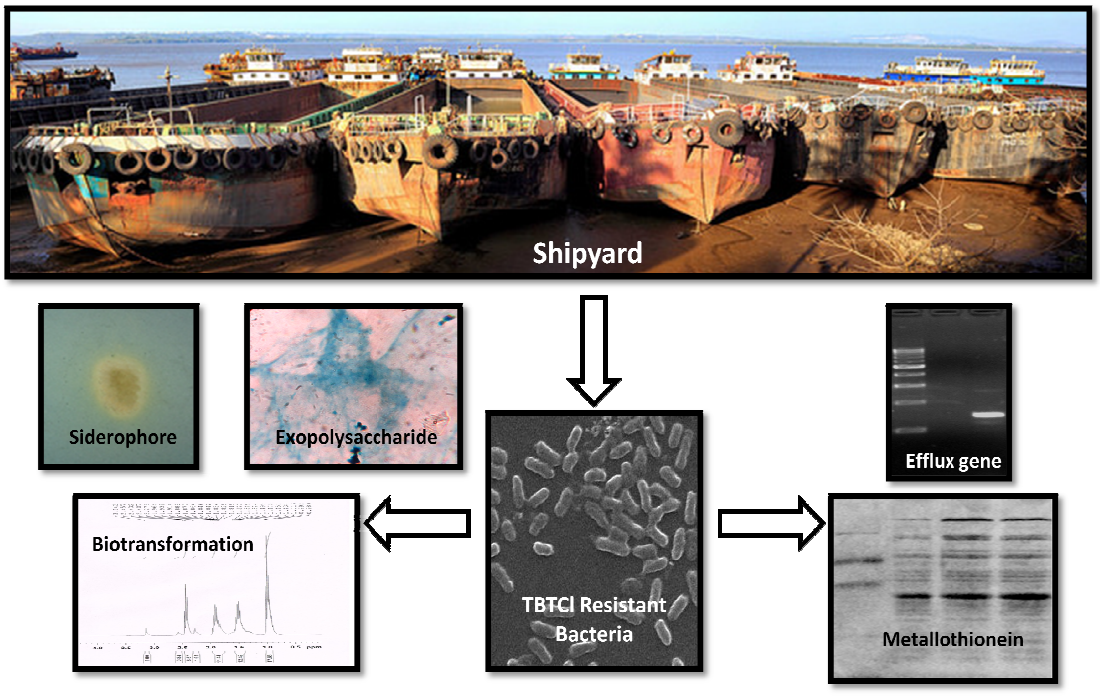
GOA UNIVERSITY
TALEIGAO PLATEAU
GOA – 403206

BIOCHEMICAL AND MOLECULAR CHARACTERIZATION OF TBTCl RESISTANT ESTUARINE BACTERIA FROM GOA TO EXPLORE THEIR RESISTANCE MECHANISMS



Ph.D. Thesis
by
Ms. Dnyanada Khanolkars

DEPARTMENT OF MICROBIOLOGY
2014

BIOCHEMICAL AND MOLECULAR CHARACTERIZATION OF TBTCl RESISTANT ESTUARINE BACTERIA FROM GOA TO EXPLORE THEIR RESISTANCE MECHANISMS

THESIS SUBMITTED TO THE

GOA UNIVERSITY

FOR THE DEGREE OF

DOCTOR OF PHILOSOPHY

IN

MICROBIOLOGY

BY

Ms. Dnyanada Khanolkar
M.Sc. Microbiology

Research Guide
Professor Santosh Kumar Dubey

Department of Microbiology
Goa University, Goa, India

2014

STATEMENT

I hereby state that this thesis for Ph.D. degree on **"Biochemical and Molecular Characterization of TBTCl Resistant Estuarine Bacteria from Goa to Explore their Resistance Mechanisms"** is my original contribution and that the thesis and any part of it has not been previously submitted for the award of any degree /diploma of any University or Institute. To the best of my knowledge, the present study is the first comprehensive work of its kind from this area.

Ms. Dnyanada Khanolkar
Ph.D. student
Department of Microbiology
Goa University
Goa

CERTIFICATE

This is to certify that Miss Dnyanada Khanolkar has worked on the thesis entitled **"Biochemical and Molecular Characterization of TBTCl Resistant Estuarine Bacteria from Goa to Explore their Resistance Mechanisms"** under my supervision and guidance.

This thesis, being submitted to the Goa University, Goa, India, for the award of the degree of Doctor of Philosophy in Microbiology is an original record of the 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.

Prof. Santosh Kumar Dubey, JSPS Fellow
Head
Department of Microbiology
Goa University

Professor Santosh Kumar Dubey
Research Guide
Goa University

Alone we can do so little; together we can do so much

- Helen Keller

This study started off as a small thought and progressed into being one of the biggest dreams of my life. In this journey to my goal, I wished to succeed as much as I wished to breathe. I am grateful to a lot of people involved in this work, as it definitely is team work that has made my dream work. Sometimes the most ordinary things could be made extraordinary simply by doing them with the right kind of people and I am glad to have found so many of them during the entire course of this study. I have experienced moments of hardship and failure and moments of euphoria and achievement too. During all these phases there have been several people standing by me like pillars of strength, support, optimism and positivity through the thick and thin, pushing my limits and motivating me to do better each day. With deep gratitude, I would like to dedicate this work to all of them as I consider myself blessed to have encountered such people in my life and without them this work wouldn't have been possible.

I am deeply grateful to my guide, ***Dr. Santosh Kumar Dubey****, my mentor, my teacher, my guiding light and the one who constantly had faith in me even at times when I didn't have in my own self. It is because of his immense patience, endurance, encouragement as well as criticism that this work has been successful. His scientific experience and innovative ideas have had a great influence on me and so also on this piece of research work. I would also like to thank him for extending the laboratory facilities required for this research work.*

I would like to express my gratitude to ***Prof. Saroj Bhosle****, Dean, Faculty of Life Sciences and* ***Dr. Sanjiv Ghadi*** *VC 's nominee, Faculty research committee, for the facilities provided and their valuable criticism towards the betterment of this research work.*

There comes a phase in everyone's life where one feels lost and left in the dark. In those moments we often pray to God and believe that he will get us out of the dreadful situation. Since God cannot be there everywhere, he sends certain people who make our lives easier. During the course of this work ***Dr. Milind Mohan Naik****, has truly been an angel in disguise for me. As a colleague, friend, philosopher, critic and advisor with his admirable problem solving approach to any given hurdle in life, he made me believe that there exists light at the end of the darkest tunnel and helped me walk towards it. This work deserves a special mention and gratitude for his enormous efforts and help extended towards its successful completion. I shall always be indebted to him for all that I have learned from him.*

I would like to thank ***Dr. Priya D'costa*** *for instilling faith and confidence in me, in my weakest moments and filling my life with positivity that shall stay with me forever. With her constant positive inputs, optimistic approach and undying faith in my potential, she helped me change my perspective towards life. It is people like her, who are truly beautiful as they always see beauty in others.*

My sincere thanks to ***Prof. Irene Furtado****,* ***Prof. Sarita Nazareth****,* ***Dr. Sandeep Garg****,* ***Dr. Lakshangy Charya*** *and* ***Dr. Vishwas Khodse*** *for their valuable suggestions during the study.*

I express my sincere gratitude to entire non-teaching staff including ***Mr. Shashikant Parab, Budhaji, Dominic, Ladu, Saraswati, Deepa*** *and* ***Narayan*** *for their constant help and support in various ways.*

I acknowledge the financial support provided by ***Goa University*** *in the form of Research Studentship. I am also thankful to* ***Dr. M S. Prasad*** *and* ***Mr. Vijay Khedekar*** *from National Institute of Oceanography, Goa, India for scanning electron microscopy. With deep gratitude I would like to appreciate the help extended by* ***Dr. Lisette Gomes****, NIO, Goa, India for permitting me to use the facilities available in her laboratory.*

I special thanks to my dear friends ***Bhakti Salgaonkar****,* ***Amrita Kharangate****,* ***Dr. Brenda D'costa*** *and* ***Akshaya Bicholkar*** *for always being there for me every time I was in dire need, for motivating me in various possible ways and boosting my spirit whenever I went through a low point in this research work.*

I am grateful to my colleagues, Drs. Anju, Vidya, Celisa, Nimali, Trelita, Subhojit, Sheryanne, Valerie, Marileou, Shweta, Mufeeda and Ph.D. students Sandesh, Cristabell, Sanika, Pramoda, Teja, Neha, Sandip Minaxi, Rahul, Kashif, Jaya and Sushma for encouraging me throughout this study. I would also like to thank my friends, Sumati, Kabilan, Meghnath, Krishna, Meera, Varsha, Chetali, Nadia, Manjita, Milind, Nilesh and Joephil for all the help and support they provided me with during this research work.

This research endeavor would have remained incomplete without the constant help and support of my beloved mother, ***Mrs. Unnati Khanolkar*** *who showed immense patience and confidence while educating me against all odds. I thank her for being there for me when no one else was and for making me what I am today.*

I have successfully compiled my creative and thoughtful research work due to genuine concern and painstaking efforts of many more well wishers whose names are not mentioned here, but may they know that they own a special place in my heart. I am in many ways also thankful to the hurdles, setbacks, challenges and all those people who refused to help me and let me down in various ways, for because of them I was able to move out of my comfort zone, explore greater avenues and become a better person. May God bless each and every one in abundance who has directly or indirectly been involved in this work.

In the entire duration of this work and even beyond that, I am thankful to the ***God*** *almighty, for meeting me in the form of different people, different situations and blessing me throughout.* ***Had he not held my hand, I wouldn't have come so far.***

ABBREVIATIONS

Abs	Absorbance	**Hg+2**	Mercuric ions
$AgNo_3$	Silver Nitrate	**Kbps**	Kilo base pairs
APS	Ammonium per sulphate	**KNO_3**	Potassium nitrate
b.p.	Boiling point	K+	Potassium ions
CFU	Colony forming unit	**kDa**	Kilo Dalton
°C	Degree Celsius	**L**	Litre
Ca^{+2}	Calcium ion	**LB**	Luria Bertani
Cd^{+2}	Cadmium ion	**$MBTCl_3$**	Monobutyltin trichloride
$DBTCl_2$	Dibutyltin dichloride	**MS**	Mass Spectrometry
d/w	Distilled water	**MSM**	Minimal salt media
EDTA	Ethylene diamine tetra acetic acid	**M**	molar
		µl	Microlitre
EPS	Exopolysaccharide	**mA**	milli ampere
Fig.	Figure	**mg**	milli gram(s)
FTIR	Fourier transform infrared spectroscopy	**mg+2**	Magnesium ions
		min	minute(s)
gm	Gram	**mM**	milli molar
GC	Gas chromatography	**ml**	milliliter
hrs	Hour(s)	**µg**	microgram
HCl	Hydrochloric acid	**µ**	Micron
H_2SO_4	Sulphuric acid	**µM**	micromolar

NA	Nutrient aga
NH_4NO_3	Ammonium nitrate
NH_4C1	Ammonium chloride
NaOH	Sodium hydroxide
nm	Nanometer
NaC1	Sodium chloride
O.D.	Optical density
PAGE	Polyacrylamide gel electrophoresis
%	Percentage
Pb+2	Lead ions
PCR	Polymerase chain reaction
rpm	Revolution per minute
RT	Room temperature
SDS	Sodium dodecyl sulphate
sec	Seconds
sp.	Species
Sn	Tin
SEM	Scanning Electron Microscopy
TEMED	Tetra methyl ethylene diamine
TMM	Tris-minimal medium
TOC	Total organic carbon
TBT	Tributyltin
TBTCl	Tributyltin chloride
UV	Ultra violet
V	Volts
v/v	Volume/Volume
w/v	Weight/Volume
ZMB	Zobell Marine Broth
Zn^{+2}	Zinc ions

INDEX

LIST OF TABLES

CHAPTER I

CHAPTER II

CHAPTER III

LIST OF FIGURES

CHAPTER I

CHAPTER II

CHAPTER III

CHAPTER IV

CHAPTER I

CHAPTER II

ISOLATION AND IDENTIFICATION OF TBTCl RESISTANT BACTERIA

CHAPTER IV

MOLECULAR BIOLOGICAL CHARACTERIZATION OF TBTCl RESISTANT BACTERIAL ISOLATES

MATERIALS AND METHODS **Page. No.**

RESULTS AND DISCUSSION **Page No.**

CHAPTER I

INTRODUCTION

1.1 Sources of Organotins in the environment

Organotins are ubiquitous and persistent organic pollutants in aquatic and terrestrial environment which include tributyltin oxide (TBTO), tributyltin chloride (TBTCl), tributyltin fluoride (TBTF), tributyltin hydroxide (TBTH), tributyltin naphthanate (TBTN), triphenyltin chloride (TPTCl) and tris(tributyl stannyl) phosphate (TBTP). Among these, Tributyltin (TBT) is a potent biocide used in marine antifouling paints to paint the submerged surface of ships and boats, cooling water pipes, docks, aquaculture cages and buoys along with fishing nets and marina platforms to prevent biofouling caused by barnacles, algae, mussels, tube worms and several other marine organisms (Corbin 1976; Clark et al. 1988; Seligman et al. 1986; 1988; Dowson et al. 1996; Dubey and Roy 2003; Bangkedphol et al. 2009; Sousa et al. 2010; Sampath et al. 2012; Ayanda et al. 2012; Lee et al. 2012; Pagliarani et al. 2013; Bernat et al. 2014). The tributyltin compounds belong to a subgroup of trialkyl organotin family and are the main active ingredients used to control growth of broad spectrum of organisms (Suzuki et al. 1994). Due to their antimicrobial activity they are used in textile industry and industrial water systems, as a biocide in agriculture, as preservative for wood, leather and paper and as a catalyst for making polyurethane foam and silicon rubber tube (Clark et al. 1988; White et al. 1999; Hoch 2001; Dubey and Roy 2003; Antizar-Ladislao 2008). It is interesting to note that TBT significantly inhibits biofouling caused by

adhesion of invertebrates (crustaceans), macroalgae and microbes on ship hulls and their submerged surfaces which otherwise would significantly reduce the speed of ships and boats subsequently increasing the fuel consumption and severely affecting the economy of a country. Organotins are tetra or divalent tin compounds having or more organic group(s). They form chemically stable compounds with with aliphatic as well as aromatic groups (Fig. 1.1). TBTCl used in antifouling paints is chemically bound in a co-polymer resin system via an organotin-ester linkage but there is a slow release of this biocide as the link gets hydrolysed when sea water comes in contact with paint's surface. This antifoulant biocide finally ends up in marine environment as a result of hydrolytic leaching and adversely affects the marine biota (Clark et al. 1988; Suzuki et al. 1994; Ayanda et al. 2012)

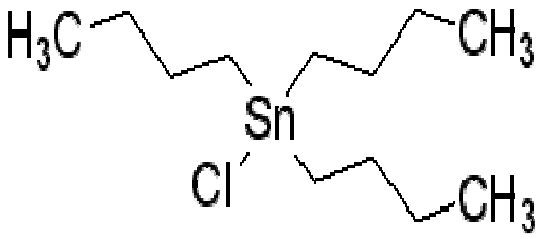


Fig. 1.1 Chemical structure of Tributyltin Chloride (TBTCl)

A large market exists for organotins in antifouling paints for ships and boats. The most common organometallics used in these antifouling paints include Tribultyltin oxide, Tributyltin chloride and Tributlytin

methacrylate. These paints can protect biofouling for more than two years and is superior to copper- and mercury-based paints. Currently TBT based paints have been manufactured as copolymers of chlorides, oxides and bromides which control the release of TBT and result in longer and effective life of the paint as an antifoulant (Clark et al. 1988; Bennett 1996; Champ and Seligman 1996; Ayanda et al. 2012). Due to these important attributes of TBT based antifouling paints, the U.S. Navy in the year 1984 proposed its application to paint hulls of naval ships. According to the U.S. Navy, use of TBT based paints as compared to other antifouling paints, would not only reduce fuel consumption by 15% but would also increase time duration between repainting from less than 5 years to 5 -7 years due to its stability in marine environments (Page et al. 1996). Such paints have been shown to be an effective and relatively long-lived deterrent to adhesion of barnacles and other fouling organisms. Thus due to its durability, high efficiency and reasonable cost, the usage of TBT in antifouling paints had increased in the past. The use of TBT in antifouling paints on ships, boats, nets, crab pots, docks, and water cooling towers probably contributes most to direct release of organotins into the aquatic environment. This proves to be a serious problem as it is released from fishing boats and nets into marine and estuarine waters along with sediments as a result of leaching and degrades slowly resulting in its global distribution in the marine environment (Seligman et al. 1986; 1988; Gadd 2000; Bangkedphol et al. 2009; Sousa et al. 2010; Sampath et al 2012; Ayanda et al. 2012).

The most important sources of organotin pollution are the ports, dockyards and marinas where TBT levels are appreciably high (Seligman et al. 1988; Page et al. 1996). Thus, the International Maritime Organization (IMO) prohibited the use of such organotins as antifouling biocides after 1st January 2008, in order to prevent terrestrial and aquatic pollution (IMO 2001). Although the use of TBT has been controlled in several European countries, United States and Japan, developing countries including India are yet to impose any such ban against the usage of TBT as an antifouling compound. Therefore TBT is still found in the marine environment in India.

Location	Concentration
Suva harbour, Fiji	38 $ug.g^{-1}$
Vancouver, Canada	10.78 $ng.g^{-1}$
Boston Harbour, U.S.A.	518 $ng.g^{-1}$
Lake Lucerne, Switzerland	400 $ng.g^{-1}$
Puget Sound, U.S.A.	380 ng. g^{-1}
Dona Paula Bay, India	133 ng. g^{-1}

Table. 1.1 Noticeable levels of organotins in the world

1.2 Toxicity of Organotins

While inorganic forms of tin are of relatively low toxicity, the more lipid soluble organotins are highly toxic to bacteria and fungi. Organotins belong to the most toxic pollutants known so far to aquatic life forms due to high toxicity, high environmental persistence, and often high mobility, resulting in groundwater contamination (Florea

and Busselbergh 2006). It is interesting to mention that the high lipophilicity of organotin compounds, resulting in its bioaccumulation in food chains and food web. Although it has proven to be effective in controlling colonization of submerged surfaces by the Zebra mussel, *Dreissena polymorpha* and barnacles, but it shows high toxicity to a variety of non–target organisms at levels as low as 1-10 parts per trillion Sn (Page 1989; Stab et al. 1995). Benthic organisms are also affected by TBT because it is preferentially adsorbed onto clays and clay rich sediments. It's persistence in the marine environment has lethal, immunosuppressive, carcinogenic and teratogenic effects on non-target organisms (Bryan et al. 1988; Clark et al. 1988; Cooney 1989; Florea 2005; Antizar-Ladislao 2008; Ayanda et al. 2012, Pagliarani et al. 2013; Arp et al. 2014). It has also been reported to induce larval malformations in oysters (Gibbs et al. 1991). TBT is toxic to both prokaryotes and eukaryotes including humans. Molluscs are unusually sensitive to TBT because they have low activities of cytochrome P-450 and mixed function oxidases, leading to TBT accumulation in tissues since TBT is metabolized slowly. Accumulated TBT in molluscs causes a significant increase in testosterone leading to a condition known as imposex, wherein female animals develop male sex organs and the population cannot reproduce.

Because organic tin compounds accumulate, or biomagnify, in the food web to some degree, they may eventually end up in humans, when food containing them, such as oysters and fish, are consumed (Gibbs et al. 1991; Maguire 2000; Hoch 2001; Antizar-Ladislao 2008). Three factors contribute to the extent of organotin

biomagnification in a food-chain: i) persistence and non-degradation in the environment; ii) food chain energetics; iii) non-degradation and non-excretion of the organotins from the internal environment of the organisms due to its hydrophobicity. The most common mode of TBT uptake and accumulation in organisms is generally thought to be via the diet or nutrition at the sediment–water interface (Maguire 2000). Human exposure in general to TBT is through seafood which is the most prominent source of TBT contamination in the marine and estuarine environment (Miller and Cooney 1994; Mendo et al 2003; Eggleton and Thomas 2004).

Organotin toxicity in microorganisms increases with the number and chain length of organic moiety bonded to the tin atom. The tri- and di-substituted derivatives are the most toxic organotins. The acute toxicity of tri-alkyltins rapidly declines with the decrease in length of the alkyl radical, mostly because of their lower gastrointestinal absorption (Stoner et al. 1955; Barnes and Stoner 1959). Tetra-alkyltins becomes toxic after the loss of one alkyl group. Tetra-organotins and inorganic tin compounds possess least toxicity.

In general organotins are membrane permeable due to their lipophilicity. Therefore, the site of action of organotins is mostly at the cytoplasmic membrane and intracellular level. Hence, surface adsorption and/or accumulation of organotins within the cell might lead to severe toxic effects in the living organisms (White et al. 1999; Florea 2005). There exist four different types of organotin toxicity

based on the target organ: neurotoxicity, hepatoxicity, immunotoxicity, and cutaneous toxicity (Snoeij et al. 1987). Organotins have several lethal effects on microorganisms thereby preventing biofouling due to them (Table. 1.2).

Effects of TBT that have commonly been reported include:

(i) Interference with biological membranes, disturbing their integrity and ultimately compromising their physiological functions in prokaryotic and eukaryotic organisms.

(ii) Disruption of endocrine system in oysters.

(iii) Inhibition of the uptake of amino acids and cell growth in bacteria. For example, *E. coli* membrane bound ATPase and energy dependent pyridine dinucleotide transhydrogenase (TH) are inhibited by TBT.

(iv) Growth inhibition, immune suppression and imposex in higher animals.

Process Affected	**Organisms**	**Inhibitory Concentration**
Respiration	Bacteria	0.04-1.7 µM
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
Photophosphoryraltion & 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

Table. 1.2 Toxic effects of TBT on microorganisms

However, a number of reports have documented that since 1980s, extremely low environmental levels of TBT can cause lethal and sub-lethal effects on non-target organisms, e.g., imposex or intersex in several species of gastropods; malformation in oysters (*Crassostrea gigas*), increased mortality and retardation of growth in larvae of blue mussels (*Mytilus edulis*) and disappearance of clams (*Scrobicularia plana*) in UK waters (Antizar-Ladislao 2008).

1.3 TBT as a Persistent Organometallic Pollutant (POP)

Antifouling paints prevent biofouling by continuous release and formation of a thin layer of highly concentrated hydrated organotin around the ship hull. Organotin compounds leach out continuously from the ship hulls irrespective of whether the ship is sailing or docked at the harbor. Rate of hydrolytic leaching of organotins is also controlled by paint characteristics, mainly due to binding chemicals (Thouvenin et al. 2002). Organotins have high tendency to adsorb onto suspended clay-rich sediments and organic matter (Poerschmann et al. 1997; Arnold et al. 1998; Hoch 2001). Approximately 95% of tributyltin in the water column is bound to suspended particles, including plankton and sediment particles, while the remainder is largely bound to specific ligands on dissolved organic matter. Prior to the total ban of TBT for painting ship hulls the concentration of TBT in the water columns ranged from 1-200 ng l^{-1} in harbors and marinas around the world (Seligman et al. 1988; Fent 1996; Harino et al. 1997; Antizar-Ladislao 2008). However, the concentration of TBT is approximately three orders of magnitude higher in sea floor sediments than in the water columns (Valkirs et al. 1986). In addition, as the hulls of ships are refinished or subjected to other physical scraping, organotins (TBTs) get detached from the ship

hulls and rapidly settle down in the marine sediment. Environmental Protection Agency (EPA), U.S.A. issued an '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 1 ng l^{-1}. Despite the bans and several other restrictions in force, this biocidal antifouling agent is still present in appreciably high concentrations in water columns and sediments of aquatic environments with high ship building activities in the coastal areas.

Ship building industry in Goa is one of the major industries and aquatic organisms viz. fish, shrimps, shell fish and oysters are the most common sea foods consumed by the people of Goa along with domestic and international tourists. It has been reported that the total concentration of butyltins from the Dona Paula Bay of Goa, India in the surface waters ranged from 21-89 ng l^{-1}, in biofilm samples 10-822 ng g^{-1} and in tissues of marine organisms 58-825 ng g^{-1} dry weight (Bhosle et al. 2004). This is almost 20-800 times higher than the toxic levels reported by EPA. Therefore the chances of TBT contamination and biomagnification are high in this region along with other coastal cities of India exposed to toxic levels of organotins. Similarly levels of organotins in Zuari estuary sediment of Goa, India (Jadhav et al. 2009) ranged from 20-7621 ng Sn g^{-1} which was 70-90% of the total butyltins. The Butyltin degradation index (BDI) for the Zuari sediments was not very impressive that ranged from 0-2.7 indicating a lot of fresh input of butyltins in the estuary and a lower degradation rate (Jadhav et al. 2009). Butyltin concentration in Mandovi and Zuari estuaries in water ranged from 12-73 and 0.5-77 ng Sn l^{-1} respectively whereas, the concentration of butyltin in sediment ranged from 15-118 and 6-119 ng Sn g^{-1} dry weight respectively (Garg et al. 2010). These reports suggest slow degradation of this biocidal antifoulant

resulting in its persistence in the marine water columns and sediments. TBT and its degradation products dibutyltin (DBT) and monobutyltin (MBT) present in water columns and sediments are of environmental concern. It is interesting to mention that these degradation products are less toxic than TBT.

