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LIST OF PUBLICATIONS

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Vidya Ramachandran and Santosh Kumar Dubey (2008). Isolation and characterization of exopolysaccharide produced by a marine *Alcaligenes* sp resistant to Tributyltin chloride. (Communicated – Journal of Microbiology and Biotechnology).

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CURRENT SCIENCE

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Isolation and biological characterization of a tributyltin chloride degrading marine bacterium, *Vibrio* sp. from Bombay High Oil Field, India

Tri-organotins have a broad range of applications with an annual world production of approximating 50,000 tons/yr. These are most commonly used in marine antifouling paints, PVC stabilizers, as a biocide in agriculture and as preservatives for wood, leather, textiles and paper. Unfortunately, these compounds finally end up in the marine environment as a result of leaching. Their persistence in the marine environment has lethal, immunological, carcinogenic and teratogenic effects on non-target organisms. There are reports of few bacteria that can tolerate and degrade tributyltin chloride (TBTC) and evidences suggest that biodegradation is the major breakdown pathway in sedimentary environment. Although little is known about the resistance mechanism with which microorganisms tolerate TBTC, several TBTC-resistant marine bacteria have been isolated and characterized, *Alteromonas* sp. M-1 being the first record of its kind, where the presence of genes conferring TBT-resistance was reported[1–11]. Despite the regulations enforced to limit their use as anti-foulants, tributyltins are still present at toxic levels in the water columns and sediments[3]. It is interesting to note that there are microorganisms predominating in sediments of decks and harbours and also colonizing antifouling paints that contain high levels of TBTC[3,5,12–14]. Though bacterial strains from these econiches are slow TBTC degraders, they may prove to be a natural tool for bioremediation of marine sediments contaminated with organotins and other heavy metals. Hence it is important to isolate and study TBTC-resistant bacteria. This correspondence presents screening and biological characterization of a TBTC degrading bacterium from Bombay High Oil Field with reference to its growth behaviour in the presence of TBTC, limit of TBTC tolerance, TBTC utilization, cross tolerance to metals, viz. Cd, Hg and Mn, effect of TBTC on pigment and exopolymer production and plasmid profile.

The sample collected from Bombay High Oil Field was serially diluted and spread plated on Zobell Marine agar with 20 µM TBTC. The isolates obtained were subcultured in Zobell Marine Broth (ZMB) and maintained. The colony characteristics of the isolates were recorded and identified using biochemical tests, according to *Bergey's Manual of Systematic Bacteriology*[15]. One isolate which was circular, convex, butyreous, lactose, glucose and sucrose-fermentative, catalase and oxidase-positive, nitrate-reductive, facultative anaerobe, indole and MR-negative, VP-positive grew on TCBS medium producing green pigment and showed TBTC optima of 50 µM which was used for further studies, although it tolerated 100 µM of TBTC. Tolerance of the isolate to TBTC was checked by an antibiotic filter-disk method[16]. Growth in ZMB and mineral salts medium (MSM) with and without TBTC was determined in terms of absorbance at 600 nm after every two hours till the stationary phase was reached, and total cell protein was estimated using Lowry's method[17]. TBTC degradation was carried out using thin layer chromatography[18]. Intra and extracellular pigment was extracted by growing the culture in MSM broth with and without TBTC at pH 7.4, sonicating the cells (pulse of 15 s for 2 min) using ice jacket in acetone and centrifuged to collect clear supernatant (pigment extract). Pigment extract was scanned in the UV–visible range (190–500 nm) using a spectrophotometer. Extracellular pigment was extracted by standard procedure and scanned spectrophotometrically in the UV–visible range (190–500 nm). Growth and EPS production by the TBTC-degrading bacterial isolate was studied in mineral salts medium supplemented with NaCl (1.5%) and glucose (0.2%). The exopolymer was recovered from the culture supernatant using the cold ethanol precipitation–dialysis procedure[19]. Stock solutions of heavy metals $CdCl_2H_2O$, $HgCl_2$ and $MnSO_4$ were prepared separately in sterile, double-distilled water and filter-sterilized by passing through Millipore membrane filter (0.45 µm). LD_{50} values of heavy metal ions were determined in terms of growth and absorbance (A_{600}) recorded at an interval of every 2 h. The plasmid was isolated from overnight-grown cells by an alkaline lysis method[20]. The culture was cured with acridine orange (10–100 µg/ml). Colonies obtained after curing were checked for their ability to grow on TBT + MSM agar. The same colonies were then checked for the presence of plasmid in order to correlate loss/retention of TBTC resistance with loss of plasmids.