1.4 Mechanisms of TBT degradation

TBT is a persistent, recalcitrant organic pollutant which is degraded very slowly in the estuarine and marine environment due to its long half life ranging from several months to years (Clark et al. 1988; Rajendran et al. 2001; Ayanda et al. 2012). It is interesting to mention that there are several factors which govern the TBT degradation process in the aquatic environment. These factors may be categorized in two major categories viz. biotic and abiotic.

1.4.1 Abiotic Factors

Abiotic factors which are responsible for degradation of TBT in the environment include UV and gamma rays, hydrolysis, high temperature (above 200 ^{o}C) and treatment with strong acids or electrophilic agents. Photolysis and hydrolysis are abiotic processes, but in the temperature- and pH-conditions of natural water environments only photolysis is significant as an abiotic degrader in breaking down organic tin compounds (Rudel 2003). The Sn-C bond is stable up to temperatures of 200°C (Kotrikla 2009). In photocatalytic degradation, UV light and the resulting hydroxyl-radicals degrade TBT. However, water turbidity often effectively blocks photocatalytic degradation. In addition to photolysis, chemical degradation is also an abiotic degradation process, having some significance in natural

conditions (Gadd 2000; Kotrikla 2009). Strong acids and electrophilic substances are capable of cleaving the bond between tin and carbon. It is interesting to note that organic tin compounds can also be transformed by methylation or dismutation (Rantala 2010).

The solubility of TBT compounds in water is influenced by factors viz. oxidation-reduction potential, pH, salinity, temperature, ionic strength, concentration and composition of the dissolved organic matter (Corbin 1976; Clark et al. 1988). The solubility of tributyltin oxide in water also varies with the highest solubility being at acidic pH (Maguire et al. 1983). An increase in NaCl concentration has also known to reduce TBT toxicity in microorganisms. The presence of Na^+ and Cl^- ions causes an osmotic response in the organisms changing their intracellular compatible solutes and membrane composition (Cooney et al. 1988). The carbon-tin covalent bond does not hydrolyze in water (Maguire et al. 1983), and the half-life for photolysis due to sunlight is greater than 89 days (Maguire et al. 1985; Seligman et al. 1986). In estuarine waters the typical half life of TBT is 6-7 days at 28^oC. However in deeper anoxic sediments degradation is much slower (i.e. 1.9 – 3.8 yrs) resulting in persistence of TBT for several years. Thermal cleavage is also one of the mechanisms of TBT degradation which occurs only above 200^oC. Whereas chemical cleavage is a rare phenomenon occurring in natural environments with tributyltin contamination. Only the near UV spectrum (300–350 nm) is likely to cause direct photolysis of TBT. Although due to the low transmittance

of UV light, this process occurs only in the upper layer of the water columns.This clearly indicates that abiotic factors also play an important role in degradation of TBT but the rate of degradation is slow and varies with reference to different abiotic factors.Since abiotic pathways of tributyltin degradation are time consuming and poor, with tributyltin having half-lives of several days to weeks in water, and from several days to months or more than a year in sediments (Maguire and Tkacz 1985; Stang and Seligman 1986; Clark et al. 1988; Seligman et al. 1989; Maguire 2000; Stang et al. 1992), biodegradation proves to be the major and most reliable breakdown pathway for detoxification of TBT in water and sediments.

1.4.2 Biotic Factors

Biotic factors play an important role in biodegradation of TBT which includes various microorganisms including bacteria. Uptake of TBT by microorganisms has been largely overlooked when considering the fate and effect of organotins in the aquatic environment (White et al. 1999; Gadd 2000). These interactions are important because microorganisms are at the base of the food web and mediate a number of important environmental processes for bioaccumulation and degradation of TBT. Few studies have focused on the uptake mechanisms of organotins even though their accumulation is a prerequisite for subsequent toxic effects. Microbial uptake mechanisms may be based on the cationic and/or lipophilic properties of organotin compounds. Non-metabolizing cells may accumulate metal ions by processes frequently termed as biosorption i.e. the binding of metals by ion exchange, adsorption, complexation, precipitation and crystallization within the cell wall (Tobin

et al. 1984; Gadd 1990; Iyer et al. 2004). Organotins also participate in lipophilic interactions with cellular membranes. Uptake of lipophilic metal complexes by membrane diffusion mechanisms may occur in addition to, or in place of biosorption processes. Microorganisms may also posess cetain proteins which may attribute to TBTCl degradation in the contaminated environments (Table. 1.4). Several such mechanisms have been proposed by researchers however, relatively little is known about the exact mechanisms of organotin-cell interactions (White and Tobin 2004; Antizar-Ladislao 2008).

1.5 TBTCl Resistant and Degrading Microorganisms

The degradation of TBTCl in natural environment appears to be mainly governed by microorganisms. These may be bacteria, fungi, cyanobacteria and green algae from terrestrial as well as aquatic environments. Microorganisms capable of TBT bioaccumulation and degradation include certain fungi, viz. *Coniophora puteana*, *Trametes versicolor*, *Chaetomium globosum*, *Aureobacidium pullulans* and *Cunninghamella elegans* and bacteria viz. *Alcaligenes faecalis*, *Flavobacterium* sp. *Vibrio* sp., *Pseudoalteromonas* sp. several *Pseudomonas* spp., *Klebsiella* sp.(Table. 1.3). Breakdown products of tributyltin include dibutyltins (DBT), monobutyltins (MBT) and tins have also been transformed into methyltins by sulfate reducing bacteria, *Desulfovibrio* sp. (Cooney 1988; Wuertz et al. 1991; Yonezawa et al. 1994; Kawai et al. 1998; Pain and Cooney 1998; Dubey and Roy 2003; Suehiro et al. 2006; Antizar-Ladislao 2008;

Bangkedphol et al. 2009; Sakultantimetha et al. 2009; Sousa et al. 2010; Ayanda et al. 2012).

It has been demonstrated that the susceptibility to TBT varies in bacteria according to the structure of the cell wall. It was shown that TBT is less toxic to Gram negative bacteria since growth is observed up to 900 ng Sn ml^{-1}, in case of Gram negative bacteria whereas Gram positive bacteria showed suppression of growth above 400 ng Sn ml^{-1} (Mendo et al. 2003). Gram negative bacteria resistant to organotins include *E. coli*, *Pseudomonas fluorescens*, *P. aeruginosa*, *Proteus mirabilis*, *Serratia marcescens* and *Alcaligenes faecalis*, and the Gram positives include *Staphylococcus aureus*, *S. epidermidis*, *Bacillus subtilis*, *Mycobacterium phlei* and *Vibrio* spp. (Dubey and Roy 2003). Microbial degradation of TBTCl has been reported as a major process in sea water (Seligman et al. 1988) therefore biological treatment of TBTCl – contaminated wastewaters has got greater potential for bioremediation.

TBT Resistant Microbes	
Bacteria	***Pseudomonas aeruginosa***
	Pseudomonas stutzeri
	Pseudomonas fluorescens
	Pseudomonas chlororaphis
	***Alteromonas* sp.**
	***Vibrio* sp.**
	Serratia marcescens
	Staphylococcus aureus
	Aeromonas molluscorum
Fungi	***Coniophora puteana***
	Trametes versicolor
	Chaetomium globosum
	Aureobacidium pullulans
	Cunninghamella elegans

Table. 1.3 TBT Resistant Microorganisms

1.6 Mechanisms Involved in TBTCl Resistance

Despite its toxicity to majority of organisms, TBT resistant bacteria have been reported from TBT contaminated estuarine and marine ecosystems (Suzuki and Fukagawa 1995; Jude et al. 2004; Krishnamurthy et al. 2007, Cruz et al. 2007). Bacteria play an important role in biogeochemical cycles along with toxic organic matter degradation and recycling in marine ecosystem. It is important to understand the TBT resistance mechanisms operational in bacteria. TBT resistant bacteria tolerate high levels of this biocide by virtue of their several inherent biochemical and molecular mechanisms which include:

(i) Transformation/ Degradation of TBT into less toxic derivatives viz. di- and mono-butyltin through debutylation process.

(ii) Exclusion/ efflux of TBT to the cell exterior mediated by membrane proteins.

(iii) Metabolic utilization of TBT as carbon source.

(iv) Intracellular sequestration/Bioaccumulation of TBT without breakdown, mediated by metallothionein like proteins.

(Blair et al. 1982; Fukagawa et al. 1994; Harino et al. 1997; Kawai et al 1998; Dubey and Roy 2003; Inoue et al. 2003a, b; Ramachandran and Dubey 2009; Sampath et al. 2012)

1.7 TBTCl Resistance Mechanisms Adopted by Microorganisms

TBT resistant microorganisms possess several inherent mechanisms to withstand high concentrations of TBT which may range from alterations in cell morphology, exclusion of TBT outside the cell, intracellular accumulation to metabolic degradation into less toxic derivatives viz. DBT and MBT and finally their utilization as sole

carbon source (Blair et al. 1982; Kawai et al. 1998; Roy et al. 2004; Jude et al. 2004; Suehiro et al. 2006; Mimura et al. 2008; Ramchandran and Dubey 2009; Shamim et al 2012; Sampath et al. 2012).

1.7.1 Degradation and Transformation of TBTCl

Microbial degradation is one of the most predominant processes for breakdown of TBT in near shore waters with dibutyltin being the major degradation product (Page 1989). Microbes may degrade TBTCl by utilizing it as a sole source of carbon. Several reports have demonstrated good growth of bacteria in minimal media supplemented with TBTCl which clearly confirms that TBTCl has been utilized as a sole carbon source (Dubey and Roy 2003; Cruz et al. 2007; Krishnamurthy et al. 2007; Sakultantimetha et al. 2010; Sampath et al. 2011).

Although several reports suggest degradation to be a crucial mechanism for TBTCl resistance in bacteria but very few studies have documented the exact chemical nature of TBTCl degradation product. It has been reported that TBTCl may be broken down into its less toxic derivatives viz. dibutyltin dichloride and monobutyltin trichloride (Krishnamurthy et al. 2007; Cruz et al. 2007). Biodegradation and biotransformation of TBTCl occurs through successive sequential removal of organic moeity attached to tin atom through debutylation steps involving removal of butyl groups and thus decreasing the toxicity of TBTCl (Cooney 1988).Enzymes such as dioxygenases present in microorganisms may play a key role in this successive biodegradation/ biotransformation process (Table 1.4).

TBTCl $\longrightarrow$ **$DBTCl_2$** $\longrightarrow$ **$MBTCl_3$** $\longrightarrow$ **Tin** (Cooney 1988)

Degradation of TBT via successive dealkylation

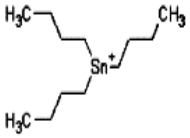
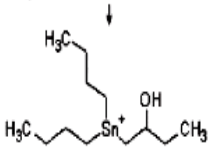
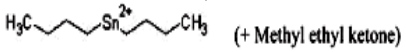
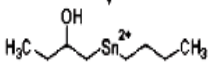
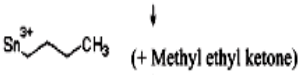
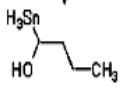
Compound	Chemical structure	Enzyme	Formula	Molecular weight
Tributyltin, TBT			$C_{12}H_{27}Sn^{+}$	290.06
		TBT dioxygenase		
β-hydroxybutyl-dibutyltin			$C_{12}H_{27}OSn^{+}$	306.06
Dibutyltin, DBT	 (+ Methyl ethyl ketone)	DBT dioxygenase	$C_8H_{18}Sn^{2+}$	232.94
β-hydroxybutyl-butyltin			$C_8H_{18}OSn^{2+}$	248.94
Monobutyltin, MBT	 (+ Methyl ethyl ketone)	MBT dioxygenase	$C_4H_9Sn^{3+}$	175.83
β-hydroxybutyl			$C_4H_{12}OSn^{3+}$	194.85
	Sn (+ Methyl ethyl ketone)		Sn^{4+}	118.71

Table. 1.4 Pathway for degradation of TBT

(Antizar-Ladislao et al. 2008)

1.7.2. Intracellular Sequestration and Biosorption of TBTCl in Microorganisms

It is interesting to note that few *Pseudomonas* spp. have been reported to bioaccumulate tributyltin up to 2% of its dry weight (Blair et al. 1982; Gadd 2000). Several gram negative bacteria possess capability to accumulate tributyltin oxide without its breakdown (Barug 1981). The high lipid solubility of organotin ensures cell penetration and association with intracellular sites, while some cell wall components also play an important role (Gadd 2000). Thus it is evident that site of action of organotins may be both at cytoplasmic membrane and at intracellular level. TBT biosorption studies in fungi, cyanobacteria and microalgae indicate that cell surface binding alone occurs in these organisms and no evidences of intracellular

sequestration is reported. While on the other hand studies on effect of TBT on certain bacterial strains indicated that it can also interact with cytosolic enzymes (White et al. 1999). Being a hydrophobic substrate, TBT uptake would depend on its dispersion/ dissolution into the aqueous phase brought about by surfactants and emulsifiers produced by the TBT resistant bacteria.

1.7.3 Molecular Basis of TBTCl Resistance in Microorganisms

Although many studies are available on the toxicity of tributyltin compounds, little is known about the genetic mechanisms governing tributyltin resistance in bacteria. Chromosomal genes have dominated tributyltin resistance in bacteria and no reports on plasmid encoded tributyltin resistance genes have been documented so far although the presence of plasmids in TBT resistant bacteria have been reported (Fukagawa and Suzuki 1993; Miller et al. 1994; Jude et al. 2004; Fukushima et al. 2009; Cruz et al. 2010; Fukushima et al. 2012).The *TbtRABM* operon of *Pseudomonas stutzeri* strain 5MP1 associated with TBT resistance regulates efflux of toxic organotin from the bacterial cells. TBT resistance in this isolate was found to be associated with the presence of the operon *TbtABM*, which is homologous to the resistance-nodulation-cell division (RND) efflux pump family. *TbtABM* was found to exhibit homology with proton dependent efflux pump belonging to the RND (analogous to *TbtB* gene), membrane fusion (analogous *TbtA* gene) and outer membrane (analogous to *TbtM* gene) proteins which function together to extrude substrates across membranes of Gram-negative bacteria (Fig. 1.2). This was the very first report of MDR

efflux pump in *P. stutzeri*, on an organotin substrate like TBT belonging to the RND family of transporters suggesting removal of this biocide out of the resistant bacterial strain (Jude et al. 2004). Similarly a chromosomal gene responsible for TBTCl resistance in *Alteromonas* sp. M-1 was cloned by Fukugawa and Suzuki (1993) which posessed one open reading frame (ORF) of 324 bps and 108 amino acids. Homology search of this ORF with reference to amino acid alignment indicated that the product is homologous to transport proteins suggesting its involvement in efflux of TBTCl.

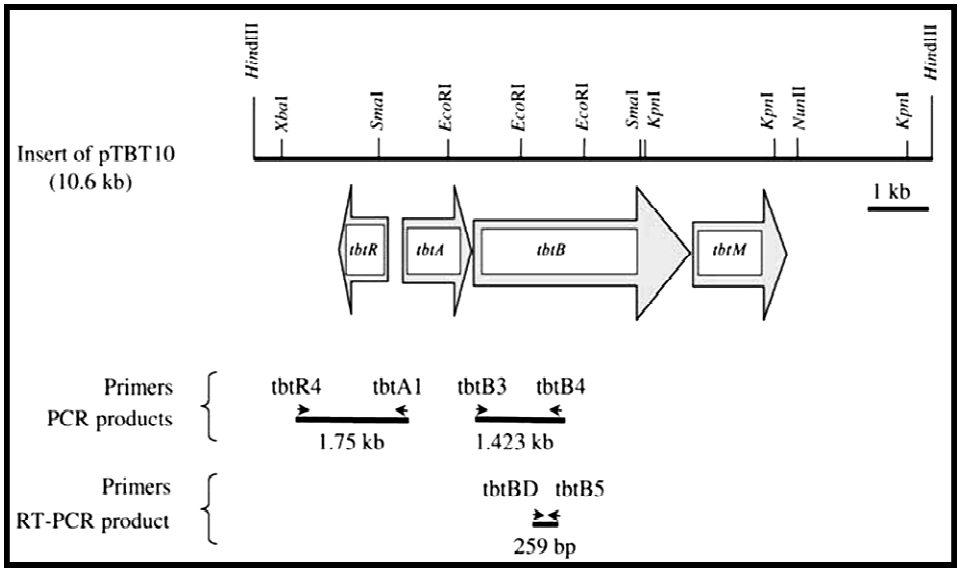


Fig. 1.2 *TbtRABM* operon in TBT resistant *Pseudomonas stutzeri* strain 5MP1 (Jude et al. 2004)

. In *Aeromonas molluscorum* Av27, *sugE* gene has been reported which encodes *sugE* protein belonging to small MDR family, a lipophillic drug transporter (Cruz et al. 2010). RT-PCR analysis

demonstrated that enhanced expression of *SugE* gene was noticed in *Aeromonas molluscorum* Av27 when the cells were grown in the presence of high concentration of TBT. Interestingly this bacterial strain uses TBT as a carbon source. In recent times, a novel TBTCl resistance gene *PA0320* of *Psuedomonas aeruginosa* 25W having an amino acid sequence homologous to *YgiW* proteins of *E. coli* and *Salmonella enteric*a has been reported to play an important role in stress tolerance against TBTCl in *Pseudomonas aeruginosa* 25W (Fukushima et al. 2012). Although reports are available on TBT resistance encoding genes in bacteria which regulate efflux of TBT but nothing is known about genes which encode enzymes for TBT degradation and/ or biotransformation. Studies in the field of molecular genetics of TBT resistant bacteria continue to be of great importance as they hold immense scientific value in devising biological systems for bioremediation and biomonitoring of TBT contaminated sites.

1.7.4 Proteomics of TBT Resistant Bacteria

Bacterial proteins specifically membrane proteins play a pivotal role in regulating resistance to heavy metals viz. Cd, Hg, Zn, Cu including TBT. Some heavy metal resistant bacterial strains are known to synthesize cysteine and histidine rich low molecular weight polypeptides which are responsible for intracellular sequestration of toxic metals, ultimately resulting in their immobilization in order to protect their vital metabolic processes catalysed by enzymes (Highman et al. 1984; Pazirandeh et al. 1995; Gadd 2000). While involvement of

microbial dioxygenases has been suggested in TBTCl breakdown (Antizar-Ladislao 2008); involvement of transglycolase enzyme in tributyltin resistance has also been demonstrated in TBTCl resistant *Alteromonas* sp (Fukagawa and Suzuki 1993). A significant change in protein profile of microorganisms resistant to TBTCl has been observed while in presence of the toxic biocide, where the resistant bacteria showed increased protein production when subjected to TBTCl stress (Sampath et al. 2012; Bernat et al. 2014). The *sugE*-like proteins show affinity towards lipophillic drugs in *Aeromonas molluscorum* Av27 thus they may regulate the transport of TBT outside the cell since TBT is a lipophillic organic biocide. The periplasmic space is involved in various biochemical pathways including nutrient acquisition, synthesis of peptidoglycan, electron transport and alteration of substances toxic to cells. Protein profiles of *Alteromonas sp.* M1 clearly showed that biosynthesis of 30 kDa and 12 kDa polypeptides increased drastically when cells were exposed to 125 µM TBTCl (Fukagawa et al. 1992). The expression of TBTCl induced three periplasmic proteins (43, 63 and 68 kDa) were also reported in a TBTCl - resistant marine sediment isolate *Alcaligenes* sp. which is responsible for TBTCl resistance (Ramachandran and Dubey 2009). Presence of an additional 52 kDa outer membrane protein in TBTCl resistant *Pseudomonas stutzeri* strain 5MP1 also indicates its involvement in transport of TBT to the cell exterior (Jude et al. 2004).

As mentioned earlier, bioaccumulation of TBT without its breakdown by metallothionein proteins is proposed as a possible mechanism of TBT resistance in

microorganisms (Barug 1981; Blair et al. 1982; Gadd 2000). Metallothioneins are low molecular weight cysteine or histidine rich proteins playing an important role in immobilisation of toxic heavy metals (Blindauer et al. 2002). Majority of the available experimental data available in literature on metallothioneins relates to cyanobacterial metallothionein, SmtA, from *Synechococcus* PCC 7942 mediating resistance to Zinc in the bacterial strain (Robinson et al. 2001). Bacteria resistant to a particular heavy metal many a times are found to show cross resistance to other metals and organometals also (Adelaja and Keenan 2012; Naik et al 2012; Shamim et al. 2012). Supposedly, by the virtue of these metallothioneins such multiple resistance may be observed in some bacteria. Similarly bacterial metallothioneins (bmtA) also confer resistance to heavy metals. Cyanobacterial and bacterial strains such as *Anabaena* PCC 7120, *Pseudomonas aeruginosa* and *Pseudomonas putida* have been reported to possess bacterial metallothioneins (bmt) to maintain cytosolic metal homoeostasis (Turner et al. 1996; Blindauer et al. 2002). A lead resistant bacterial isolate *Psuedomonas aeruginosa* strain WI-1 showed presence of *BmtA* gene encoding 11 kDa bacterial metallothionein protein responsible for sequestration of lead. Interestingly, this bacterial isolate also showed cross tolerance to other toxicants such as $CdCl_2$, $HgCl_2$ and 0.2 mM TBTCl (Naik et al. 2012). Although there is little reported on bioaccumulation of TBTCl in bacteria, these findings suggest the possible involvement of metallothioneins in conferring TBTCl resistance in them by facilitating intracellular bioaccumulation of the toxic biocide. Thus bacteria possessing metallothioneins are an ideal tool for bioremediation of heavy metal and organometal contaminated environmental sites.

1.7.5. Surface Adsorption and Morphological Alterations

Bacterial viability analysis in presence of TBT so far suggests peculiar morphological changes in the bacterial cells as shrinkage in size and clumping (Cruz et al. 2007). This is an applied energy saving mechanism exhibited by resistant bacteria when exposed to TBT for long duration. It has been noted that TBT stimulates an increment in cell number. It has also been reported, that after exposure to lethal concentration (10mM) of TBTCl, certain bacterial cells became wrinkled and rough in appearance, as opposed to untreated cells (control) showing a smooth surface (Mimura et al. 2008). Changes in cell surfaces appear to contribute to an increase in surface area of the cells resulting in an increase in the adsorption capacity of the cell surface towards TBT. It is the function of the cell surface rather than its structure that plays an important role in adsorption of TBT (Mimura et al. 2008) as proven by reports of accelerator analysis of TBT adsorbed onto the bacterial cell surface. It has also been observed that when cells of *Aeromonas caveyii* were exposed to high levels of TBT a very unique response as long chain formation was noticed (Shamim et al. 2012).

1.7.6 Production of Siderophores

Various other mechanisms have been revealed in bacteria like *Pseudomonas* under TBT stress which showed enhanced production of extracellular pigment which could possibly be a defense mechanism for cells against TBT stress. The role of pyoverdine in *Pseudomonas*

chlororaphis CNR15 in organotin degradation, and pyochelin and pyoverdine involving Triphenyltin decomposition in *Pseudomonas aeruginosa* CGMCC 1.860 has confirmed the potential of such extracellular products in decomposition of organotins (Inoue et al. 2000; 2003 a; Sun et al. 2006). Although, siderophores are mainly responsible for chelating iron but they also play a major role in conferring tributyltin resistance in bacteria.Similar to iron binding, they also chelate many other heavy metals and even organotins thereby aiding in survival of bacteria in environments with high tributyltin contamination. Tributyltin is taken up by siderophore structures of *Aeromonas* sp. and then gradually exported as DBT after possible degradation inside the cells (Cruz et al. 2007). Similar findings were reported by Inoue et al. (2003b) in *Burkholderia* sp. where role of siderophores was significant in tributyltin degradation. These reports suggest that extracellular substances like siderophores secreted by TBTCl resistant bacteria may also play a role in the degradation process of tributyltin in the environment.

1.7.7 Production of Exopolysaccharides

Many bacteria produce EPS under various environmental stresses viz. metals, toxins and nutrient limitations, thus provide a mechanism to protect cells from its toxic effects. Besides, Exopolysaccharides create a microenvironment around the organisms allowing it to metabolize and reproduce more efficiently. Being a hydrophobic substrate, TBT uptake would depend on its dispersion/ dissolution into

the aqueous phase. This may be brought about by surfactants or emulsifiers. Microorganisms are known to produce surfactants and emulsifiers to increase the bioavailability of organic compounds. TBT resistance by sequestration is proposed as one of the important mechanisms rendering many bacteria the capacity to thrive in TBT contaminated sites. In the marine environment, binding of heavy metals by microbial EPS (dissolved or particulate) can have far-reaching implications on its dynamics, removal of these metals from water column and subsequent transfer to marine organisms (Buffle et al. 1998). These exopolysaccharides are chemically diverse and are mostly acidic heteropolysaccharides with functional groups viz. hydroxyl, carboxyl, amides and phosphoryl which exhibit high affinity towards heavy metals (Bhaskar and Bhosle 2006; Bramhachari et al. 2007; Braissant et al. 2007; Morillo et al. 2008). EPS have greater binding capacity for metals than any other known mineral sorbent (Quigley et al. 2002) and form multiple complexes with metal ions. Microbial exopolymers chelate heavy metals present in the water columns as well as sediments thus regulate the bioavailability of toxic heavy metals in the aquatic environment. Interestingly, TBT resistant bacteria are known to show enhanced production of EPS when exposed to high levels of this biocide. It has also been observed, that maximum yield of EPS was obtained from TBT degrading *Vibrio* sp. in a medium containing TBT as compared to tributyltin deficient media (MSM) (Krishnamurthy et al. 2007; Ramachandran and Dubey 2009). Several reports have demonstrated that majority of bacteria produce

exopolymers (EPS) which are involved in heavy metal sequestration outside the cell, cell adhesion, biofilm formation and cell survival (Bhaskar and Bhosle 2006). Enhanced EPS production by organotin resistant bacterial strains may play an important role in immobilization of TBT outside the cell through binding of TBT with several functional groups of EPS. Thus EPS production in TBT resistant bacteria is one of the several interesting resistance mechanisms.

1.7.8 Heavy Metal and Antibiotic Resistance

As it is known that most of the antifouling paints contain many biocides including heavy metals, it is always expected and observed that these organotin resistant bacteria would develop a mechanism of resistance to heavy metals that are commonly present in these paints. Bioremediation involving bacterial strains needs that the microorganism should be resistant to target contaminant as well as other contaminants. Many reports of tributyltin resistant bacteria have so far been documented showing cross tolerance to several heavy metals including one organometal viz. Hg, Pb, Cd, Cu, Zn, methyl mercury (Wuertz et al. 1991; Suzuki et al. 1992; Fukagawa et al. 1994; Pain and Cooney 1998; Cruz et al. 2007; Shamim et al. 2012). It may be thus presumed that the resistance mechanisms adapted by bacteria against tributyltin could be very similar to those adapted against other heavy metals. The heavy metal resistance genes are generally located on plasmids and transposons with their counterparts on chromosomal genomes as well (Naik and Dubey 2011; Naik et al. 2011).

Antibiotics are essential drugs to treat bacteria producing infectious diseases. Majority of currently used antibiotics are no longer effective because of emerging drug resistance, which is mainly caused by uncontrolled, haphazard and extensive use of antibiotics and anti-microbial drugs in hospitals, aquaculture farms prawn hatcheries and poultry farms has become a major environmental problem due to their ultimate release and persistence in the natural terrestrial and aquatic environments (Khetan and Collins 2007). Long term exposure of microbes to these residual antimicrobial compounds leads to evolution of drug resistance which is conferred by genetic determinants on plasmids, transposons or integrons (Okeke et al. 2000; Martinez 2008). These mobile elements are known to carry several antibiotic resistance genes, and thus their transfer results in immediate multidrug resistance in recipient strains (Horizontal DNA transfer). *L. monocytogenes* isolated from environment and food products showed multi-drug efflux pump (*MdrL*) which governed resistance to multiple antibiotics (Mereghetti et al. 2000). The same efflux pump was reported to mediate antibiotic as well as lead resistance in bacteria (Naik et al. 2013). In *Enterobacteriaceae*, the expulsion of antibiotics is currently associated with efflux pump encoded by *AcrAB-TolC* gene family. This pump contributes to a multi-drug resistance (MDR) phenotype often associated to modification of the outer membrane permeability (Baucheron et al. 2004). The *MdrL* efflux pump has been reported in lead resistant *Pseudomonas stutzeri*strain M-9 and *Vibrio harveyi* strain M-11 which clearly revealed significant involvement of genes encoding multi-drug efflux pumps in heavy metal resistance also (Naik et al. 2012). Organotin resistant natural bacterial communities invariably demonstrate resistance to commonly used antibiotics. Although it is yet to be known how these bacteria develop resistance to antibiotics which are not commonly

present in organotin contaminated waters. It could be attributed to the fact that water bodies serve as reservoirs for the release of industrial and domestic effluents containing inorganic and organic pollutants. The presence of *TbtABM* operon which encodes the proteins regulating MDR efflux pump is associated with TBT resistance as well as antibiotic resistance in *Psuedomonas stutzeri* showed resistance to antibiotics viz. nalidixic acid and chloramphenicol (Jude et al. 2004). The mechanism of antibiotic resistance in bacteria is through efflux pump which is facilitated by membrane proteins excluding out the antibiotics. Similar type of resistance mechanism is also found in TBT resistant bacteria. Thus it is evident that resistance to both metals and antibiotics are wide spread among TBT resistant organisms possibly due to common location of genes encoding heavy metal and antibiotic resistance.

Tributyltin is one of the most toxic compounds widely used as antifouling paints and wood preservatives due to its biocidal property. Despite of the ban on its usage as a biocide in ship paints in many countries, it still persists in the water columns and sediments of ship docks. Of particular significance, tributyltin chloride is toxic even at nano molar concentrations to non-target organisms. A great deal of work describing various bacteria resistant to tributyltin chloride is available in literature. However, there are few defined reports on the mode of resistance employed by the bacteria against these antifouling biocides. Ban on the extensive use of tributyltin in antifouling paints

for ships and boats is essential and its release into the marine environment needs to be restricted/avoided. The concentration of tributyltin chloride persistent in marine environments needs to be reduced and this can be done by imposing effective bioremediation measures involving several highly TBT resistant bacteria as biological tools in organotin contaminated sites. In order to accomplish this objective, biochemical and genetic resistance mechanisms of these TBT resistant microorganisms need to be explored and understood so that they can be employed to combat its seriously dangerous effects to aquatic biota and subsequently to human beings. Although it is much evident that bacteria serve as promising tools to bioremediate tributyltin chloride contaminated environmental sites, but the possible preventions and remediation strategies using these bacteria are yet to be implemented. The scope for the removal of this dangerous biocide from environmental sites is still ample and new approaches employing biological tools including microbes and microbial products would facilitate environmental clean-up of this toxic biocide. Very little research work has been done to study resistance mechanisms of TBT resistant microbes including bacteria (Fukugawa and Suzuki 2003; Jude et al. 2004; Cruz et al. 2010; Fukushima et al. 2012). Therefore it is imperative and promising to isolate and characterize TBTCl resistant bacteria from estuarine sites of Goa, India with reference to their biochemical and molecular mechanisms of TBT resistance.

Keeping in view these interesting facts and findings, I have isolated and biologically characterized several potential TBTCl resistant and degrading estuarine bacterial isolates from Goa with reference to their biochemical and molecular mechanisms of TBTCl resistance.

1.8 Specific objectives of research

- Isolation and identification of TBTCl resistant bacteria.
- Morphological and biochemical characterization of selected TBTCl resistant bacterial isolates
- Molecular biological characterization of TBTCl resistant bacterial isolates

CHAPTER II

ISOLATION AND IDENTIFICATION OF TBTCl RESISTANT BACTERIA

MATERIALS AND METHODS

2.1 Collection of environmental samples

2.1.1 Details of sampling sites

In the present study five sampling sites were selected for collecting water and sediment samples from Zuari estuary of Goa. These include, Western India Shipyard Ltd. (WISL), Goa Shipyard Ltd. (GSL), Dempo Shipyard, Marmon Shipyard, Garson Shipyard and Abhishek Shipyard, located in Goa, India (Fig. 2.1, 2.2).

Surface water and sediment samples were collected from different sites of Zuari estuary viz. ship building yards, ship painting yards and vicinity of anchored ships. All the samples were collected in sterile polycarbonate bottles and used within 24 hours of collection for bacteriological analysis. The bottles containing water samples were thoroughly shaken and kept for 10 minutes to allow the heavy particles to settle down prior to use. Physicochemical analysis of water samples was done to determine pH (using digital pH meter), temperature (using mercury thermometer) and salinity (using salinometer) as per the standard procedures. The appropriate volume (100 µ1-10 ml) of sample was taken for physiochemical and bacteriological analysis.

2.2 Isolation of TBTCl resistant bacteria

TBTCl-resistant bacteria were isolated from surface water and sediment samples from various sampling sites of Zuari estuary Goa, India viz. Western India Shipyard Ltd. (WISL), Goa Shipyard Ltd., Dempo Shipyard, Marmon Shipyard, Garson Shipyard and Abhishek Shipyard. Water samples were spread plated on Zobell Marine Agar (ZMA) plates and Minimal Salt Media (MSM) agar plates (Appendix A.3) amended with 0.1 mM TBTCl (Sigma, Aldrich, USA) by dilution plating technique (Zobell 1941). A total volume of 2.7 µl of filter sterilized TBTCl stock (3.2

M) was added to the growth media (100 ml) subsequent to media sterilization to attain the desired concentration of 1 mM TBTCl. Plates were incubated at 28 °C ± 2 for 48 hours. Based on morphological differences, the isolated bacterial colonies which appeared on plates were further spot inoculated on fresh Zobell marine agar plates amended with different concentrations of TBTCl (0 mM - 6 mM) and the colonies which appeared on the plate with the highest concentration of TBTCl were selected for further characterization. Viable count of TBTCl resistant bacteria was also determined as colony forming units (cfu)/ ml in Zobell marine as well as MSM agar plates supplemented with 0.1 mM TBTCl.

2.3 Screening of potential TBTCl resistant bacteria

Bacterial isolates showing conspicuous growth on agar plates amended with highest concentration of TBTCl in ZMA were selected to study their growth response in liquid media viz. Zobell marine broth (ZMB) and MSM broth supplemented with 1 mM TBTCl at 28 °C ± 2, 120 rpm, and growth was recorded after 48 hrs as absorbance at 600 nm. Media without amendment of TBTCl served as control. Bacterial isolates showing highest absorbance after 48 hrs of incubation were selected for identification and further characterization.

2.4 Identification of potential TBTCl resistant bacterial isolates

Identification of the potential TBTCl-resistant bacterial isolates which showed best growth in solid as well as liquid media amended with TBTCl was subsequently carried out based on morphological and biochemical characteristics following Bergey's manual of systematic bacteriology (Krieg and Holt 1984) (Appendix C) and 16S rDNA sequencing followed by BLAST search (Altschul et al.1990).

Identification of one of the isolates was confirmed by Fatty acid methyl ester (FAME) analysis (Sherlock version 6.0B).

2.4.1 Identification based on morphological and biochemical tests

Morphological characterization of bacterial isolates is based on colony morphology, pigmentation and motility. Further identification of isolates is based on gram staining followed by several biochemical tests. Staining revealed cell wall composition whereas oxidative-fermentative test determines whether organism is oxidative, fermentative or facultative anaerobe. Carbohydrate fermentation test confirms fermentative utilization of sugars with production of acid and gas. Methyl Red test detects formation of large quantities of acid in the medium resulting from fermentation of glucose and Voges-Proskauer's test detects the production of non-acidic or neutral end products i.e. butanediol and acetoin. Citrate utilization test determines that a bacterium can use citrate as the sole carbon source and indole production test determines ability of bacteria to convert tryptophan into indole. Production of several microbial enzymes viz. urease, amylase, catalase, gelatinase, oxidase and nitrate reductase by the test bacterial isolates was also determined. Based on morphological, biochemical characteristics and following Bergey's manual of systematic bacteriology (Krieg and Holt, 1984) the potential TBTCl resistant isolates were identified tentatively.

2.4.2 Molecular identification of the potential TBTCl resistant isolates

Molecular identification of the potential TBTCl resistant bacterial isolates was performed using 16S rDNA. Total genomic DNA was extracted as per procedure by Jones and Barlet (1990) and 16S rDNA of the bacterial isolates was PCR amplified.

The PCR primers used for amplification of 16S rDNA are as follows:

Forward Primer: 8F (5'-AGAGTTTGATCCTGGCTCAG-3')

Reverse Primer: 1492R (5'-ACGGCTACCTTGTTACGACTT-3')

PCR amplification reaction was performed using PCR amplification kit (Bangalore Genei, India) and sequencing was done at Xcelris laboratories, Ahmedabad, Gujarat, India. The16S rDNA sequence data of bacterial isolates was compared with 16S rDNA data base of GenBank using NCBI- BLAST search (Altschul et al. 1990).

2.4.3 Identification of TBTCl resistant bacterial isolate based on FAME analysis

Bacterial isolate DN2 was grown in trypticase soy broth (TSA) at 28 °C ± 2, 120 rpm for 24 hrs. Whole cell fatty acids were extracted from cells according to the MIDI protocol (Sasser 1990). Overnight grown bacterial culture was harvested (8000 rpm, 4 °C) and cell pellet (approximately 40 mg) was transferred in a clean screw capped glass tube after adding 1 ml of reagent I (Appendix B.3). The tube sealed with teflon lined screw cap was vortexed gently and immersed in a boiling water bath for 5 minutes. The tube was vigorously vortexed again for 10 seconds and immersed in the water bath (100°C) again for 30 minutes (Saponification step). The tube was taken out of the water bath, incubated at room temperature and 2 ml of Reagent II was added (Appendix B.3). The tube was capped again and briefly vortexed for few seconds. After vortexing, the tube was incubated in water bath for 10 minutes at 80° C. This methylation step is critical with time and temperature. Addition of 1.25 ml of Reagent III (Appendix B.3) to the cooled tube was followed by recapping and gentle tumbling on a clinical rotator for about 10 minutes. The tube was uncapped, organic phase was retained in a clean tube and the aqueous phase was discarded (extraction step).

Approximately 3 ml of Reagent IV (Appendix B.3) was added to the organic phase, the tube was recapped, tumbled on rotator for 5 minutes. Approximately, 2/3rd volume of the organic phase was pipette out into a GC vial and GC analysis was performed on a GC Sherlock microbial identification system 4.0 (New York, USA) fitted with cross-linked methyl silicon fused capillary column (25 m, 0.2 mm i.d.), flame ionization detector (FID) and a sampler. Helium was used as carrier gas. The sample was injected at oven temperature of 50°C. After 1 min, the oven temperature was raised to 170°C at the rate of 30°C/min and then to 270°C at the rate of 2°C/min and finally to 300°C at 5°C/min.

2.4.4 Phylogenetic analysis of TBTCl resistant isolates

Based on 16S rDNA sequence data phylogenetic analysis of the TBTCl resistant isolates was done to study their phylogenetic relationship with already known bacterial isolates using ClustalW and MEGA 4.0.2 (Saitaou and Nei 1987; Tamura et al 2007). The phylogenetic tree was constructed using the neighbor joining and bootstrap methods which are commonly used to demonstrate evolutionary relatedness between different taxonomic groups. The percentage of replicate trees in which associated taxa are clustered together in bootstrap test are shown next to the branches at the joint.

RESULTS AND DISCUSSION

2.5 Collection of environmental samples

Coastal region of Goa has been extensively explored over several years as an area with high potential for mining, shipping and ship building activities. Ten major mines are located in the Zuari basin of Goa generating 1,000–4,000 tons of mine

rejects/day/mine (Dessai and Nayak, 2009). The environmental pollutants generated by Goan mines and shipyards include heavy metals viz. Fe, Mn, Hg, Cr, As, Pb, Zn, Sn, and organometals such as Tributyltin (TBT) and Dibutyltin (DBT) (Meena et al. 2003; Alagarsamy 2006; Bhosle 2007; Dessai and Nayak 2009; Turner 2010; Atri and Kerkar 2011). Goa shipyard ltd. (GSL) and Western India shipyard ltd. (WISL) situated at Vasco-da-gama, Goa (Zuari estuary) are among the biggest shipyards in the west coast of India, which substantially contribute several heavy metal and organometal pollutants (eg. TBT) in the surrounding estuarine and marine environment of Goa (Fig. 2.1, 2.2). These environmental pollutants are often found to be toxic with high persistence levels and are commonly non biodegradable thus entering food chains and higher food webs causing biomagnifications of serious toxic compounds. The TBTCl resistant bacteria were isolated from coastal waters and sediments of the Zuari estuary, Goa, India since it is reported to be highly contaminated with TBTCl (>1.3 ng Sn/g) (Jadhav et al. 2009; Garg et al. 2011). These TBTCl contaminated estuarine sediments with extensive ship building activities, are responsible for natural enrichment of TBTCl resistant microbes including bacteria. There have been reports demonstrating presence of potential TBTCl resistant bacteria in the estuarine ecosystems worldwide (Miller and Cooney 1994; Mendo et al. 2003; Krishnamurthy et al. 2007; Ramachandran and Dubey 2009; Sampath et al. 2012). As microbial degradation is observed as a predominant biological process for breakdown of TBTCl in coastal waters (Suzuki et al. 1992; Fukagawa et al. 1994; Dowson et al. 1996; Harino et al 1997; Krishnamurthy et al. 2007; Maguire 2008; Dubey and Roy 2003; Mendo et al. 2003; Suehiro et al. 2006; Ayanda et al. 2012; Sampath et al. 2012), it is imperative and interesting to investigate the mechanisms of resistance

adapted by the bacterial microflora towards TBTCl, thriving in such unique econiches.

Physicochemical analysis of estuarine samples clearly revealed variation in pH ranging from 7.3 to 7.8, temperature from 28 ° C to 31 ° C and salinity from 19.34 PSU to 20.46 PSU respectively at different sampling stations (Table. 2.2). It is interesting to note that pH of estuarine water samples ranged from neutral to slightly alkaline and maximum alkalinity i.e. 7.8 ± 2 was observed for samples collected from Dempo Shipyard Ltd. and Goa Shipyard Ltd. possibly due to the fact that these sites are situated close to open sea. Similarly salinity was also comparatively higher for these samples (i.e. 20.43 - 20.67 ± 2 PSU).

2.6 Viable Counts of TBTCl resistant bacteria

2.6.1 Viable count of bacteria in estuarine water samples

The total viable count ofTBTCl resistant bacteria in all the water samples from Zuari estuary ranged from 20 – 44 ± 2 × 10^3 cfu/ml when plated on Zobell marine agar amended with 0.1 mM TBTCl whereas viable count in Zuari estuary water samples on Zobell marine agar in the absence of TBTCl ranged from 50 - 70 ± 3 x 10^8 cfu/ml (Table. 2.3). These studies clearly demonstrated that more than 50 % of natural bacterial population from Zuari estuary is TBTCl resistant due to TBTCl contamination through ship building, mining, industrial and other anthropogenic activities. Several reports on TBTCl resistant bacteria have been documented till date, which include TBTCl resistant bacteria viz. *Pseudomonas* spp., *Alteromonas* sp., *Aeromonas* sp, and *Vibrio* sp. among many others (Fukagawa and Suzuki 1993; Cruz et al. 2007; Krishnamurthy et al. 2007; Ramachndran and Dubey 2009; Sakultantimetha et al. 2009; Sampath et al. 2012). The viable count of TBTCl

resistant bacteria on MSM agar supplemented with 0.1 mM TBTCl ranged from 2 – 11 ± 2 x 10^3 cfu/ml which clearly revealed that approximately 19.6 % of isolated TBTCl resistant bacterial population is capable of degrading TBTCl since they utilize it as a sole source of carbon (Table. 2.3). Exposure of bacterial isolates to high levels of TBTCl facilitates selective enrichment of TBTCl resistant isolates in estuarine environment and culminates in evolution of novel resistance mechanisms to counteract TBTCl stress (Blair et al. 1982; Wuertz et al. 1991; Dubey and Roy 2003; Ramachandran and Dubey 2009; Sampath et al. 2012).

2.6.2 Viable count of bacteria in estuarine sediment samples

The total viable count of TBTCl resistant bacteria in all the sediment samples from Zuari estuary ranged from 18 - 40 ± 2 × 10^3 cfu/ml when plated on Zobell marine agar amended with 0.1 mM TBTCl whereas the viable count of bacteria in Zobell marine agar without TBTCl ranged from 28 - 50 ± 3 x 10^8 cfu/ml (Table. 2.3). These studies clearly indicated that approximately 69.4 % of natural bacterial population from sediments of Zuari estuary is resistant to TBTCl. Interestingly, the viable count on MSM agar supplemented with 0.1 mM TBTCl ranged from 2 – 10 ± 2 x 10^3 indicating that approximately 21.9 % of bacterial isolates was capable of degrading TBTCl by utilizing it as a sole carbon source. Utilisation of TBTCl as a sole source of carbon is one of the resistant mechanisms commonly observed in TBTCl resistant bacterial strains (Barug 1981; Roy and Dubey 2003; Cruz et al 2007; Krishnamurthy et al. 2007; Sakultantimetha et al. 2010; Sampath et al. 2011).These studies have clearly demonstrated that population density of TBTCl resistant bacteria is higher in the sediment samples as compared to the water samples of this estuarine econiche. Sediments often serve as enrichment zones of TBTCl with clay and sediment particles

being bound to the organic toxicant. Hence bacteria isolated TBTCl rich sediments may show greater adaptability to TBTCl rich environment (Miller and Cooney 1994; Page et al. 1996; Eggleton and Thomas 2004). This could be attributed to the presence of TBTCl in estuarine sediments for longer duration due to their hydrophobicity and occurrence of extensive ship building activities resulting in enrichment of TBTCl resistant bacteria.

2.6.3 TBTCl tolerance limits of bacteria

Bacteriological analysis of several water and sediment samples of Zuari estuary interestingly revealed 36 morphologically different TBTCl resistant bacterial isolates. Out of these, 17 isolates tolerated even up to 6 mM TBTCl on Zobell marine agar plates (Table. 2.4) which clearly demonstrated that these isolates possess biochemical and molecular mechanisms to withstand such a high concentration of this antifouling biocide. There are several other reports of TBTCl resistant bacteria from different terrestrial and aquatic environments worldwide (Barug 1981; Cooney and Miller 1994; Suzuki and Fukagawa 1995; Dubey and Roy 2003; Inoue et al. 2002; Jude et al. 2004; Cruz et al. 2007; Sakultantimetha et al. 2009). It has been reported that *Aeromonas molluscorum* Av27 is capable of tolerating TBTCl in Trypticase Soy Broth (TSB) upto 3 mM (Cruz et al. 2010). TBTCl resistant bacteria with capability of utilizing it as sole source of carbon even at 2 mM have been isolated from a marine ecosystem (Bhosle et al. 2004). Sediment and water samples from Bowling Basin, Glasgow, UK interestingly showed presence of bacteria capable of degrading TBTCl upto 135µM in glycerol containing media (Sakultantimetha et al. 2009). Similarly, a TBTCl degrading *Vibrio* sp. was isolated from Bombay High Oil Field, India which showed tolerance to TBTCl upto 0.1 mM in ZMB (Krishnamurthy et al. 2007). Thus

it is evident that tolerance to TBTCl is variable in different bacterial isolates possibly due to variation in resistance mechanisms operational in them.

2.7 Screening of potential TBTCl resistant bacteria

Out of the 17 selected bacterial isolates that showed good growth on ZMB at concentration of TBTCl up to 6 MM, eight isolates viz. SD5, SD9, DP1, DP2, DP3, DP4, DP5 and DN2 showed best growth in presence of 1 m MTBTCl in ZMB as well as MSM broth as compared to the remaining isolates (Table. 2.4). Thus it is evident that these bacterial isolates are resistant to TBTCl present in liquid media where exposure to this biocide is maximum.It is interesting to note that these bacterial isolates showed better growth on Zobell marine agar and MSM agar plates supplemented with 1 mM TBTCl than in liquid media. This conspicuous variation in the growth response is due to less bioavailability of TBTCl in solid media as compared to liquid media.There are similar reports that bioavailabilty of heavy metal toxicants in liquid media is higher as compared to solid media since the solidifying agent, agar binds to metal cations thereby making it less bioavailable to the bacteria, resulting in reduction in its toxicity (Jonas et al 1984; Wuertz et al. 1991; Rathnayake et al. 2013). Therefore the potential TBTCl resistant bacteria were screened in liquid media (ZMB and MSM broth) which was supplemented with 1 mM TBTCl.

2.8 Identification of TBTCl resistant bacterial isolates

2.8.1 Identification of potential TBTCl resistant isolates based on morphological and biochemical characteristics

Based on morphological characteristics and biochemical tests as per Bergey's manual of systematic bacteriology (Kreig and Holt 1984), the eight potential TBTCl

resistant bacterial isolates were tentatively identified. Bacterial isolate SD9 was tentatively identified as *Klebsiella* sp. while isolate SD5 was tentatively identified as *Alteromonas* sp. TBTCl resistant bacterial isolated DN2, DP1 and DP4 were tentatively identified as *Pseudomonas* sp. respectively. Bacterial isolate DP3 was tentatively identified as *Vibrio* sp. while isolates DP2 and DP5 were tentatively identified as *Aeromonas* sp. respectively.

2.8.2 Molecular identification of potential TBTCl resistant isolates

TBTCl resistant bacterial isolates were identified as *Aeromonas salmonicida* strain DP2, *Pseudomonas mendocina*strain DP4, *Pseudomonas stutzeri* strain DP1, *Vibrio* sp. DP3, *Alcaligenes faecalis* strain SD5, *Klebsiella pneumoniae* strain SD9 and *Chromohalobacter salexigens* strain DP5 based on morphological, biochemical characteristics along with 16S rDNA sequence analysis (Table. 2.8)

The BLAST search data obtained after comparing the 16S rDNA sequences of these TBTCl resistant bacterial strain to already known sequences of other bacterial strains showed a distinct homology with bacteria belonging to same genera respectively. Bacterial strains SD5 and SD9 showed homology of 99 % and 98 % with *Alcaligenes faecalis* strain AU02 and *Klebsiella pneumoniae* strain ARCC13884T respectively**.** Bacterial strain DP1 showed 98 % homology with *Pseudomonas stutzeri* strain HMGM while strain DP2 showed 91 % homology with *Aeromonas salmonicida* strain TDR17. While bacterial strain DP3 was homologous to *Vibrio* sp. VIB 411, showing 98 % homology with the strain, bacterial strains DP4 and DP5 showed homology of 98 % and 91 % to *Pseudomonas mendocina* strain PMLR1 and *Chromohalobacter salexigens* strain SSB-7 respectively (Table. 2.8).