The pigmented isolate has been tentatively identified as *Vibrio* sp. according to *Bergey's Manual of Systematic Bacteriology*[15]. The growth pattern in terms of protein content[17] at different concentrations of TBTC revealed that 50 µM of TBTC was the optimal, and was hence used for further studies. Study of growth pattern of the isolate in ZMB with TBTC, showed an initial lag of 4 h. This is a new organic compound for which there is no known metabolic pathway for breakdown (Figure 1). The TLC profile of the deg-

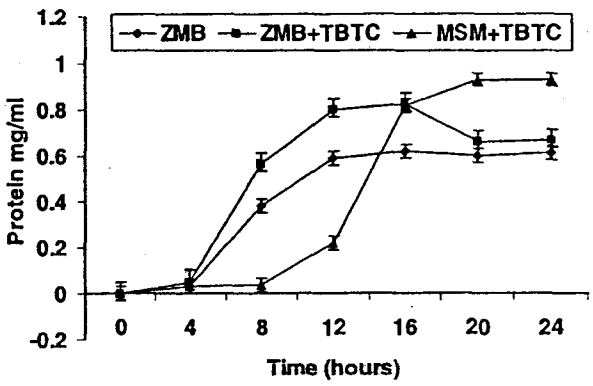

Figure 1. Growth of *Vibrio* sp. in media containing TBTC.

radation product clearly reveals depletion of TBTC and gradual transformation of this biocide into dibutyltin chloride (DBTC; Figure 2). It is interesting to note that pigment production was enhanced four-fold under TBTC stress. In a separate study exopolymer production in different media, viz. ZMB only, ZMB–TBTC (50 μM), and MSM–TBTC (50 μM), showed maximum yield in nutrient-rich media. The isolate showed a tolerance limit of 4.5 mM ($CdCl_2$), 4.0 mM ($MnSO_4$) and 0.2 mM ($HgCl_2$). The plasmid profile of the isolate showed the presence of a supercoiled plasmid. Loss of plasmid and concurrent presence of TBTC resistance in colonies of the isolate confirmed that TBTC resistance genes are not plasmid-borne.

Morphological characteristics and biochemical tests indicate the isolate to be a marine *Vibrio* sp., which produced blue-green extracellular pigment on TCBS, a selective medium. Though this marine isolate could tolerate up to 100 μM of TBTC, the optimum level of TBTC was found to be 50 μM. Interestingly, a lag of 8 h was noticed in the culture grown in MSM + TBTC (50 μM), whereas in ZMB it showed a lag of only 4 h. This lag might be due to the time taken by the isolate to acclimatize and utilize TBTC as a sole carbon source. The TLC profile of the chloroform extract of the cell pellet obtained after 24, 48 and 72 h of incubation revealed the presence of the degradation product which may be attributed to DBTC. The *Rf* values of TBTC and the transformed compound were 0.8 (solvent front – 15 ± 2, TBTC – 12.5 ± 1.5) and 0.94 (solvent front – 15 ± 2, product – 14.1 ± 2) respectively. This shows that the organism has some inherent mechanism to degrade TBTC[21]. The pigment produced by this isolate under TBTC stress showed that TBTC enhances the production of pigment, which could possibly act as a defense mechanism for cells against TBTC. Inoue (2000) reported the involvement of pyoverdin in co-metabolism of triphenyltin (TPT). It has been reported that pyoverdin from *Pseudomonas chlororaphis* CNR15 has a major role in TPT degradation. Interestingly, the isolate showed maximum yield of EPS when grown in ZMB + 50 μM of TBTC than in MSM + 50 μM of TBTC. As many reports suggest, most bacteria use carbohydrates as a carbon and energy source and the increased production may be attributed to TBTC/toxic metal sequestration. The isolate tolerated up to 4.5 mM $CdCl_2$, 4.0 mM of $MnSO_4$ and 0.2 mM $HgCl_2$. Plasmid-mediated bacterial heavy metal resistance has been extensively reviewed[22]. This TBTC-resistant marine isolate revealed the presence of a supercoiled plasmid. It is interesting to note that even after acridine orange curing of plasmid DNA, the bacterial isolate was able to grow on MSM agar with 50 μM TBTC. Thus, it clearly confirms that TBTC resistance is not plasmid-mediated.

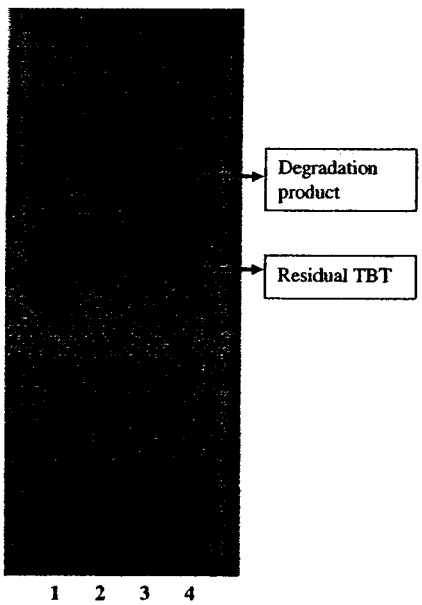

Figure 2. TBTC degradation by *Vibrio* sp. TLC profiles: Lane 1, crude TBTC; lane 2, Chloroform extract of cells after 24 h; lane 3, Chloroform extract of cells after 48 h and lane 4, Chloroform extract of cells after 72 h.

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R. KRISHNA MURTHY
L. CABRAL
R. VIDYA
SANTOSH KUMAR DUBEY*

Laboratory of Bacterial Genetics and Environmental Biotechnology, Department of Microbiology, Goa University, Goa 403 206, India
**For correspondence.*
e-mail: dubey_santoshI@rediffmail.com