The data obtained after 16S rDNA sequencing of these seven TBTCl resistant isolates has already been submitted to GenBank and accession numbers have been allotted (Table. 2.8).

2.8.3 Identification of TBTCl resistant bacterial isolate based on FAME analysis

Based on FAME analysis one TBTCl resistant bacterial isolate (DN2) was identified as *Pseudomonas stutzeri* strain DN2 as it showed a similarity index of 0.987 to *Pseudomonas stutzeri* (Fig 2.4).

Several other bacterial isolates showing resistance to TBTCl have been extensively studied and documented. These include, *Alteromonas* sp. M1, *Pseudomonas aeruginosa* 25W, *Pseudomonas stutzeri, Vibrio* sp., *Aeromona smolluscorum* Av27, *Citrobacterbracii, Burkholderia* sp., *Klebsiella* spp. (Suzuki and Fukagawa 2003; Dubey and Roy 2003; Jude et al. 2004; Krishnamurthy et al. 2007; Sakultantimetha et al. 2009; Cruz et al. 2010; Ayanda et al. 2012; Sampath et al. 2012)

2.8.4 Phylogenetic analysis of TBTCl resistant isolates

Phylogenetic analysis of the TBTCl resistant bacterial isolates using neighbor joining method showed evolutionary relationship with other closely related species of bacteria belonging to the same genera. *Alcaligene sfaecalis* strain SD5 showed closest evolutionary relationship with *Alcaligenes faecalis* strain AU02, *Klebsiella pneumoniae* strain SD9 with *Klebsiella pneumoniae* strain ARCC13884T, *Pseudomonas mendocina* strain DP4 with *Pseudomonas mendocina* strain PMLR1, *Chromohalobacter salexigens* strain DP5 with *Chromohalobacter salexigens* strain SSB-7, *Vibrio* sp. strain DP3 with *Vibrio* sp. VIB 411, *Aeromonas salmonicida* strain

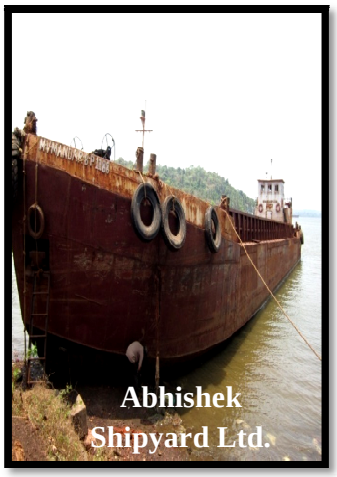
DP2 with *Aeromonas salmonicida* strain TDR17 and *Pseudomonas stutzeri* strain DP1 with *Pseudomonas stutzeri* strain HMGM respectively (Fig. 2.5 a-g).

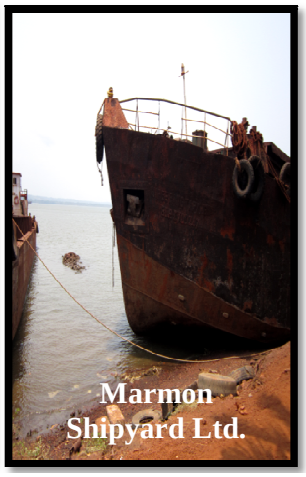
Fig. 2.1 Map of Goa showing various sampling sites

Site Symbol	Sampling Site	Sample Number	Sample Type
●	Dempo Shipyard Ltd.	1	Water Sample
		2	Sediment Sample
●	Western India Shipyard Ltd.	3	Water Sample
		4	Sediment Sample
●	Goa Shipyard Ltd.	5	Water Sample
		6	Sediment Sample
●	Garson Shipyard Ltd.	7	Water Sample
		8	Sediment Sample
●	Marmon Shipyard Ltd.	9	Water Sample
		10	Sediment Sample
●	Abhishek Shipyard Ltd.	11	Water Sample
		12	Sediment Sample

Table. 2.1 Details of sampling sites

Fig. 2.2 Estuarine ship building sites of Goa





Sr. No.	Sampling Stations	pH	Temperature (°C)	Salinity (PSU)
1	Dempo Shipyard Ltd.			
	Water Sample	7.8 ± 0.2	28 ± 2	20.43 ± 2
	Sediment Sample			
2	Western India Shipyard Ltd.			
	Water Sample	7.5 ± 0.2	30 ± 2	21.46 ± 2
	Sediment Sample			
3	Goa Shipyard Ltd.			
	Water Sample	7.8 ± 0.2	31 ± 2	20.67 ± 2
	Sediment Sample			
4	Garson Shipyard Ltd.			
	Water Sample	7.4 ± 0.2	28 ± 2	19.34 ± 2
	Sediment Sample			
5	Marmon Shipyard Ltd.			
	Water Sample	7.4 ± 0.2	28 ± 2	19.44 ± 2
	Sediment Sample			
6	Abhishek Shipyard Ltd.			
	Water Sample	7.3 ± 0.2	30 ± 2	19.49 ± 2
	Sediment Sample			

Table. 2.2 Physicochemical characteristics of samples

Sample Nos.	Viable Count in cfu/ml (Mean ± SD)			
	ZMA + 0.1mM TBTCl	ZMA	MSM + 0.1mM TBTCl	MSM
1	$44 \pm 5 \times 10^3$	$50 \pm 5 \times 10^8$	$11 \pm 2 \times 10^3$	No Growth
2	$20 \pm 2 \times 10^3$	$39 \pm 2 \times 10^8$	$6 \pm 2 \times 10^3$	No Growth
3	$20 \pm 4 \times 10^3$	$70 \pm 5 \times 10^8$	$4 \pm 2 \times 10^3$	No Growth
4	$21 \pm 3 \times 10^3$	$48 \pm 2 \times 10^8$	$2 \pm 2 \times 10^3$	No Growth
5	$40 \pm 5 \times 10^3$	$62 \pm 3 \times 10^8$	$4 \pm 2 \times 10^3$	No Growth
6	$32 \pm 3 \times 10^3$	$30 \pm 2 \times 10^8$	$6 \pm 2 \times 10^3$	No Growth
7	$30 \pm 2 \times 10^3$	$45 \pm 5 \times 10^8$	$3 \pm 2 \times 10^3$	No Growth
8	$40 \pm 5 \times 10^3$	$50 \pm 3 \times 10^8$	$10 \pm 2 \times 10^3$	No Growth
9	$22 \pm 4 \times 10^3$	$55 \pm 4 \times 10^8$	$2 \pm 2 \times 10^3$	No Growth
10	$18 \pm 2 \times 10^3$	$28 \pm 2 \times 10^8$	$3 \pm 2 \times 10^3$	No Growth
11	$21 \pm 2 \times 10^3$	$53 \pm 3 \times 10^8$	$11 \pm 2 \times 10^3$	No Growth
12	$33 \pm 3 \times 10^3$	$41 \pm 4 \times 10^8$	$9 \pm 2 \times 10^3$	No Growth

Table. 2.3 Viable count of bacterial population in estuarine water and sediment samples

Table. 2.4 Tolerance of bacterial isolates to TBTCl concentrations in ZMA

Isolate Nos.	TBTCl Concentration in mM in ZMA								
	0.5	1	1.5	2	2.5	3	4	5	6
SD1	+++	+++	+++	+++	+++	+	+	+	-
SD2	+++	+++	+++	+++	+++	+	+	+	+
SD3	+++	+++	+++	+++	++	+	+	+	-
SD4	+++	++	++	++	++	+	+	+	+
SD5	+++	+++	+++	+++	+++	+++	+++	+++	++
SD6	+++	+++	++	++	+	+	+	+	+
SD7	+++	++	++	++	+	+	+	+	+
SD8	+++	++	++	++	+	+	+	+	+
SD9	+++	+++	+++	+++	+++	+++	++	++	++
SD10	+++	+++	++	++	++	+	+	+	+
SD11	+++	+++	++	++	++	++	+	+	-
SD12	+++	+++	++	++	+	-	-	-	-
SD13	+++	+++	++	++	+	+	+	-	-
SD14	+++	+++	++	++	+	+	-	-	-
SD15	+++	+++	++	++	+	+	+	-	-
SD16	+++	+++	++	++	+	-	-	-	-
SD17	+++	+++	++	++	+	-	-	-	-
SD18	+++	+++	++	++	+	-	-	-	-
SD19	+++	+++	+	-	-	-	-	-	-
SD20	+++	+++	+	-	-	-	-	-	-
SD21	+++	+++	+	-	-	-	-	-	-
SD22	+++	+++	+	-	-	-	-	-	-
SD23	+++	+++	+	-	-	-	-	-	-
SD24	+++	+++	+	-	-	-	-	-	-
SD25	+++	+++	+	-	-	-	-	-	-
SD26	+++	+++	+	-	-	-	-	-	-
SD 27	+++	+++	++	+	+	-	-	-	-

Isolate Nos.	TBTCl Concentration in mM in ZMA								
	0.5	1	1.5	2	2.5	3	4	5	6
SD28	+++	+++	+	-	-	-	-	-	-
SD29	+++	+++	+	-	-	-	-	-	-
SD30	+++	+++	+	-	-	-	-	-	-
DP1	+++	+++	+++	++	++	++	++	++	++
DP2	+++	+++	+++	++	++	++	++	++	++
DP3	+++	+++	+++	++	++	++	++	++	++
DP4	+++	+++	+++	++	++	++	++	++	++
DP5	+++	+++	+++	++	++	++	++	++	++
DN2	+++	+++	+++	++	++	++	++	++	++

Table. 2.4 Tolerance of bacterial isolates to TBTCl concentrations in ZMA

+++	: Excellent Growth	+	: Poor Growth
++	: Good Growth	-	: No Growth

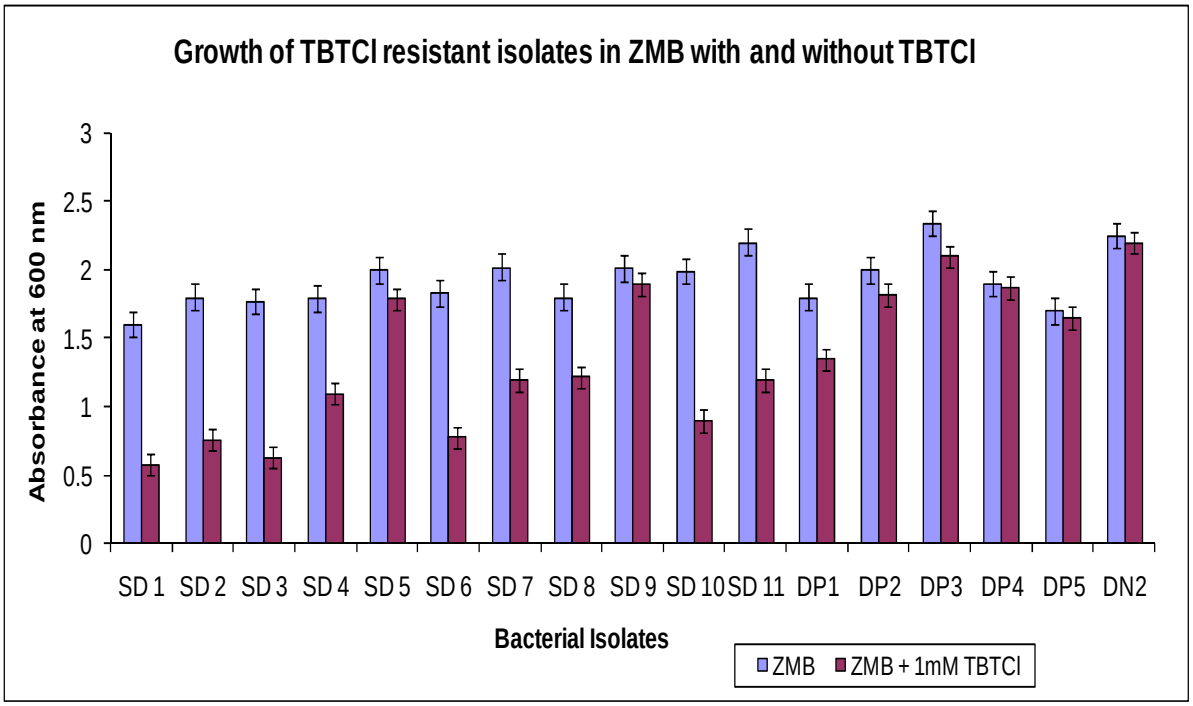


Fig. 2.3 (a) Growth of TBTCl resistant bacterial isolates in liquid media ZMB

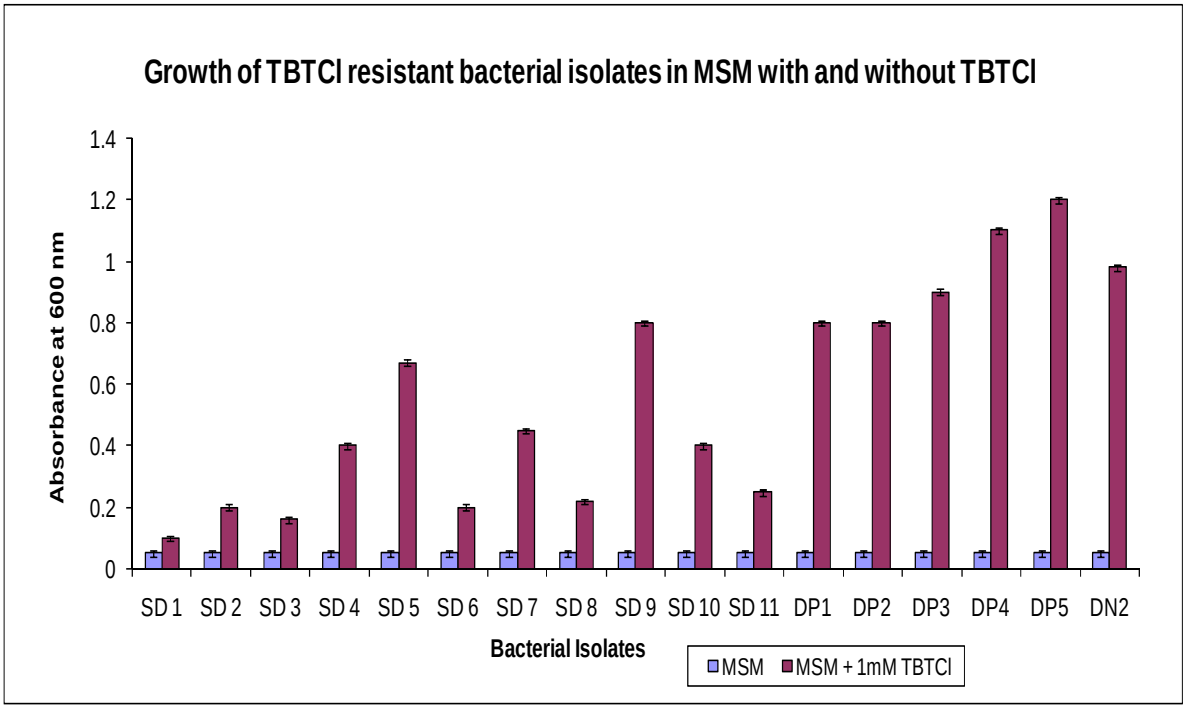


Fig. 2.3 (b) Growth of TBTCl resistant bacterial isolates in liquid media MSM broth

Colony morphology	SD5	SD9	DP1	DP2	DP3	DP3	DP4	DN2
Size (cm)	0.2	0.4	0.4	0.1	0.2	0.2	0.2	0.2
Shape	Circular	Circular	Circular	Circular	Circular	Circular	Circular	Circular
Colour	Cream	White	Cream	Cream	Cream	Cream	Cream	Cream
Opacity	Translucent	Translucent	Opaque	Translucent	Translucent	Translucent	Translucent	Translucent
Elevation	Convex	Convex	Convex	Convex	Convex	Convex	Convex	Convex
Surface	Smooth	Smooth	Smooth	Smooth	Smooth	Smooth	Smooth	Smooth
Margin	Entire	Entire	Entire	Entire	Entire	Entire	Entire	Entire
Consistency	Butyrous	Butyrous	Butyrous	Butyrous	Butyrous	Butyrous	Butyrous	Butyrous
Pigment production	-	-	-	-	-	-	-	-
Motility	Motile	Motile	Motile	Motile	Motile	Motile	Motile	Motile
Gram's reaction	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative
Arrangement	Short rods	Short rods	Short rods	Short rods	Short rods	Short rods	Short rods	Short rods

Table. 2.5 Morphological tests of TBTCl resistant bacterial isolates

TESTS	SD5	SD9	DP1	DP2	DP3	DP4	DP5	DN2
Sugars								
Arabinose	-	+	+	-	+	+	-	-
Xylose	-	+	+	-	+	-	-	-
Adonitol	-	+	-	-	-	-	-	-
Rhamnose	+	+	+	-	+	-	+	-
Cellobiose	-	-	+	-	+	-	-	-
Melibiose	+	+	+	-	+	-	+	-
Saccharose	+	+	+	-	+	-	+	-
Raffinose	+	+	+	-	+	-	+	-
Trehalose	-	+	+	-	+	-	-	-
Glucose	+	+	+	+	+	+	+	+
Lactose	+	+	+	-	+	-	+	-

Table. 2.6 Sugar fermentation by TBTCl resistant bacterial isolates

TESTS	SD5	SD9	DP1	DP2	DP3	DP4	DP5	DN2
ONPG	+	+	+	+	+	+	+	+
Lysine Utilization	-	+	-	-	-	-	-	-
Ornithine Utilizations	-	-	+	-	-	-	-	-
Urease	-	-	-	-	-	-	-	-
Phenylalanine Deaminase test	-	-	-	-	-	-	-	-
Nitrate Reduction	+	+	+	+	-	+	+	+
H2S Production	-	-	-	-	-	-	-	-
Citrate Utilizations	+	+	+	+	-	+	+	+
Voges Proskauer	+	+	-	+	+	-	+	+
Methyl Red	-	-	-	-	-	-	-	-
Indole	-	-	+	-	-	+	-	-
Malonate Utilizations	+	+	+	+	+	+	+	+
Esculin Hydrolysis	-	+	+	-	+	+	-	-
Amylase	+	+	+	+	+	+	+	+
Gelatin	-	+	-	-	-	-	-	-
Mac conkey's	+	+	+	+	+	+	+	+
Oxidase	+	+	+	+	+	+	+	+
Catalase	+	+	+	+	+	+	+	+
O/F Test	O	O	O	O	OF	O	O	O
Tentative Identification	*Alteromonas* sp.	*Klebsiella* sp.	*Pseudomonas* sp.	*Aeromonas* sp.	*Vibrio* sp.	*Pseudomonas* sp.	*Aeromonas* sp.	*Pseudomonas* sp.

Table. 2.7 Biochemical tests of TBTCl resistant bacterial isolates

- Negative result
+ Positive result

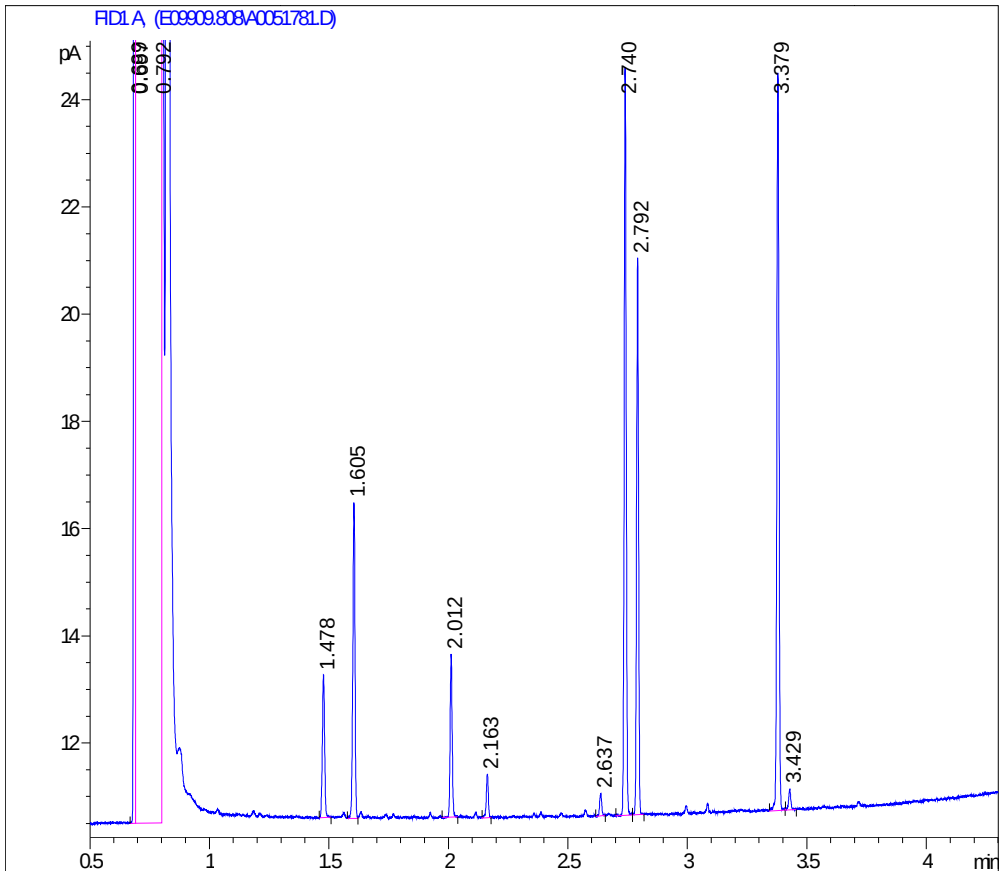
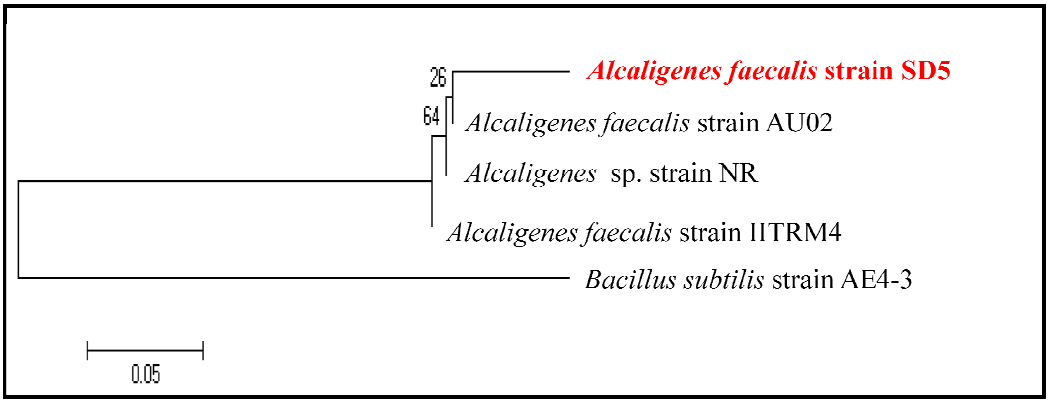


Fig. 2.4 FAME profile of TBTCl resistant *Pseudomonas stutzeri* strainDN2

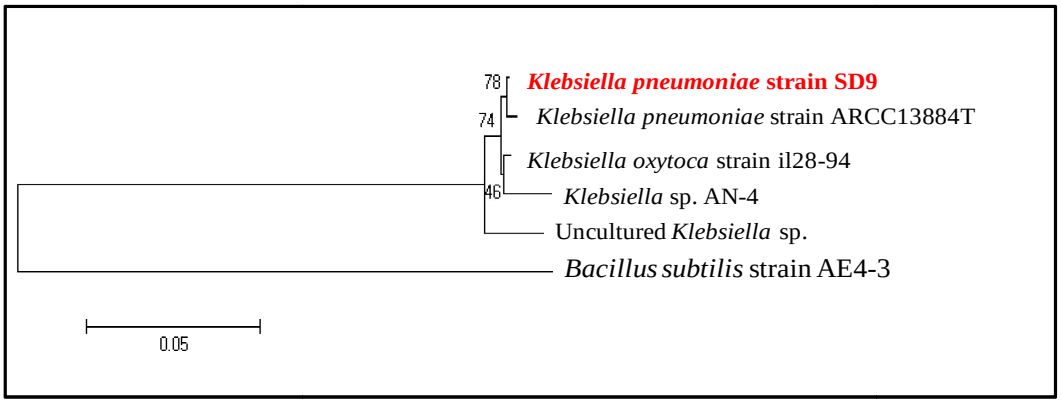
Bacterial Isolates	Homology with	% Homology	GenBank Accession Number
DP1	*Pseudomonas stutzeri* strain HMGM	98	GU596989
DP2	*Aeromonas salmonicida* strain TDR17	91	GU596990
DP3	*Vibrio* sp. VIB411	98	GU596991
DP4	*Pseudomonas mendocina* strain PMLR1	98	GU596992
DP5	*Chromohalobacter salexigens* strain SSB-7	91	GU596993
SD5	*Alcaligenes faecalis* strain AU02	99	JQ993101
SD9	*Klebsiella pneumoniae* strain ARCC13884T	98	JQ993102

Table. 2.8 TBTCl resistant bacterial isolates showing maximum homology with other bacterial strain and their allotted GenBank accession numbers

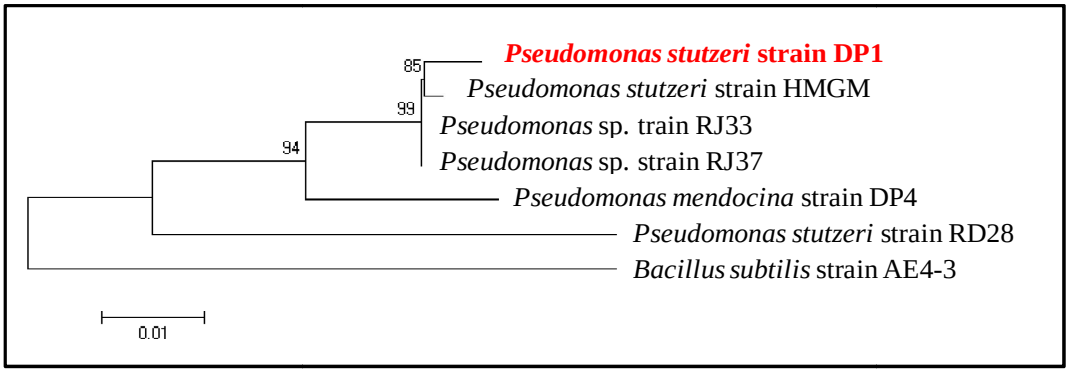
Fig. 2.5 (a-g) Dendrograms of TBTCl resistant bacterial strains showing phylogenetic relationship with other closely related bacterial strains (Neighbor-joining method shows the percentage of replicate trees in which the associated taxa are clustered together in a bootstrap test which is shown next to the branches. The number at the node indicates the bootstrap value at which a given branch was supported)



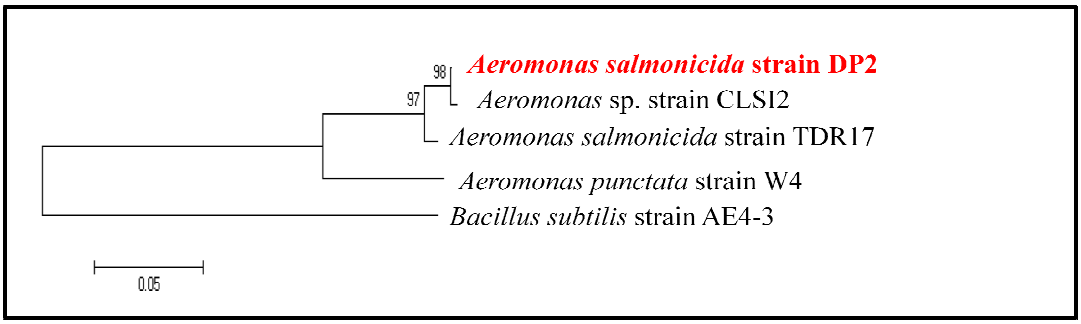
(a)



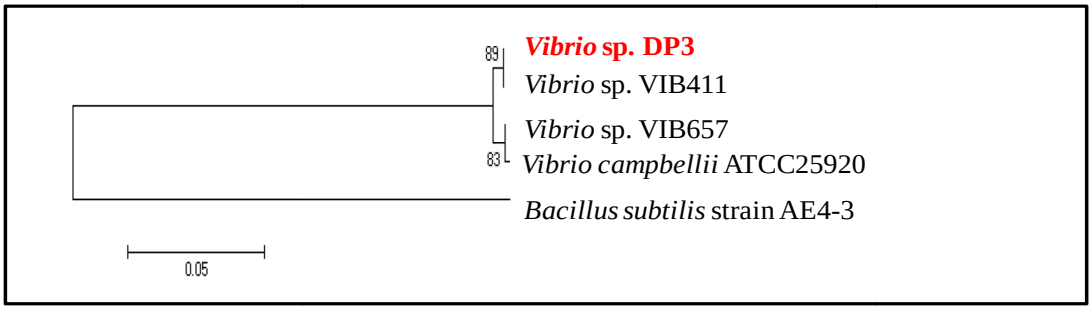
(b)



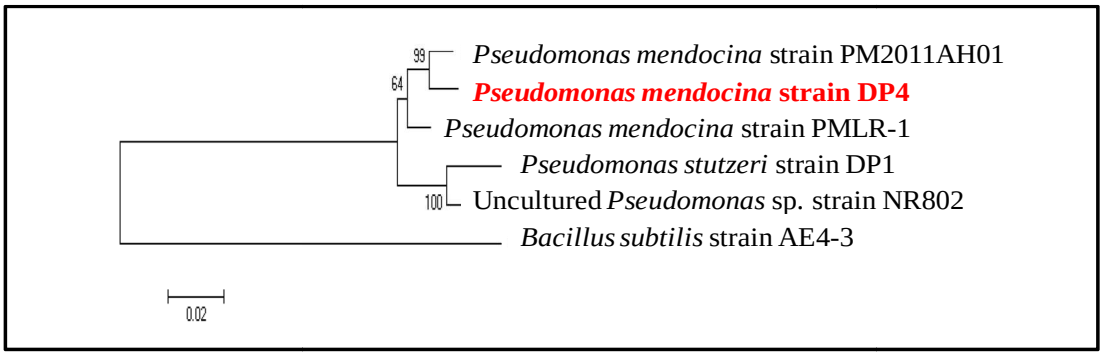
(c)


98
97
Aeromonas salmonicida strain DP2
Aeromonas sp. strain CLSI2
Aeromonas salmonicida strain TDR17
Aeromonas punctata strain W4
Bacillus subtilis strain AE4-3
0.05

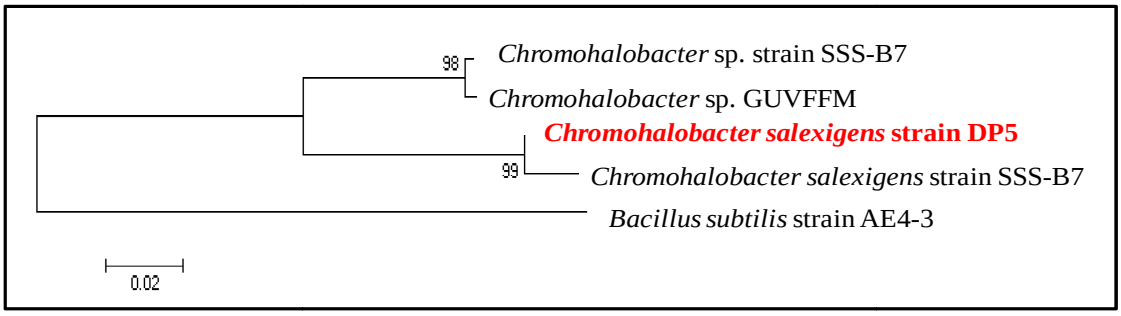
(d)


89
83
Vibrio sp. DP3
Vibrio sp. VIB411
Vibrio sp. VIB657
Vibrio campbellii ATCC25920
Bacillus subtilis strain AE4-3
0.05

(e)


99
64
100
Pseudomonas mendocina strain PM2011AH01
Pseudomonas mendocina strain DP4
Pseudomonas mendocina strain PMLR-1
Pseudomonas stutzeri strain DP1
Uncultured Pseudomonas sp. strain NR802
Bacillus subtilis strain AE4-3
0.02

(f)


98
99
Chromohalobacter sp. strain SSS-B7
Chromohalobacter sp. GUVFFM
Chromohalobacter salexigens strain DP5
Chromohalobacter salexigens strain SSS-B7
Bacillus subtilis strain AE4-3
0.02

(g)

CHAPTER III

MORPHOLOGICAL AND BIOCHEMICAL CHARACTERIZATION OF TBTCl RESISTANT BACTERIAL ISOLATES

MATERIALS AND METHODS

3.1 Growth studies of TBTCl resistant bacteria

3.1.1 Growth response of TBTCl resistant bacterial strains in MSM

Growth behavior of the selected eight potential TBTCl resistant bacterial strains was studied in MSM broth (100 ml) supplemented with different concentrations of TBTCl. Inoculum of bacterial cells (2%) was added in MSM broth amended with varying concentrations (0.5 – 4 mM) of TBTCl and flasks were incubated at 28 °C ± 2 at 120 rpm. Growth response was recorded for 48 hrs at regular intervals of 4 hrs as absorbance at 600 nm using UV-Vis spectrophotometer (Shimadzu, UV-2450, Japan). Similarly, bacterial isolates were also inoculated in MSM broth (100 ml) using equal inoculums without any carbon source which served as a control. A graph was plotted between absorbance at 600 nm v/s time in hrs.

3.1.2 Growth response of TBTCl resistant bacterial strains in ZMB

Growth behavious of the selected eight potential TBTCl resistant bacterial strains was also studied in ZMB supplemented with different concentrations of TBTCl. Inoculum of bacterial cells (2%) was added in ZMB amended with varying concentrations (0 – 4 mM) of TBTCl and flasks were incubated at 28 °C ± 2 at 120 rpm. Growth was recorded at regular intervals of 2 hrs as absorbance at 600 nm using UV-Vis spectrophotometer (Shimadzu, UV-2450, Japan). Bacterial isolates grown in ZMB with 0 mM TBTCl served as control. A graph was plotted between absorbance at 600 nm v/s time in hrs.

3.2 Morphological characterization of TBTCl resistant bacterial strains under TBTCl stress

In order to reveal whether any morphological alterations and adaptations occur in bacteria when subjected to TBTCl stress, scanning electron microscopy (SEMJEOL JSM-5800LV, Japan) was performed on bacterial cells of *Pseudomonas stutzeri* strain DN2 and *Klebsiella pneumoniae* strain SD9 which were grown separately in MSM supplemented with 2mM TBTCl. Culture smear was prepared on glass slide, air dried and then fixed in 3 % glutaraldehyde overnight with 50 mM Potassium phosphate buffer. The slide was gently washed thrice with phosphate buffer (Appendix B.2) and dehydrated in gradually increasing concentrations of ethanol i.e., 10, 20, 50, 70, 80, 90, 95, and 100% for 15 min each, air dried and finally stored in vacuum chamber prior to SEM analysis (Naik et al. 2011).

3.3 Analysis of TBTCl Degradation Product

3.3.1 Extraction and thin layer chromatography (TLC) of degradation products

TBTCl resistant bacteria viz. *Pseudomonas stutzeri* strain DN2 and *Klebsiella pneumoniae strain* SD9 were grown separately in 250 ml Ehrlenmeyer flasks containing 100 ml MSM (starting inoculum size; showing absorbance at 600 nm of 0.025 which is equivalent to 0.007 gms/l) with TBTCl as a sole source of carbon at 28 °C in an incubator shaker at 150 rpm for 1 week. The concentration of 2 mM TBTCl for growth was selected for *Klebsiella pneumoniae strain* SD9 since it grew best at this concentration. Similarly, an uninoculated flask containing MSM (100 ml) with 2 mMTBTCl was used as a control. The same was repeated for TBTCl resistant *Alcaligenes faecalis* strain SD5 with concentration of TBTCl used being 3 mM, as this strain showed best growth in presence of 3 mM TBTCl. After incubation the cell

pellet was harvested by centrifugation at 8000 rpm and the cell free supernatant was separated. The cell free supernatants were extracted using double volume of distilled chloroform. The organic layer was collected in a conical flask. The chloroform extract was reduced under vacuum and the concentrated samples were then loaded on a pre activated TLC plate. Similarly a mixture containing TBTCl, $DBTCL_2$ and $MBTCl_3$ served as control which was loaded on a separate pre activated TLC plate. All the samples were run using the solvent system, petroleum ether : acetic acid (9.5:0.5). The TLC plates were subsequently exposed to iodine vapors to develop the spots. The locations of the spots ware marked and recorded.

The concentrated residual extract was further purified by silica gel H-20 column chromatography (SIGMA, 30 x 2 cm glass column). Slurry of silica gel H-20 was prepared by mixing 9 gm of silica in 20 ml of ether (40 – 60 °C). A glass column (15.5 cms) was packed by adding the slurry with the help of a glass rod and gently tapped to avoid any void volume. The concentrated residual extractwas added to the column and chloroform was passed through the column. The same procedure was repeated for the control flask containing uninoculated MSM broth with TBTCl. The eluent obtained after purification by column chromatography was further concentrated under vacuum and dried. The purified product was stored in a glass screw capped vial at 4^oC until use subsequently used for further identification studies.

3.3.2 FTIR analysis of purified TBTCl biotransformation products

The purified biotransformation product (2 mg) of *Pseudomonas stutzeri* strain DN2 was analyzed in the region 400 – 4,000 cm^{-1} using (SHIMADZU-FTIR 8201 PC instrument, Japan) in order to find out different transformation products of TBTCl.

3.3.3 NMR spectrometric analysis of purified TBTCl biotransformation products

Nuclear magnetic resonance spectrum analysis (H^1-NMR) of the control product and purified biotransformation products of *Pseudomonas stutzeri* strain DN2, *Alcaligenes faecalis* strain SD5 and *Klebsiella pneumoniae* strain SD9 were recorded with the help of an NMR spectrometer (BRUKERWT, 300 MHz) in deuterated chloroform ($CDCl_3$) with tetramethylsilane (TMS) as an internal standard. Sample (5 mg) to be analyzed was dissolved in 0.6 ml of deuterated $CDCl_3$ and placed in an NMR tube (5 mm diameter). The NMR tube was then appropriately positioned in a spinner and introduced into the NMR spectrometer. All the reagents were purchased from Sigma Aldrich,USA (Devi et al. 2010).

3.3.4 Mass spectrometric analysis of purified TBTCl bioransformation products

Subsequent to NMR spectroscopic analysis, the column purified biotransformation products of *Alcaligenes faecalis* strain SD5 and *Klebsiella pneumoniae* strain SD9 were further analyzed by mass spectrometer to confirm the presence of biotransformation products. In a typical experiment, purified biotransformation product was mixed with cyclohexane prior to injection of 2 µl into Mass Selective Detector (Applied Biosystem QSTAR XL Canada) for analysis. In Mass spectrometry, a sample is ionized, for example by bombarding it with electrons. This may cause sample molecules to break into charged fragments. These ions are then separated according to their mass-to-charge ratio, typically by accelerating them and subjecting them to an electric or magnetic field: ions of the same mass-to-charge ratio will undergo the same amount of deflection. The ions are detected by a mechanism

capable of detecting charged particles, such as an electron multiplier. Results are displayed as spectra of the relative abundance of detected ions as a function of the mass-to-charge ratio. The atoms or molecules in the sample can be identified by correlating known masses to the identified masses or through a characteristic fragmentation pattern and comparing this pattern with mass library.

3.4 Detection of antibiotic resistance in TBTCl resistant bacteria

Antibiotic susceptibility test was performed following Kirby–Bauer disc diffusion method (Bauer et al. 1966), using Mueller–Hinton agar and antibiotic discs (Himedia, India). Bacterial suspension (0.1 ml) of different TBTCl resistant strains having concentration was spread plated on Mueller–Hinton agar plates. Discs (6 mm) containing antibiotics were placed in the centre of the agar plates with growth of cells. Antibiotic resistance or susceptibility was determined as per the procedure of Bauer et al. (1966). Zones of inhibition of cell growth were measured and bacteria showing antibiotic inhibition zones of size ≤ 10 mm were considered to be resistant.

3.5 Production of (EPS) byTBTCl resistant, *Alcaligenes faecalis* strain SD5 and *Klebsiella pneumoniae* strain SD9

3.5.1 Qualitative analysis of EPS

3.5.1.1 Congo red agar assay

In order to qualitatively assess production of EPS, TBTCl resistant bacteria viz. *Klebsiella pneumoniae* strain SD9 and *Alcaligenes faecalis* strain SD5 were spot inoculated on Congo red agar (Appendix A.8) supplemented with and without 2mM TBTCl and 3 mM TBTCl respectively. Plates were incubated for 48 hrs at 28 ℃ ± 2. Bacterial colonies producing EPS distinctively pick up congo red from the medium

and turn pinkish-maroon in color. Bacterial colonies without EPS do not pick up congo red from the medium (Friedman and Kolter 2004).

3.5.1.2 Alcian blue staining

Alcian blue staining of EPS produced by bacterial strains viz. *Klebsiella pneumoniae* strain SD9 and *Alcaligenes faecalis* strain SD5 was performed to investigate its acidic or basic nature. Alcian blue is a cationic dye used to stain acidic polysaccharides (Naik et al. 2012). *Klebsiella pneumoniae* strain SD9 was grown in MSM with 2 mM TBTCl, while *Alcaligenes faecalis* strain SD5 was grown in MSM with 3 mM TBTCl for 48 hrs at 28 °C $\leq$ 2 respectively. Both the bacterial strains were also grown separately in MSM amended with 0.1% glucose which served as control. Bacterial cells were smeared on a glass slide, air dried and hydrated with distilled water. The slide with bacterial smear was flooded with 20 µl of 0.1% Alcian blue dye in acetic acid (pH 2.5) for 5 min. The slide was destained gently with running distilled water followed by air drying. The slide was observed under oil immersion lense using light microscope (Alldredge et al. 1993; Bhaskar and Bhosle 2006). Acidic EPS is expected to stain blue while bacterial cells are stained red.

3.5.2 Purification and quantification of bacterial EPS

EPS production was monitored by growing bacterial cells in MSM with TBTCl in order to study role of EPS in resistance against TBTCl. *Klebsiella pneumoniae* strain SD9 was grown in MSM with 2 mM TBTCl, while *Alcaligenes faecalis* strain SD5 was grown in MSM with 3 mM TBTCl for 48 hrs at 28 °C ± 2 respectively. Both the bacterial strains were also grown separately in MSM amended with 0.1% glucose which served as control. Purification of EPS was done using modified ice cold ethanol

precipitation method (Bramhachari and Dubey 2006). Culture grown in the presence of TBTCl and without TBTCl (0.1% glucose) in MSM (500 ml) was harvested separately at 10,000 rpm for 15 min at 4°C, when culture reached stationary growth phase. Culture supernatant was filtered through 0.22 µ cellulose nitrate filters (Millipore Filters, Bangalore, India). EPS was precipitated from the final filtrate by addition of three volumes of ice cold ethanol and kept at 4°C overnight. Resulting precipitate was centrifuged and washed with 70–100 % ethanol–water mixture. EPS was dissolved in distilled water and dialysed for 24 h at 4°C (molecular weight cut off of 13 kDa; Sigma Aldrich, Germany) against distilled water. EPS was then lyophilized and EPS production was recorded as dry weight of EPS in control and TBTCl exposed conditions. Each experiment was performed in triplicates.

3.5.3 Quantitative analysis of total carbohydrates (TOC) in EPS

Quantitative analysis of total carbohydrate (TOC) in EPS produced was carried out by phenol sulphuric acid method (Dubois et al. 1956). Bacterial cells viz. *Klebsiella pneumoniae* strain SD9 and *Alcaligenes faecalis* strain SD5 were grown separately in MSM with TBTCl concentrations of 2 mM and 3 mM respectively, as well as in MSM supplemented with 0.1% glucose which served as control. Bacterial cells were incubated for 48 hrs at 28 °C at 120 rpm. Lyophilized EPS (30 mg) was weighed and dissolved in 10ml of deionised water. EPS solution (1ml) was mixed with 1 ml of 5% aqueous phenol and tubes were placed in ice. In this sample 5 ml of concentrated H_2SO_4 was added and kept at room temperature for 10 minutes to develop colour and absorbance was measured at 490 nm using UV–Vis spectrophotometer (Shimadzu, UV-2450, Japan). Glucose was used as standard and the absorbance of test and

control was compared with a standard glucose curve (Appendix E) in order to estimate TOC in EPS. Each experiment was performed in triplicates.

3.6 Productionof siderophores by TBTCl resistant, *Alcaligenes faecalis* strain SD5 and *Klebsiella pneumoniae* strain SD9

3.6.1 Qualitative analysis of siderophores

In order to detect siderophores, Chrome azurol sulfonate (CAS) agar assay (Schwyn and Neilands 1987) was used since it is universal, comprehensive, exceptionally responsive, and most convenient assay. The bacterial culture was spot inoculated on the CAS agar plate (blue) amended with and without TBTCl and incubated at 28 °C ± 2 for 48 hrs. The concentration of TBTCl used was 2 mM for *Klebsiella pneumoniae* strain SD9 and 3 mM for *Alcaligenes faecalis* strain SD5 respectively. Siderophore production was confirmed by color change on the agar plate which occurs due to transfer of ferric ions from intense blue complex to the siderophores resulting in formation of yellow-orange halo around the bacterial colony. The size of halo around the bacterial colony indicates the quantum of siderophore production.

3.6.2 Biochemical characterization of siderophores

In order to determine the chemical nature of bacterial siderophore produced by TBTCl resistant bacteria, Csaky's assay and Arnow's assay were performed.

3.6.2.1 Csaky's Assay

Hydroxamate type siderophores were characterized by mixing 1 ml supernatant of overnight grown bacterial culture with 1 ml, 6 N H_2SO_4 in boiling water bath for 6 hrs or 130°C for 30 min. This hydrolyzed sample was buffered using 3 ml of sodium acetate solution and 0.5 ml iodine was added to allow it to react for 3-5 min. After completion of reaction, the excess iodine was destroyed with 1 ml of sodium arsenate solution. Finally 1 ml of α–Naphthyl-amine solution was added to this mixture and allowed to develop the color. Wine red color formation indicates production of hydroxamate type of siderophores (Gillan et al.1981).

3.6.2.2 Arnow's assay

Catecholate type siderophores produced by bacteria may be detected following Arnow's assay (Arnow 1937). Overnight grown bacterial cells were harvested at 9000 rpm at 4 ^{0}C and 1ml cell-free supernatant was mixed with equal volumes of nitrite-molybdate reagent and NaOH solution. Finally, 1 ml of 0.5 N HCl was added to this sample which results in yellow colour. Development of yellow color in the sample indicates confirmed production of catecholate type siderophores.

3.7 Determination of intracellular bioaccumulation of TBTCl by *Pseudomonas mendocina* strain DP4

The TBTCl resistant *Pseudomonas mendocina* strain DP4 was grown in 100 ml MSM with 1 mM TBTCl and with 0.1% glucose (Control) and cells were harvested at 8000 rpm and 4 ^{0}C for 10 min. The cell pellet was washed with 20 mM Na_2-EDTA for 3 min to remove cell surface bound TBTCl. Cell pellets were dried in a oven at 100 °C and 0.1gm of dried pellet was digested with concentrated HNO_3 using

microwave digestion system and tin content was analysed using Atomic absorption spectroscopy (Varian AA 240-FS model, Australia) following method of Joshi et al. (2008). Intracellular bioaccumulation of TBTCl by resistant cells was estimated in terms of concentration of Sn/gm cell dry weight.

RESULTS AND DISCUSSION

3.8 Growth studies of TBTCl resistant bacteria

3.8.1 Growth response of TBTCl resistant bacterial strains in MSM

Growth of all the eight TBTCl resistant bacterial isolates in MSM broth without TBTCl or any other carbon source was nil. Maximum growth of bacterial strains viz. *Klebsiella pneumoniae* strain SD9 and *Pseudomonas stutzeri* strain DN2 was noticed in MSM with 2mM TBTCl (Fig 3.1 b,c). While *Alcaligenes faecalis* strain SD5 showed best growth in presence of 3 mM TBTCl (Fig. 3.1 a) whereas in TBTCl resistant bacteria such as *Pseudomonas salmonicida* strain DP2, *Pseudomonas stutzeri* strain DP1, *Vibrio* sp. strain DP3, *Pseudomonas mendocina* strain DP4 and *Chromohalobacter salexigens* strain DP5 showed best growth in MSM with 1mM TBTCl (Fig. 3.1 d-h). It is interesting to note that at higher TBTCl levels (> 1 mM) significant lag (of almost 16 – 20 hrs) in growth phase was observed in all the bacterial strains which indicates low adaptability of bacterial strains to toxic levels of TBTCl (Fig. 3.1 a-h). Metabolic breakdown of TBTCl is a time consuming process and bacteria may take time to adjust to the toxic levels of this biocide subsequently adapting to it with the help of multiple resistance mechanisms put into action. In a nutrient deficient medium like MSM having TBTCl as the sole source of carbon, bacteria may synthesizing certain enzymes to utilize TBTCl as a carbon source and further degrade or sequester it. Thus a longer lag phase in growth may be observed.

TBTCl resistant bacteria, *Pseudomonas stutzeri* strain DP1, *Vibrio* sp. DP3 *and Chromohalobacter salexigens* strain DP5 could not tolerate TBTCl stress of 2 mM concentrations as they were observed to reach death phase within 40 hrs of time interval Fig. 3.1 d,f,h).

These TBTCl resistant bacterial isolates thus clearly demonstrated their capability to utilize TBTCl as a sole carbon source. Utilization of TBTCl as a sole carbon source through it's degradation confirmed that biodegradation is one of the several possible resistance mechanisms manifested by these TBTCl resistant bacteria. This phenomenon of degradation of TBTCl by utilizing it as sole source of carbon has been earlier demonstrated in *Pseudomonas* sp., *Vibrio* sp., *Enterobacter* sp. and *Aeromonas* sp. (Kawai et al. 1998; Dubey and Roy 2003; Roy et al. 2004; Krishnamurthy et al. 2007; Ramchandran and Dubey 2009; Sakultantimetha et al. 2009; Cruz et al. 2010; Sampath et al. 2012).

3.8.2 Growth response of TBTCl resistant bacterial strains in ZMB

TBTCl resistant bacterial strains showed best growth in ZMB without TBTCl. With gradual increase in TBTCl level in ZMB, a longer lag phase was observed in all the bacterial strains. In absence of TBTCl, the lag phase varied from of 0 - 4 hrs which gradually increased upto 6 – 10 hrs as the concentration of TBTCl in the growth medium increased for the analyzed bacterial strains (Fig. 3.2 a-h). As the concentration of TBTCl in the growth medium increased, growth of bacterial cells was also found to gradually decrease. This suggests that TBTCl is toxic to the cells and in a nutrient rich medium like ZMB, the bacterial cells may only be tolerating TBTCl with extremely slow degradation of the biocide. Interestingly, tolerance to TBTCl in ZMB was as high as 4 mM in all the bacterial strains (Fig. 3.2 a-h). Better

growth response in ZMB may be due to the fact that bacterial cells grown in this nutrient rich medium assimilate nutrients favorably which include, yeast extract and peptic digests of animal tissue and possibly sequester TBTCl also. Interstingly TBTCl resistant *Alcaligenes faecalis* strain SD5 showed good growth even in presence of 3 mM TBTCl in the growth medium with comparatively shortest lag (Fig. 3.2 c). Although the growth of TBTCl resistant bacterial strains in ZMB was found to be better as compared to in MSM broth, tolerance to TBTCl was more in ZMB (i.e. 4 mM) as compared to MSM broth (i.e. 1mM – 3 mM). It is therefore evident that TBTCl is not a preferred source of energy and bacteria may derive their energy from other complex sources of carbon and nitrogen present in ZMB.

Nutrient rich media viz. Zobell marine broth (ZMB), Trypticase soy broth (TSB) and Nutrient broth have commonly been used to isolate and study the growth of TBTCl resistant bacteria (Suzuki et al. 1992; Roy et al. 2004; Krishnamurthy et al 2007; Cruz et al 2007). It is interesting to mention that *Vibrio* sp. could tolerate TBTCl up to 0.1 mM in ZMB, *Aeromonas molluscorum* Av27 could tolerate TBTCl concentration as high as 3 mM in TSB (Krishnamurthy et al. 2007; Cruz et al. 2007).

3.9 Morphological characterization of TBTCl resistant strains under TBTCl stress

Scanning electron microscopy (10,000X magnification) of cells of *Pseudomonas stutzeri* strain DN2 exposed to 2 mM TBTCl clearly revealed significant morphological alterations as cell elongation,wrinkling and shrinkage, whereas cells grown in the absence of TBTCl showed normal morphological characteristics (Fig. 3.3a). Similar response was also observed in *Klebsiella pneumoniae* strain SD9 where exposure of cells to 2mM TBTCl caused cell shrinkage and reduction in size (Fig.

3.3b). Alterations in cell morphology viz. cell elongation, wrinkling and shrinkage leads to possible decrease in the surface area of the cells thereby resulting in reduced adsorption capacity of the cells to this biocide (Mimura et al. 2008). Similar findings have also been reported in *Aeromonas* sp. in presence of TBTCl, since exposed cells were smaller than normal size and appeared aggregated (Cruz et al. 2007). In our research laboratory a study on *Aeromonas caviae* strain KS-1 exposed to TBTCl revealed a very interesting response as formation of long chains of bacterial cells which reduces the surface : volume ratio and results in reduction of exposed cell surface for TBTCl (Shamim et al. 2012). It has also been reported that certain components on the cell surface of TBTCl resistant *Pseudoalteromonas* sp. may adsorb TBTCl present in the marine environment (Mimura et al. 2008).Thus this bacterial isolate may serve as a biological tool for remediation of marine sites contaminated with this biocide. These studies have clearly demonstrated that alteration in cell morphology in *Pseudomonas stutzeri* strain DN2 due to TBTCl exposure may prove to be an important resistance mechanism to withstand high concentrations of TBTCl.

3.10 Analysis of TBTCl degradation products

3.10.1 Thin Layer Chromatographic (TLC) analysis of TBTCl degradation products

Thin layer chromatography analysis of the extracts of supernatants of TBTCl resistant bacteria when compared to the standards revealed interesting results. Cell free extract of supernatant of *Pseudomonas stutzeri* strain DN2 showed presence one spot on the TLC plate with Rf value 0.3 cm which is characteristic of pure $DBTCl_2$ (Fig. 3.4).

In case of *Alcaligenes faecalis* strain SD5 two spots were observed with Rf values of 0.3 cm and 0.8 cm corresponding to $DBTCl_2$ and TBTCl respectively (Fig. 3.4). Whereas in case of *Klebsiella pneumoniae* strain SD9 three spots with Rf value of 0.12 cm, 0.3 cm and 0.8 cm were observed which were corresponding to pure $MBTCl_3$, $DBTCl_2$ and TBTCl respectively (Fig. 3.4). These results obtained on thin layer chromatography plates clearly demonstrated presence of degradation products of TBTCl in the supernatant of different TBTCl resistant strains. Biodegradation of TBTCl by different bacteria into it's less toxic derivates DBT and MBT is a well known mechanism often proposed by microbial ecologists but it has been rarely demonstrated (Barug 1981; Ramachandran and Dubey 2009; Cruz et al. 2010). Since TLC analysis revealed tentative identity of TBTCl derivatives post-degradation, further purification and analysis of degradation products is essential to reveal exact chemical nature of the degradation products.

3.10.2 FTIR analysis of purified TBTCl biotransformation products

The FTIR spectrum of the purified TBTCl degradation products of *Pseudomonas stutzeri* strain DN2 clearly revealed presence of butyl groups giving characteristic bands at 2958.80 cm^{-1}, 2926.01 cm^{-1}, 2872.01 $cm^{-1}$and 2856.58 cm^{-1} respectively (Fig.3.5). One prominent peak was noticed around 1450 cm^{-1} which is due to C–H bending vibrations of butyl group. A doublet at 705.95 cm^{-1} and 669.30 cm^{-1} is also characteristic of $DBTCl_2$. Comparison of FTIR spectrum of biodegradation product with standard TBTCl and $DBTCl_2$ (Sigma-Aldrich,USA) with Pubchem substance ID # 24900253 and 24852336 clearly demonstrated that the biodegradation product is different from pure TBTCl and matched interestingly with standard $DBTCl_2$ (Fig. 3.5).

3.10.3 NMR spectrometric analysis of purified biotransformation products

In case of *Psedomonas stutzeri* strain DN2, ^{1}H-NMR spectrum of the control product showed characteristic peaks at 1.582 cm^{-1}, 1.313 cm^{-1}, 1.265 cm^{-1} and 0.910 cm^{-1} respectively, whereas ^{1}H-NMR spectrum of purified biotransformation product showed peaks at 0.960 cm^{-1}, 1.392 cm^{-1}, 1.463 cm^{-1} and 1.800 cm^{-1} respectively (Fig. 3.6 a). ^{1}H -NMR spectral data of control and purified biodegradation products were compared against standard ^{1}H - NMR of TBTCl and $DBTCl_2$ respectively (Fig. 3.7 a), using NMR spectral database for organic compounds (SDBS).The purified biodegradation products significantly matched with standard $DBTCl_2$ (SDBS- ^{1}H NMR No. 3555 HSP-00-035) which clearly confirmed it to be $DBTCl_2$. The ^{1}H-NMR of control used in my experiment also matched with standard TBTCl (SDBS-^{1}H-NMR No. 6438HSP-01-479). The peak at 1.800 cm^{-1} specifically corresponds to the biodegradation product, $DBTCl_2$ whereas a peak at 1.582 cm^{-1} corresponds to TBTCl. ^{1}H-NMR spectrum of purified degradation product showed slight difference in the chemical shift of protons from standard TBTCl which further confirmed that TBTCl degradation product was $DBTCl_2$ (Fig. 3.6 a).

Whereas, in case of *Alcaligenes faecalis* strain SD5, NMR spectroscopic analysis revealed presence of $DBTCl_2$ along with residual TBTCl persistent in the medium. Biotransformation product showed characteristic peaks at 1.247 cm^{-1} and 1.573 cm^{-1} (Fig. 3.6 b) when compared to standard ^{1}H-NMR of TBTCl and $DBTCl_2$ in the Spectral database for organic compounds (SDBS). (http://sdbs.riodb.aist.go.jp/sdbs/cgi-bin/direct_frame_top.cgi) Interestingly these peaks matched with standard peaks of TBTCl and $DBTCl_2$ respectively (Fig. 3.7b).

NMR spectroscopy of biotransformation product of *Klebsiella pneumoniae* strain SD9 clearly revealed presence of both $DBTCl_2$ and $MBTCl_3$ (Fig. 3.6 c). NMR spectrum of control sample showed characteristic peaks at 1.562 cm^{-1}, 1.313 cm^{-1}, 1.265 cm^{-1} and 0.910 cm^{-1} respectively (Fig. 3.6 c), which was compared to the peaks of degradation products obtained after one week incubation (Fig. 3.6 c). The biodegradation product gave peaks at 3.143 cm^{-1}, 2.407 cm^{-1}, 1.834 cm^{-1} and 1.573 cm^{-1} (Fig. 3.6 c). ^{1}H-NMR data of TBTCl used in this experiment and purified biotransformation products were compared against standard ^{1}H - NMR of TBTCl, $DBTCl_2$ and $MBTCl_3$ in the Spectral database for organic compounds (SDBS) (http://sdbs.riodb.aist.go.jp/sdbs/cgi-bin/direct_frame_top.cgi). The ^{1}H - NMR of TBTCl (Control) matched with standard TBTCl having SDBS-^{1}H NMR No. 6438HSP-01-479. ^{1}H-NMR spectrum of biodegradation product showed significant difference in chemical shift from standard TBTCl. Peak of the degradation product at 1.573 cm^{-1} corresponds to TBTCl while peak at 1.834 cm^{-1} specifically corresponds to $DBTCl_2$ (SDBS-^{1}H-NMR No. 3555HSP-00-035) and a singlet at 3.143 cm^{-1} and peak at 2.407 cm^{-1} are attributed to $MBTCl_3$ (Fig. 3.6 c). The singlet peak at 3.143 cm^{-1} revealed presence of an organic compound bonded to a Sn (Tin) moiety, whereas peak at 2.407 cm^{-1} revealed further attachment of Sn to three chloride atoms, thereby confirming final degradation or biotransformation product to be a **Monobutyltin trichloride**. Interestingly, the peaks corresponding to $DBTCl_2$ and $MBTCl_3$ were not seen in the spectrum of control sample besides a peak at 1.562 cm^{-1} corresponding to TBTCl which confirmed that TBTCl was not degraded in the control sample due to absence of bacterial cells (Fig. 3.6 c).

NMR spectroscopy of degradation products clearly demonstrated that these TBTCl resistant bacterial isolates from estuarine environment of Goa significantly

transformed toxic TBTCl into it's less toxic derivatives viz. $DBTCl_2$ and $MBTCl_3$ respectively. Since microbial degradation/ transformation of TBTCl is very slow due to its long half life, traces of TBTCl remain in the growth medium (Wuertz et al. 1991). TBTCl resistant bacterial isolates viz. *Pseudomonas* sp., *Alcaligenes* sp., *Aeromonas* sp. and *Vibrio* sp. are known to utilize TBTCl as sole carbon source through step wise degradation into DBT and MBT. Several strains of TBTCl degraders from marine and estuarine environment have already been reported and characterized (Cooney 1988; Yonezawa et al. 1994; Dubey and Roy 2003; Krishnamurthy et al. 2007; Sampath et al. 2012). But there are very few reports which elucidate about biodegradation of TBTCl by bacteria and it's degradation/ biotransformation products viz. DBT and MBT (Cruz et al. 2007).

3.10.4 Mass spectrometric (MS) analysis of purified TBTCl biotransformation products

Mass spectrometric analysis of the purified degradation products further confirmed the identity of less toxic derivatives of TBTCl which were produced by the TBTCl degrading bacterial isolates. In case of *Alcaligenes faecalis* strain SD5 mass/ charge (m/z) value of 616 and 234 revealed presence of TBTCl and $DBTCl_2$ respectively (Fig. 3.7 a). In case of *Klebsiella pneumoniae* strain SD9 mass to charge ratio (m/z) value of 178.9055 clearly revealed presence of Sn^+ moiety whereas m/z values of 234.9403 and 290.9760 revealed presence of $DBTCl_2$ and $MBTCl_3$ respectively (Fig. 3.7 b). These findings go hand in hand with earlier findings of Centineo et al. (2004).

The biodegradation products of TBTCl produced by different TBTCl resistant isolates were variable in chemical nature. The degradation products of TBTCl, analysed thoroughly using TLC, FTIR, NMR, and MS clearly revealed that various

less toxic derivatives of TBTCl were produced by different resistant bacterial isolates. Thus these findings confirm biotransformation/biodegradation of TBTCl by the TBTCl resistant bacterial strains, *Pseudomonas stutzeri* strain DN2, *Klebsiella pneumoniae* strain SD9 and *Alcaligenes faecalis* strain SD5. Therefore it is evident that biotransformation/degradation is one of the important resistance mechanisms adapted by these bacterial strains to withstand TBTCl stress.

3.11 Detection of antibiotic resistance in TBTCl resistant bacteria

Antibiotic susceptibility test clearly demonstrated resistance of TBTCl resistant bacterial isolates to a variety of commonly used antibiotics viz. penicillin, tetracycline, chloramphenicol and others (Table 3.1). TBTCl resistant *Pseudomonas stutzeri* strain 5MP1 has been reported to show resistance to antibiotics viz. nalidixic acid, chloramphenicol, and sulfamethoxazole (Jude et al. 2004). Majority of antibiotic resistant bacterial strains also exhibit metal tolerance to various toxic metals and organometals (McArthur and Tuckfield 2000; Jude et al. 2004; De Souza et al. 2006; Naik and Dubey 2010; Matyar et al. 2010). Antibiotic resistance and heavy metal resistance in bacteria is known to follow similar kind of mechanisms. Mercury, lead and TBT resistant bacteria have already been isolated from west coast of India which are also reported to be resistant to cadmium and lead along with common antibiotics (Dubey and Roy 2003; Roy et al. 2004; Bramhachari 2006; Dubey et al. 2006; Krishnamurthy et al. 2008; De et al. 2008; Ramachandran 2009; Naik et al. 2013). In majority of microorganisms antibiotic as well as heavy metal resistance is generally conferred by same genes which regulate both efflux of metals as well as antibiotics (Mereghetti et al. 2000; Baucheron et al. 2004; Jude et al. 2004; Naik et al. 2013).

3.12 Production of EPS by TBTCl resistant, *Alcaligenes faecalis* strain SD5 and *Klebsiella pneumoniae* strain SD9

3.12.1 Qualitative analysis of EPS

Extracellular polymeric substances (EPS) are biosynthetic polymers produced by both prokaryotic and eukaryotic microorganisms growing in natural as well as artificial environments either as single species, in binary association or in heterogenous communities. Irrespective of their origin, EPS are localized at or outside the bacterial cell surface and comprised of a variety of high molecular weight organic macromolecules such as polysaccharides, proteins, nucleic acids, phospholipids along with other non-polymeric constituents of low molecular weight (Wingender et al. 1999; Bhaskar and Bhosle 2006; Bramhachari and Dubey 2006; Bramhachari et al. 2007). During growth under natural environment, bacterial EPS play important roles in cell adhesion, formation of microbial aggregates viz. biofilms, flocs, sludges and biogranules (Sutherland 2001; Tay et al. 2001; Comte et al. 2006) and protect cells from hostile environments. They are also involved in the degradation of particulate substances, sorption of dissolved materials including heavy metals (Gutnick and Bach 2000; Iyer et al. 2004; Morillo et al. 2006), leaching of minerals from sulphide ores as well as biocorrosion (Gehrke et al. 1998). They are also known to act as surfactants and bioemulsifiers by solubilising the non biodegradable recalcitrants and making them assimilable and bioavailable to the bacteria (Bhaskar and Bhosle 2005).

In our studies with *Alcaligenes faecalis* strain SD5 and *Klebsiella pneumoniae* strain SD9, both congo red agar assay and alcian blue staining revealed production of EPS in presence as well as absence of TBTCl in the growth medium. Congo red agar assay clearly revealed that *Klebiella pneumoniae* strain SD9 *and Alcaligenes faecalis* strain SD5 produced EPS under both conditions i.e. in presence as well as absence of

TBTCl, as bacterial colonies were observed to be pinkish on both the plates (Fig. 3.8 a, b). Formation of pink colony in presence of TBTCl indicates that EPS production was carried out by the bacterial cells even in presence of this toxic biocide.

Bacterial cells of *Klebiella pneumoniae* strain SD9 and *Alcaligenes faecalis* strain SD5 observed after alcian blue staining showed red coloured bacterial cells and blue coloured EPS (Fig. 3.9 a,b). Interestingly cells exposed to TBTCl showed a dense sheath of EPS indicating its over production in presence of TBTCl as compared to control This clearly indicates that EPS production is enhanced due to TBTCl. Enhanced production of EPS by TBTCl resistant isolates may also serve as a resistance mechanism possibly by trapping heavy metals along with TBTCl.

Over the last few decades studies on the use of microorganisms for environmental restoration have primarily focused attention towards exploiting microbial potential for remediation of heavy metal contamination in both terrestrial and aquatic environments. Bioremediation of toxic metals and radionuclides from polluted sediments and waste stream employ living and / or non-living microbial biomass or isolated biopolymers as agents for biosorption (Gadd 2000; Iyer et al. 2004; Braissant et al. 2007**;** Morillo et al. 2008; Pal and Paul 2008).

3.12.2 Quantitative analysis of EPS

EPS production by *Alcaligenes faecalis* strain SD5 showed significant increase from 283 ± 1.8 mg/L without TBTCl (control) to 868 ± 3.7 mg/L in presence of 3 mM TBTCl in MSM. Thus it was confirmed that approximately 3 times higher EPS was produced in the presence of 3 mM TBTCl in MSM than control (Fig. 3.10).

Production of EPS by *Klebsiella pneumoniae* strain SD9 was found to have increased approximately by 2.5 times in presence of 2 mM TBTCl as compared to

control (without TBTCl). EPS production by *Klebsiella pneumoniae* strain SD9 was recorded as 221 ± 2 mg/L in control flask while 530 ± 3.1 mg/L in presence of 2 mM TBTCl (Fig. 3.10).

3.12.3 Quantitative analysis of total carbohydrates (TOC) in EPS

It is interesting to note that in absence of TBTCl the TOC in EPS produced by estuarine bacteria *Alcaligenes faecalis* strain SD5 was 0.7±0.1 mg ml^{-1} whereas in presence of 3 mM TBTCl an increase of TOC in EPS to 2.2 ±0.3 mg ml^{-1} was recorded (Fig. 3.11). Thus it was confirmed that roughly three times (3x) higher TOC in EPS than its control was observed in presence of TBTCl, in TBTCl degrading estuarine *Alcaligenes faecalis* strain SD5.

In absence of TBTCl, the TOC in EPS produced by *Klebsiella pneumoniae* strain SD9 was 0.5 ± 0.1 mg ml^{-1} whereas in presence of 2 mM TBTCl an increase of TOC in EPS to 1.21 ± 0.3 mg ml^{-1} was recorded. This again confirmed an approximate increase of 2.5 folds in TOC in EPS in presence of TBTCl than in control (Fig. 3.11).

In our studies, congo red agar assay, alcian blue staining and EPS quantification revealed production of EPS in presence as well as absence of TBTCl amendment in the growth medium. Although, Alcian Blue staining showed a dense layer of EPS when cells were grown in presence of TBTCl, the same was evidently confirmed through EPS quantification. This helps in concluding that EPS production is enhanced in response to TBTCl toxicity. Increase in production of exopolysaccharides as a response to TBTCl stress may be a resistance mechanism adapted by the bacteria to counteract TBTCl toxicity. TBT resistant bacteria are known to show enhanced production of EPS when exposed to high levels of this biocide. It has also been observed, that maximum yield of TBTCl degrading *Vibrio* sp. in a medium containing

TBTCl as opposed to tributyltin deficient media (i.e. MSM) (Krishnamurthy et al. 2007; Ramachandran and Dubey 2009). Several reports have demonstrated that majority of bacteria produce EPS which are involved in heavy metal sequesteration outside the cell, cell adhesion, biofilm formation and cell survival (Decho 1990; Friedman and Kolter 2004; Iyer et al. 2004; Bhaskar and Bhosle 2005; 2006; Pal and Paul 2008; Naik and Dubey 2011). Hexadecane degradation in *Enterobacter cloacae* strain TU is governed by an exopolysaccharide which is acts as a hexadecane bioemulsifier (Hua et al. 2010). Similarly enhanced EPS production by organotin resistant bacterial strains may play an important role in emulsification of TBTCl or immobilization of TBTCl outside the cell through binding of TBTCl with several functional groups of EPS viz. hydroxyl, carboxyl, amides and phosphoryl which exhibit high affinity towards heavy metals (Bhaskar and Bhosle 2006; Bramhachari et al. 2007; Braissant et al. 2007; Ramachandran and Dubey 2009). The overproduction of EPS by the TBTCl resistant strains thus confirms that it plays a significant role in the cell survival under TBTCl stress through extracellular sequestration of this biocide. The EPS produced, adheres to TBTCl in the environment and shields the bacteria from its toxicity. Role of EPS against heavy metal toxicity has been documented extensively in earlier studies (Friedman and Kolter 2004; Iyer et al. 2004; Pal and Paul 2008; Naik et al. 2011; 2012). But nothing is known about role of EPS in TBT detoxification so far. Thus it seems that EPS over production by TBTCl resistant bacteria important mechanism of resistance to combat TBTCl stress.

3.13 Production of siderophores by TBTCl resistant *Alcaligenes faecalis* strain SD5 and *Klebsiella pneumoniae* strain SD9

3.13.1 Qualitative analysis of siderophores

TBTCl resistant bacterial strains were found to produce siderophores in presence as well as absence of this antifouling biocide which was evidently revealed by change in colour of CAS agar from blue to yellow-orange halo (Fig. 3.12 a,b). Bacterial strains produced siderophores to sequester iron along with TBTCl present in the growth medium. Enhanced production of siderophores was recorded in *Alcaligenes faecalis* strain SD5 and *Klebsiella pneumoniae* strain SD9 as increase in size of yellow-orange halo around the bacterial colony on CAS agar plates containing 3 mM TBTCl and 2 mM TBTCl respectively (Fig. 3.12 a,b).

Our earlier studies have confirmed that *Alcaligenes faecalis* strain SD5 and *Klebsiella pneumoniae* strain SD9 are TBTCl degraders as they utilised TBTCl as a sole carbon source after degrading it into its less toxic derivatives. It may be hypothesized that as a result of complete degradation of TBTCl, tin ions (Sn^{2+}) are released in the growth medium, which may be sequestered by the siderophores and EPS. Thus an increase in siderophore production may also be due to an increase in demand of iron by the TBTCl stressed bacterial cells. Therefore enhanced siderophore production was recorded when the bacterial cells were exposed to high levels of TBTCl (Fig. 3.12 a,b). These studies have clearly demonstrated important role of siderophores in TBTCl resistance in *Alcaligenes faecalis* strain SD5 and *Klebsiella pneumoniae* strain SD9.

Microbial siderophores are mainly responsible for chelating iron from the environment but they also play a major role in conferring tributyltin resistance in bacteria (Inoue et al. 2000; 2003 a; b; Sun et

al. 2006). These siderophores also chelate many other heavy metals viz. Pb, Zn, Cd, Cu, Hg along with organotins thereby reducing the bioavailability of these toxic metals and organometal TBT to microbial cells. They may also protect bacterial strains in aquatic environments contaminated with high levels of tributyltin. It has been reported that TBT is taken up by siderophores of *Aeromonas* sp. which gradually gets exported as DBT after its possible degradation inside the cells (Cruz et al. 2007). Similarly *Burkholderia* sp. showed siderophore production and degradation of TBT indicating possible role of siderophore in TBT degradation (Inoue et al. 2003 b). There are also other reports on involvement of bacterial siderophores in degradation of other organotins such as triphenyltin (Inoue et al. 2000; Sun et al. 2006). The role of pyoverdine in *Pseudomonas chlororaphis* CNR15 in organotin degradation, and pyochelin and pyoverdine involving triphenyltin decomposition in *Pseudomonas aeruginosa* CGMCC 1.860 has clearly demonstrated the potential of siderophores in degradation of organotins (Inoue et al. 2003 a; Sun et al. 2006).

TBT degradation marine *Pseudomonas* spp. isolated from indian coastal waters is a two-step process: (a) dispersion of TBT in the aqueous phase by emulsification activity of EPS and (b) tin–carbon bond cleavage by siderophores affecting debutylation of TBT (Sampath et al. 2012). In the light of all above already published reports which suggest that EPS and siderophores have a major role in toxic heavy metal and organometal resistance in bacteria and the findings of our research work validates that in presence of TBTCl stress EPS helps in sequestration and subsequent

emulsification of TBTCl and siderophores cleaves tin-carbon bond, resulting in degradation of TBTCl.

3.13.2 Biochemical characterization of siderophores

Csaky's and Arnow's assays clearly revealed that siderphores produced by *Alcaligenes faecalis* strain SD5 were catecholate type and from *Klebsiella pneumoniae* strain SD9 were hydroxamate type respectively (Fig. 3. 13 a,b). Both *Alcaligenes faecalis* and *Klebsiella pneumoniae* are commonly known to produce siderophores (Gokarn and Pal 2010; Sayyed and Chincholkar 2010).

Interestingly, we have observed enhanced production of siderophores by these bacterial strains in presence of high levels of TBTCl. Thus these bacterial strains have a great potential to clean up TBTCl contaminated sites since extracellular siderophores may serve as biosorptive entities to faciltate removal of TBTCl.

Siderophores are known to chelate iron and facilitate bacterial survival in iron deficient conditions. Besides Fe^{+3} the microbial siderophores also form stable complexes with other metals viz. Cd^{+2}, Pb^{+2}, and Zn^{+2} (Namiranian et al. 1997; Gilis et al. 1998; Hepinstall et al. 2005). Induction of bacterial siderophore synthesis in response to Cd^{+2}, Zn^{+2} and Cu^{+2} stress has also been reported which is responsible for detoxification of these heavy metals as a consequence of chelation (Clarke et al. 1987; Rossbach et al. 2000; Sinha and Mukherjee 2008) and thus proved to be an important strategy of microorganisms to sequester non-toxic (micronutrients) as well as toxic metals.

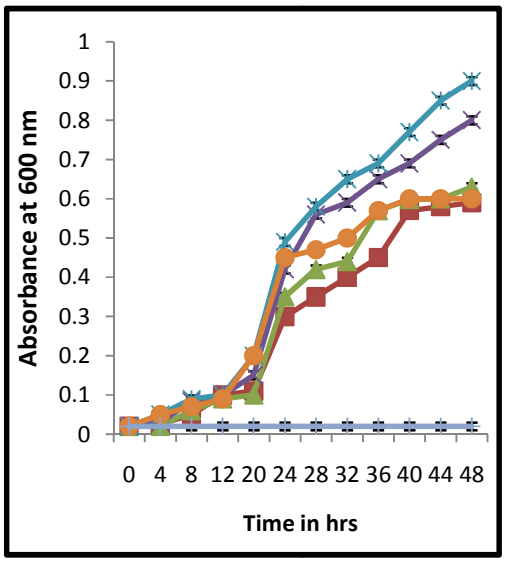
3.14 Intracellular bioaccumulation of TBTCl by *Pseudomonas mendocina* strain DP4

Bacterial cells of *Pseudomonas mendocina* strain DP4 grown in MSM with 0.1 % glucose and 1mM TBTCl for 48 hrs when homogenized and analysed by AAS, clearly demonstrated significant bioaccumulation of TBTCl. It is interesting to mention that bioaccumulation was 8 µg ± 0.1 Sn/gm cell dry weight. Interestingly, few *Pseudomonas* spp. have already been reported to bioaccumulate tributyltin up to 2 % of their dry weight (Blair et al. 1982; Gadd 2000). Several gram negative bacteria possess capability to accumulate tributyltin oxide without its breakdown (Barug 1981). The high lipid solubility of organotin ensures cell penetration and association with intracellular sites, while some cell wall components also play an important role in their intracellular bioaccumulation (Blair et al. 1982; Gadd 2000). Although intracellular bioaccumulation of heavy metals in bacteria is a well known resistance mechanism against heavy metals (Harwood-Sears and Gordon 1990; Nies and Silver 1995; Rensing et al. 1999; Roane 1999; Iyer et al. 2004; Naik et al. 2012 a, b), but this is the first report confirming TBTCl resistance mediated by intracellular bioaccumulation of TBTCl in *Pseudomonas mendocina* strain DP4. These studies emphasize that intracellular bioaccumulation of TBTCl without its breakdown is the major resistance mechanism adopted by *Pseudomonas mendocina* strain DP4.

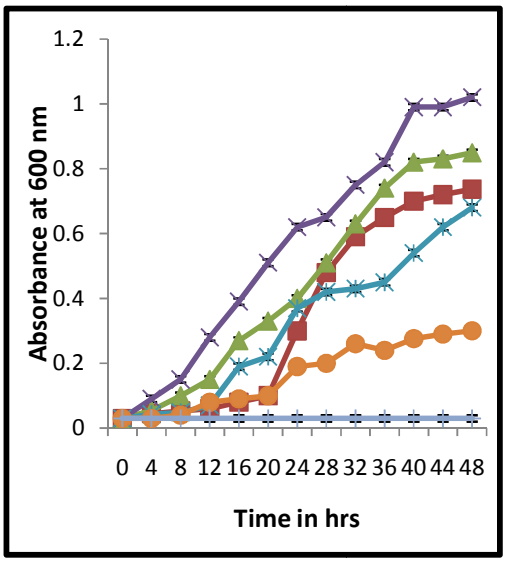
In order to maintain heavy metal homeostasis, intracellular level of toxic heavy metal ions has to be tightly controlled (Nies 1999). Soft metal transporting P-type ATPases which are group of proteins involved in transport of heavy metals outside the cell membrane governing bacterial heavy metal resistance have earlier been reported (Nies and Silver 1995; Rensing et al. 1999; Naik et al. 2013). These transporter proteins are known to prevent over-accumulation of highly toxic and

reactive metal ions viz. Pb (II), Cu (I), Ag (I), Zn (II) and Cd (II). Other proteins commonly referred to as metallothioneins viz. bmt and smt also play a crucial role in intracellular bioaccumulation and transport of heavy metals across bacterial cell membranes (Naik et al. 2012a; b). Similarly, intracellular bioaccumulation of TBTCl in bacteria may also be a characteristic resistance mechanism attributed by presence of certain transport proteins.

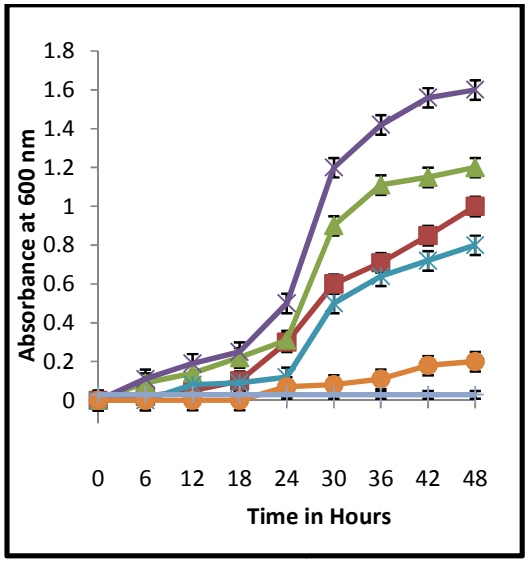
Fig. 3.1 (a-h) Growth response of TBTCl resistant strains in MSM broth with varying concentrations of TBTCl



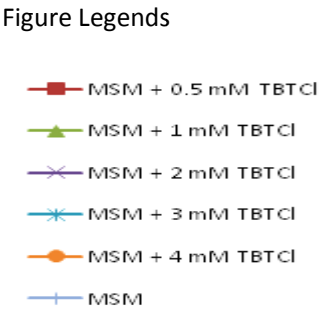
(a) *Alcaligenes faecalis* strain SD5

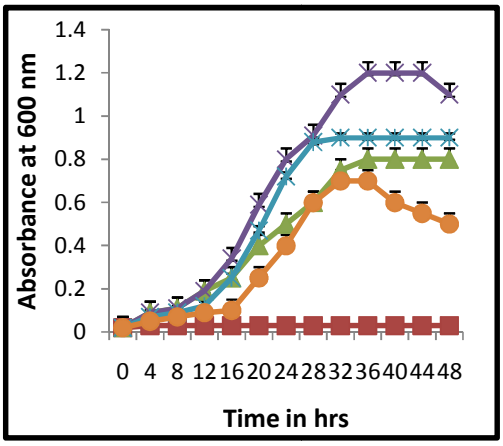


(b) *Klebsiella pneumoniae* strain SD9

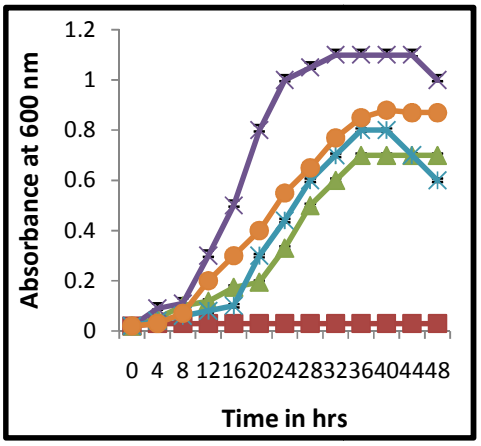


(c) *Pseudomonas stutzeri* strain DN2

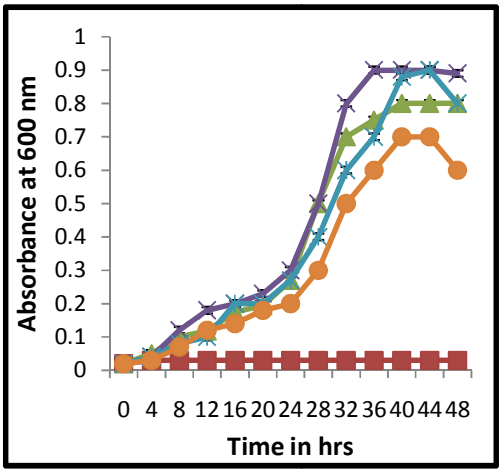




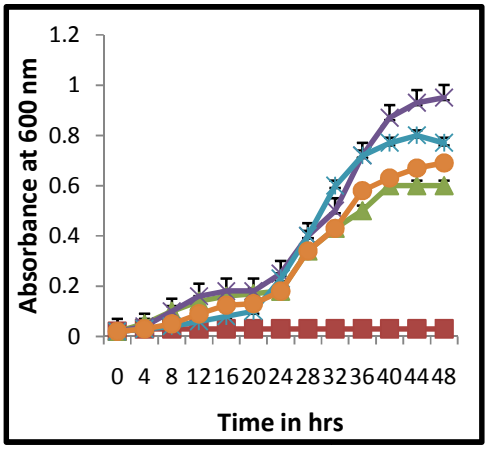
(d) *Pseudomonas stutzeri* strain DP



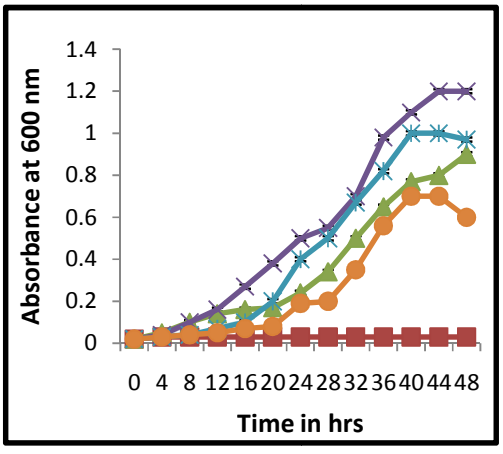
(e) *Aeromonas salmonicida* strain DP2



(f) *Vibrio* sp. DP3



(g) *Pseudomonas mendocina* strain DP4



(h) *Chromohalobacter salexigens* strain DP5

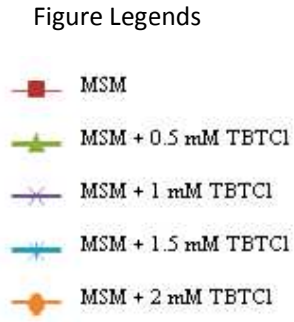
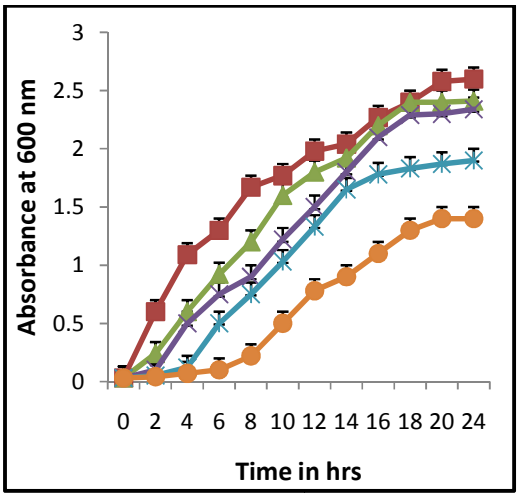
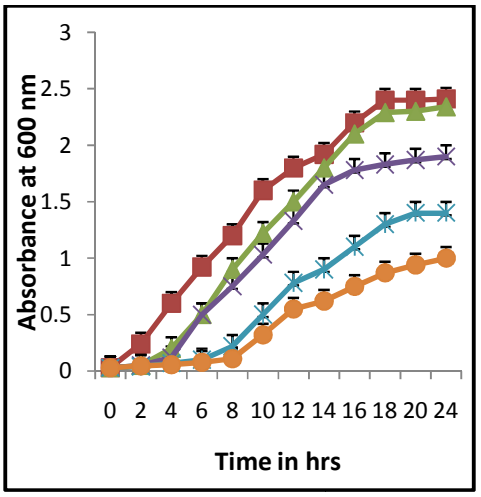


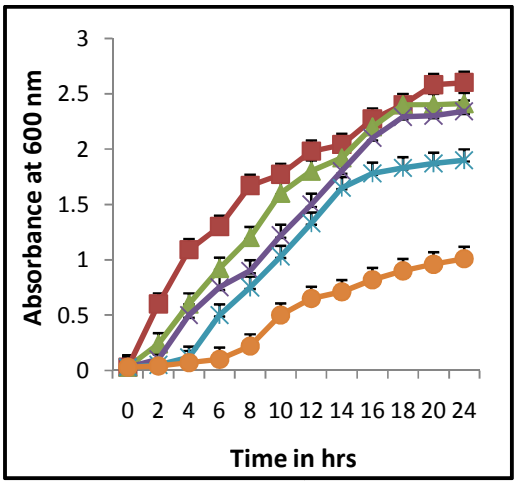
Fig. 3.2 (a-h) Growth response of TBTCl resistant strains in ZMB with varying concentrations of TBTCl



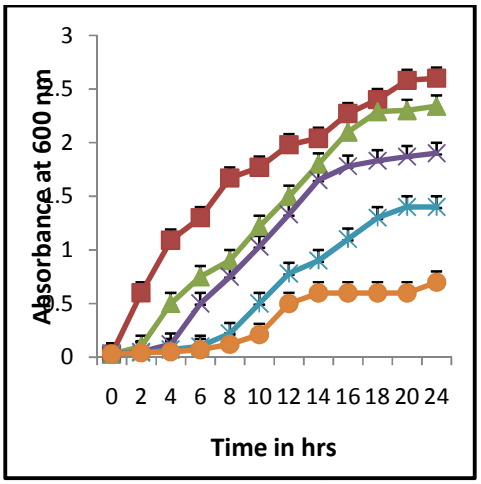
(a) ***Klebsiella pneumoniae*** **strain SD9**



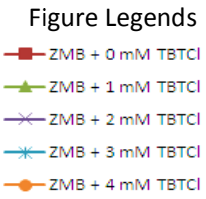
(b) ***Pseudomonas stutzeri*** **strain DN2**

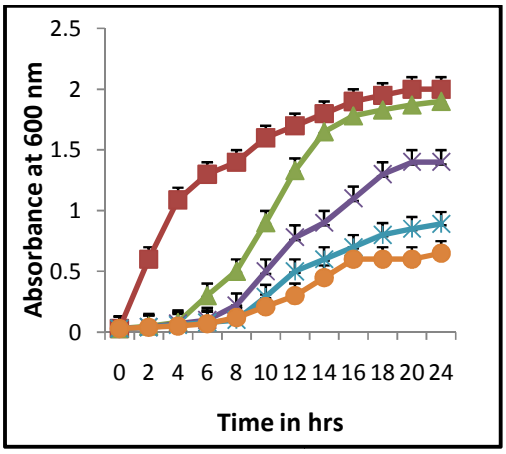


(c) ***Alcaligenes faecalis*** **strain SD5**

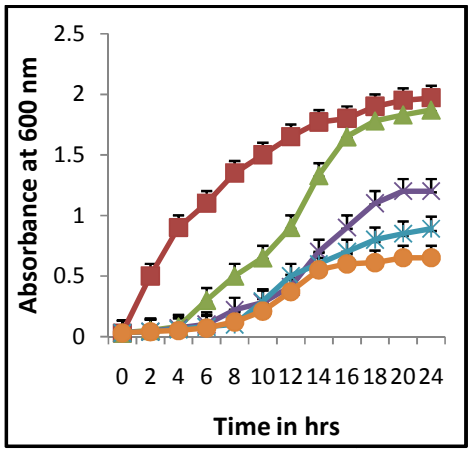


(d) ***Pseudomonas stutzeri*** **strain DP1**

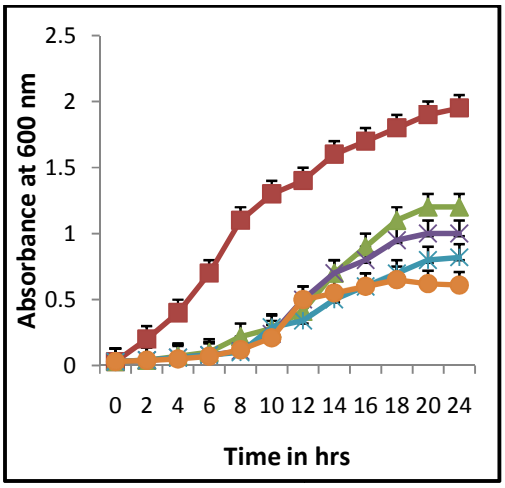




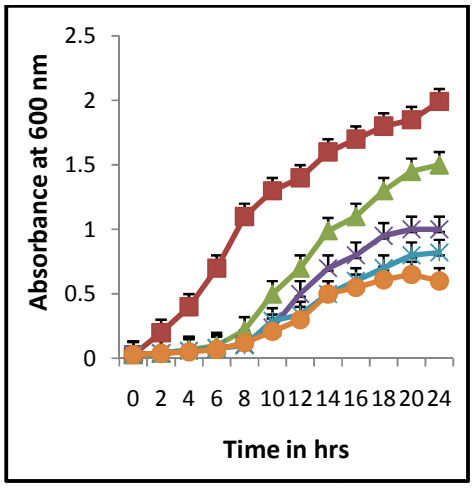
(e) ***Pseudomonas mendocina*** **strain DP4**



(f) ***Vibrio*** **sp. DP3**



(g) ***Chromohalobacter salexigens*** **strain DP5**



(h) ***Aeromonas salmonicida*** **strain DP2**

Figure Legends

ZMB + 0 mM TBTCl

ZMB + 1 mM TBTCl

ZMB + 2 mM TBTCl

ZMB + 3 mM TBTCl

ZMB + 4 mM TBTCl

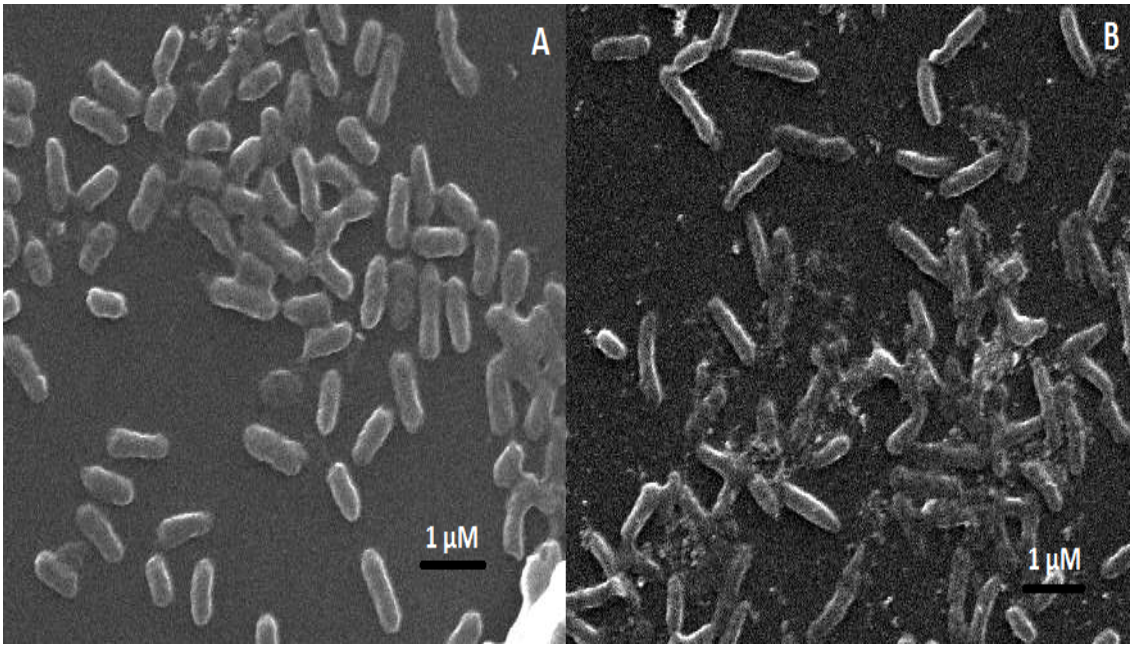


Fig. 3.3 (a) SEM micrographs of TBTCl resistant, *Psedomonas stutzeri* strain DN2 [Bacterial cells grown in A. Absence of TBTCl (MSM + 0.1% glucose) and B. Presence of TBTCl (MSM + 2 mM TBTCl)]. (Magnification: 10,000X)

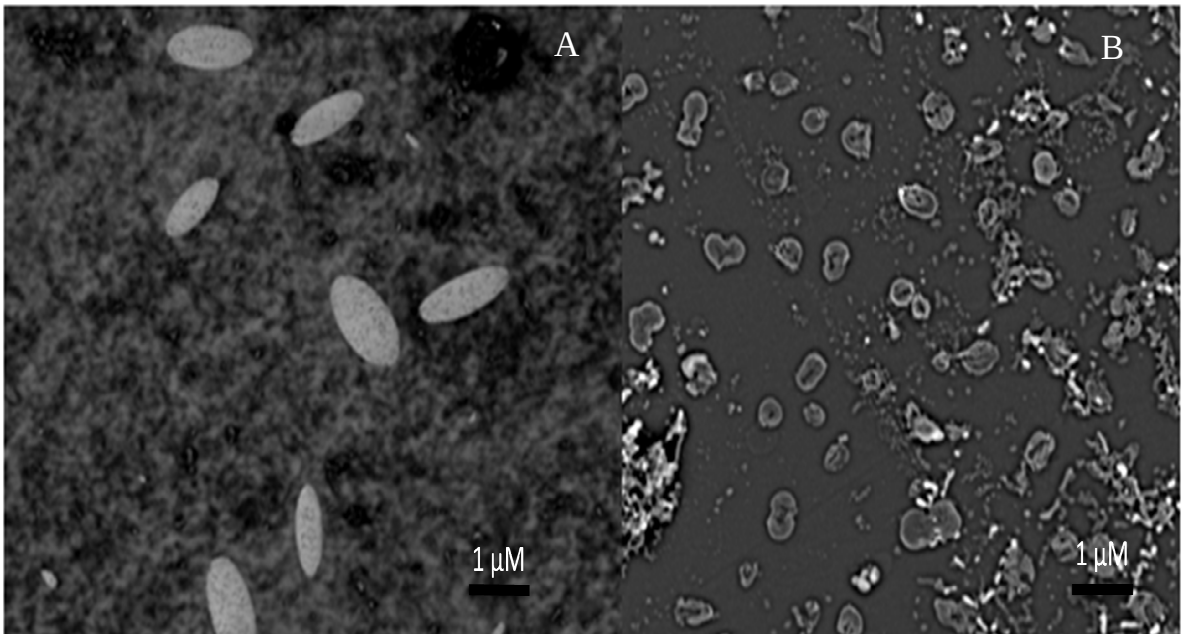


Fig. 3.3 (b) SEM micrographs of TBTCl resistant, *Klebsiella pneumoniae* strain SD9 [Bacterial cells grown in A. Absence of TBTCl (MSM + 0.1% glucose) and B. Presence of TBTCl (MSM + 2 mM TBTCl)]. (Magnification: 10,000X)

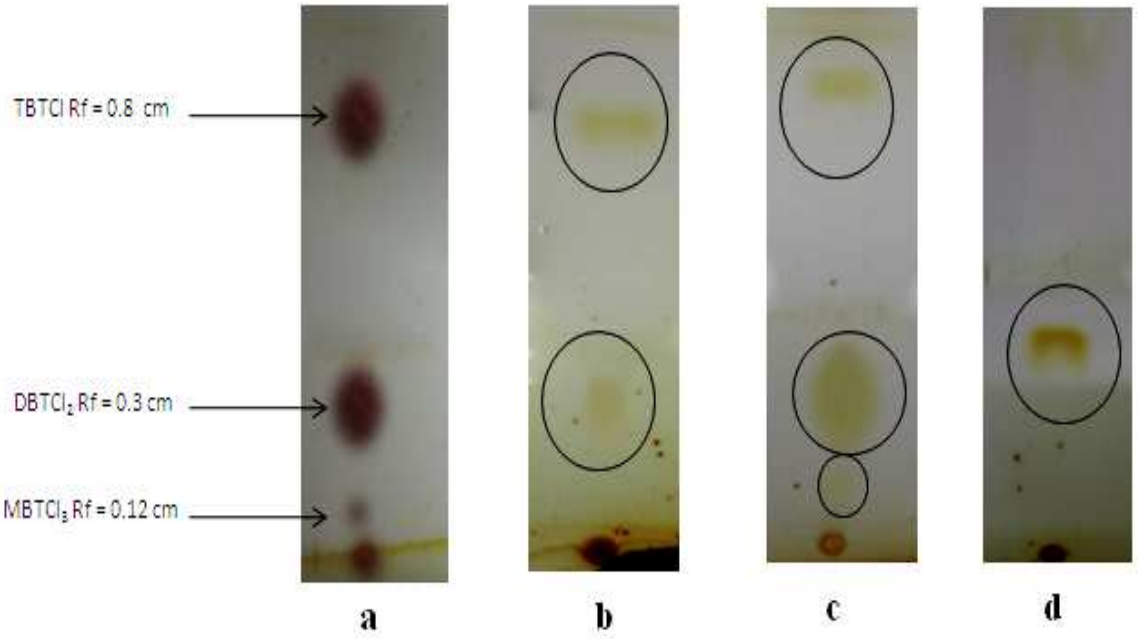


Fig. 3.4 TLC profiles of cell free supernatants of TBTCl resistant bacterial strains

a. (Control) mixture of commercially available TBTCl, $DBTCl_2$ and $MBTCl_3$

b. Cell free supernatant of *Alcaligenes faecalis* strain SD5 grown in 3 mM TBTCl

c. Cell free supernatant of *Klebsiella pneumoniae* strain SD9 grown in 2 mM TBTCl

d. Cell free supernatant of *Pseudomonas stutzeri* strain DN2 grown in 2 mM TBTCl

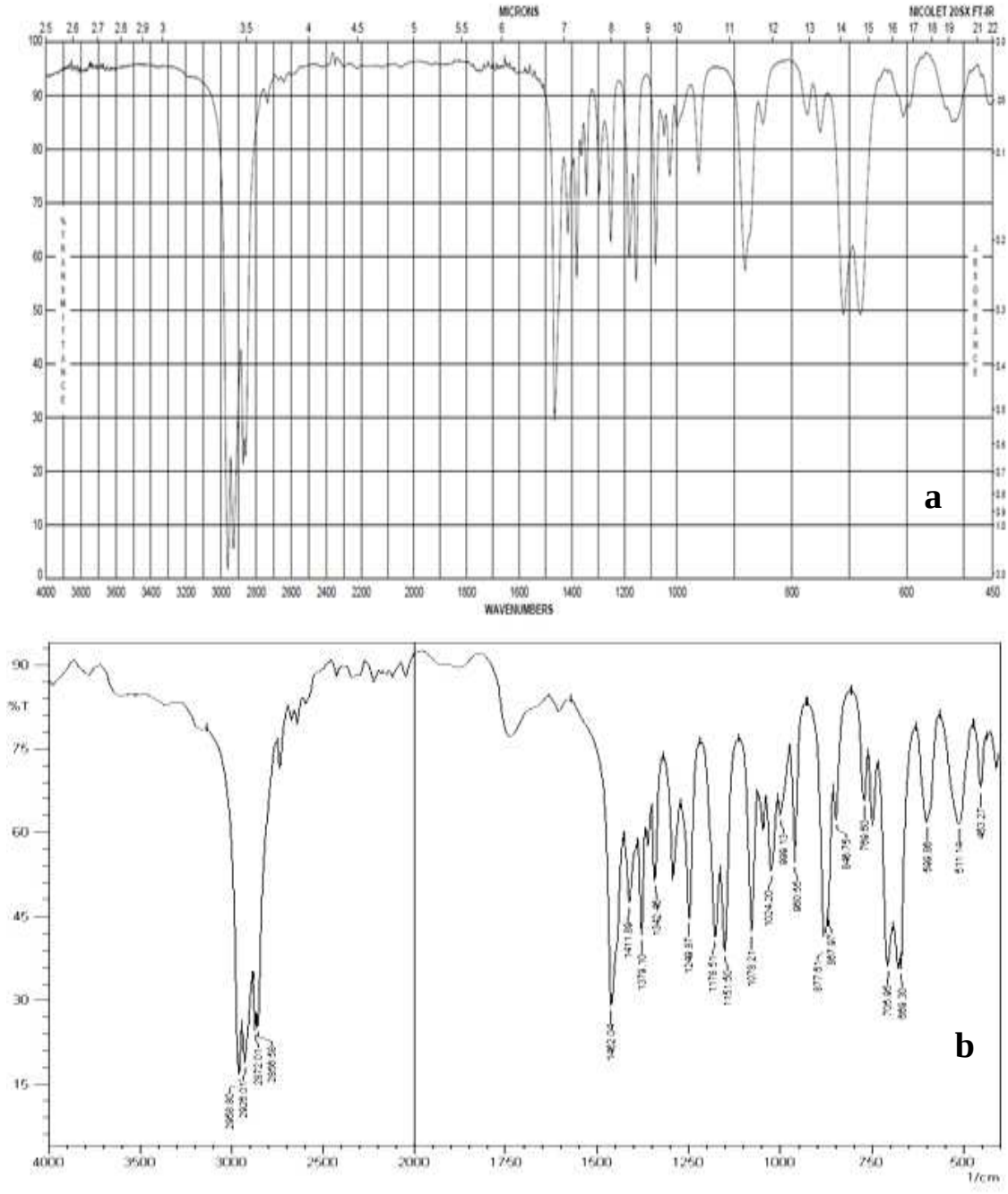


Fig. 3.5 FTIR analysis of purified cell free supernatant of TBTCl resistant, *Pseudomonas stutzeri* strain DN2 (a. standard $DBTCl_2$ and b. biotransformation product)

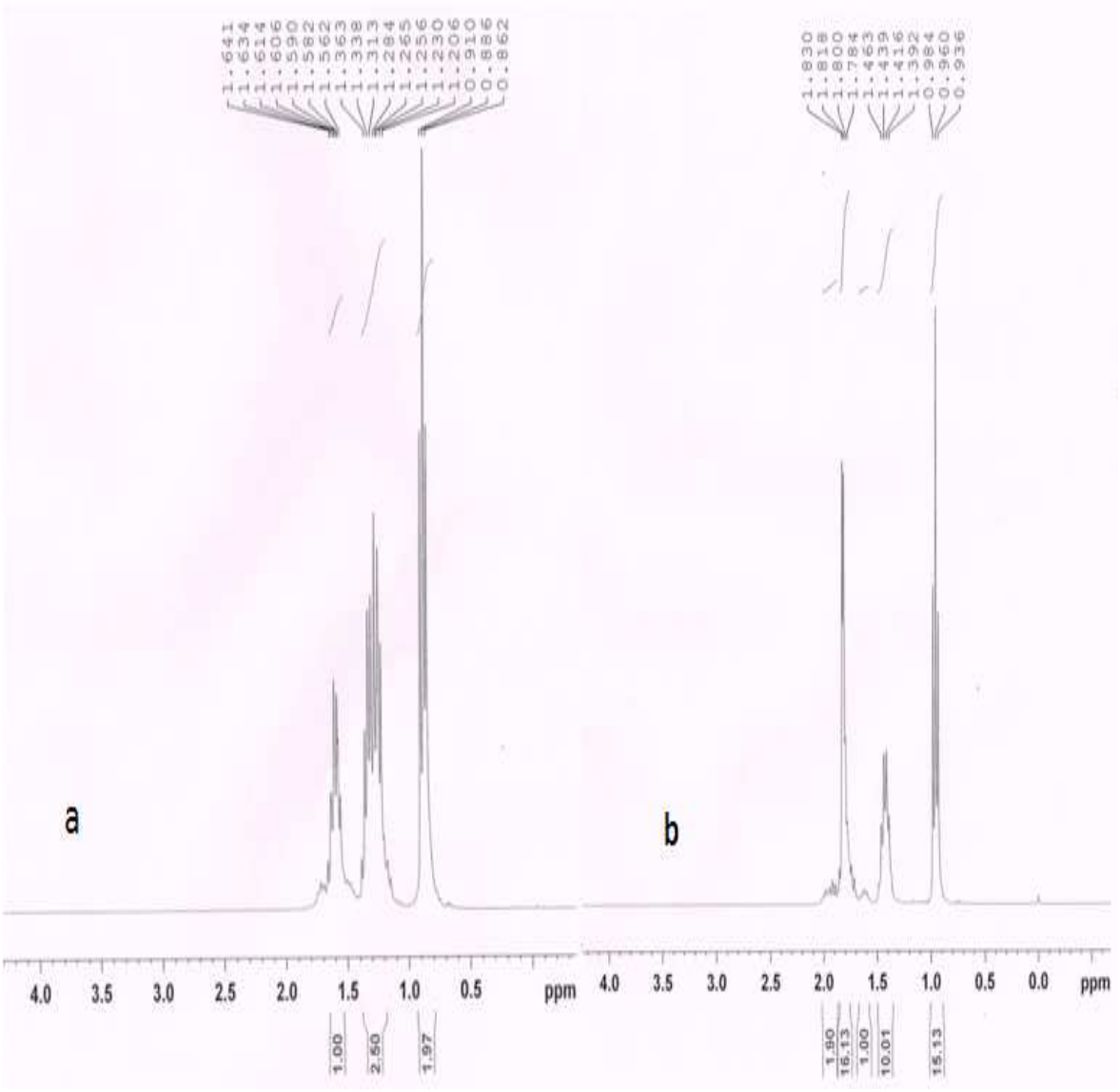


Fig. 3.6 (a) NMR spectrometric analysis of purified cell free supernatant of TBTCl resistant, *Pseudomonas stutzeri* strain DN2 (a. Control b. biotransformation product showing presence of $DBTCl_2$)

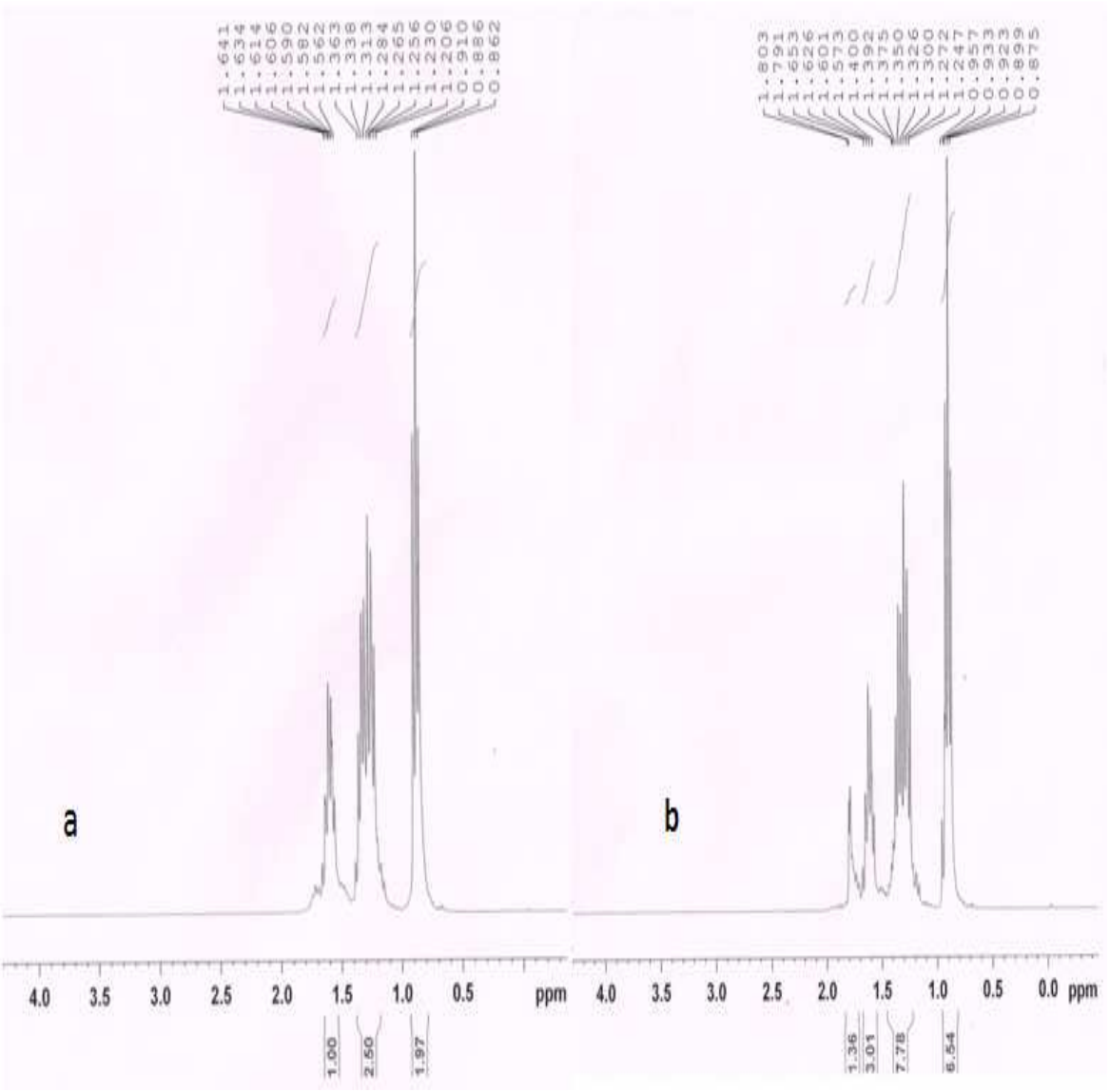


Fig. 3.6 (b) NMR spectrometric analysis of purified cell free supernatant of TBTCl resistant *Alcaligenes faecalis* strain SD5 (a. Control b. biotransformation product showing presence of TBTCl and $DBTCl_2$)

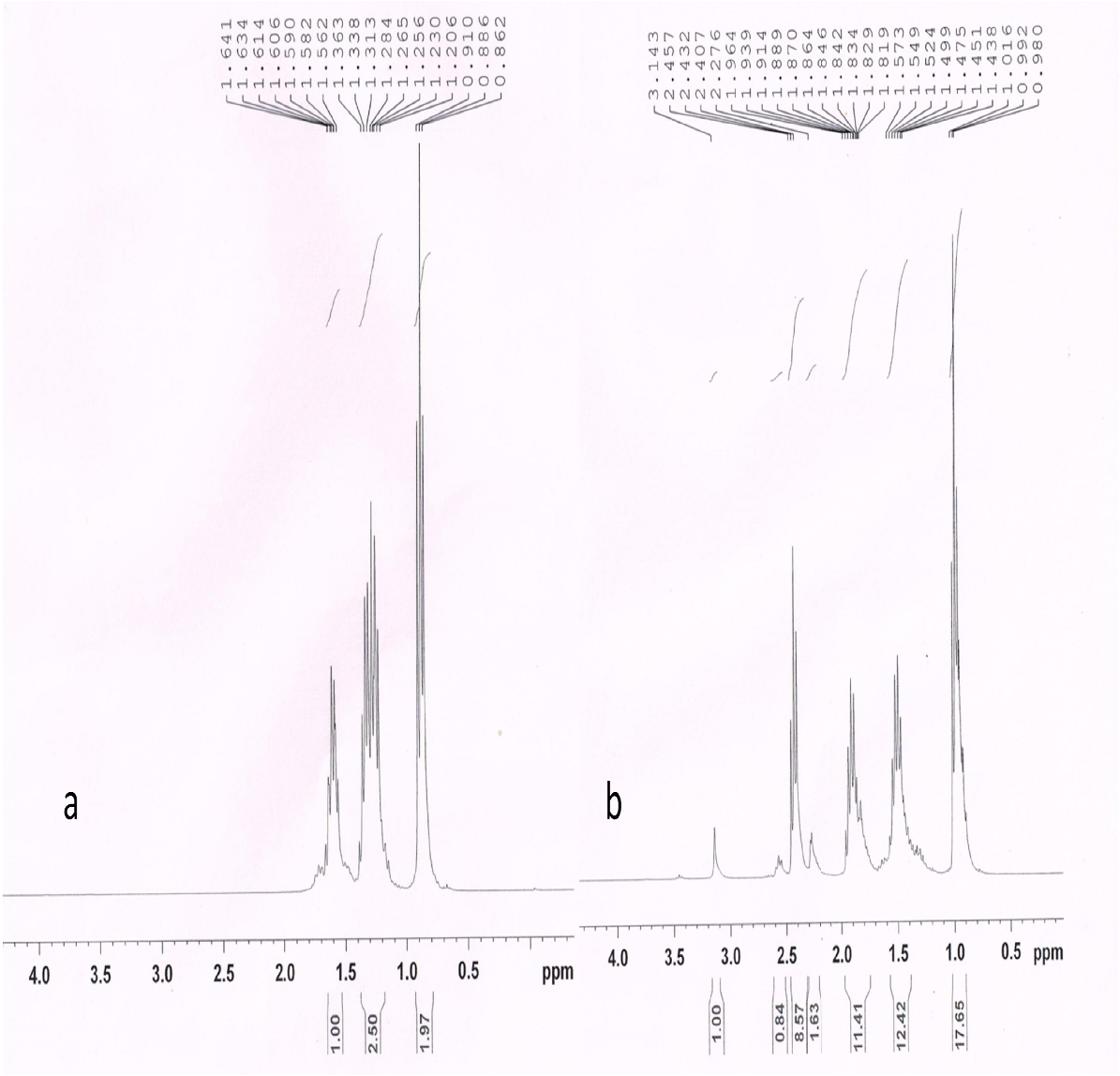
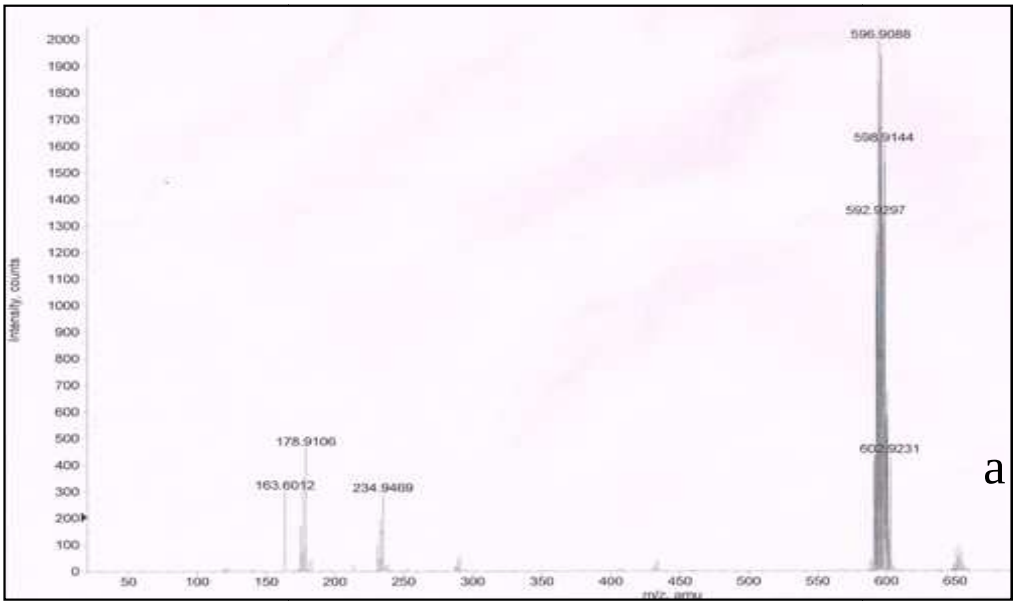


Fig. 3.6 (c) NMR spectrometric analysis of purified cell free supernatant of TBTCl resistant *Klebsiella pneumoniae* strain SD9 (a. Control and b. Degradation product showing presence of $DBTCl_2$ and $MBTCl_3$)



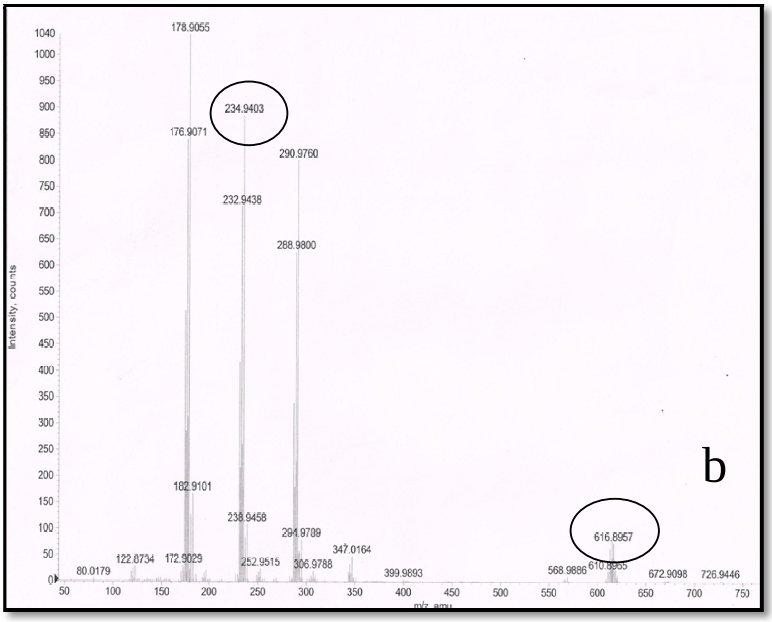
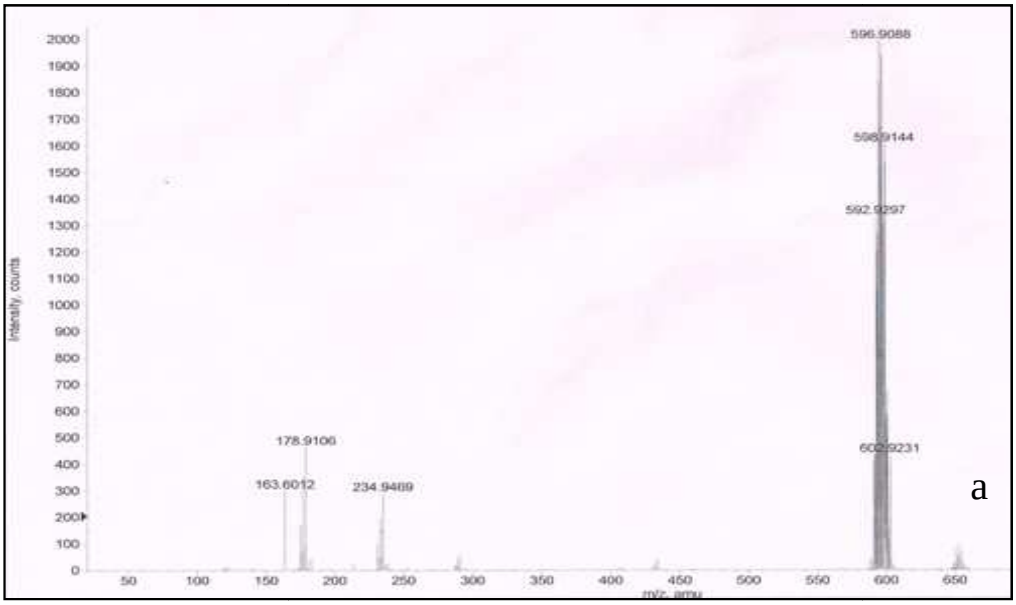


Fig. 3.7 (a) Mass spectrometric analysis of purified degradation product of TBTCl resistant, *Alcaligenes faecalis* strain SD5 (a. Control and b. Degradation product showing presence of $DBTCl_2$)



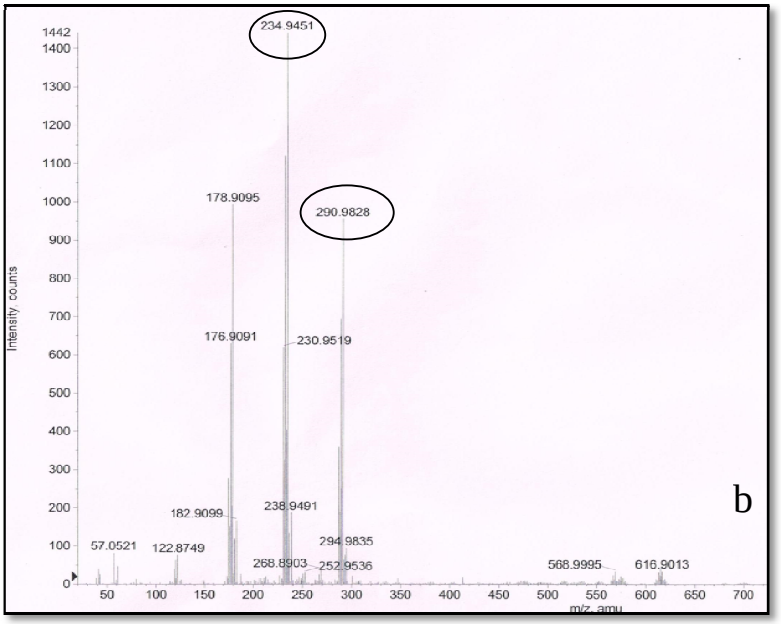
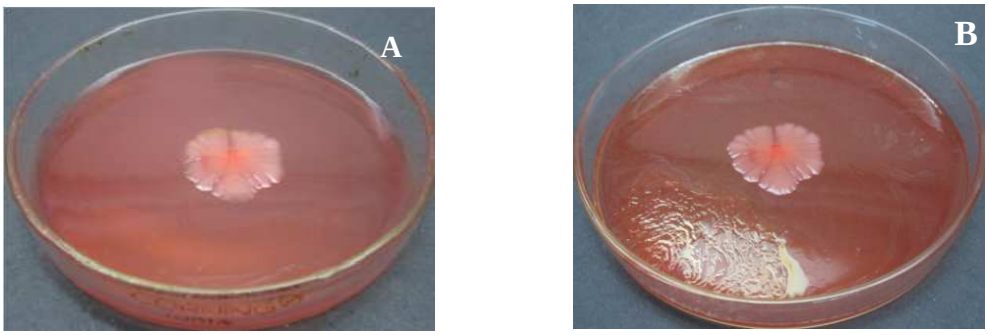


Fig. 3.7 (b) Mass spectrometric analysis of purified degradation product of TBTCl resistant, *Klebiella pneumoniae* strain SD9 (a. Control and b. Degradation product showing presence of $DBTCl_2$)

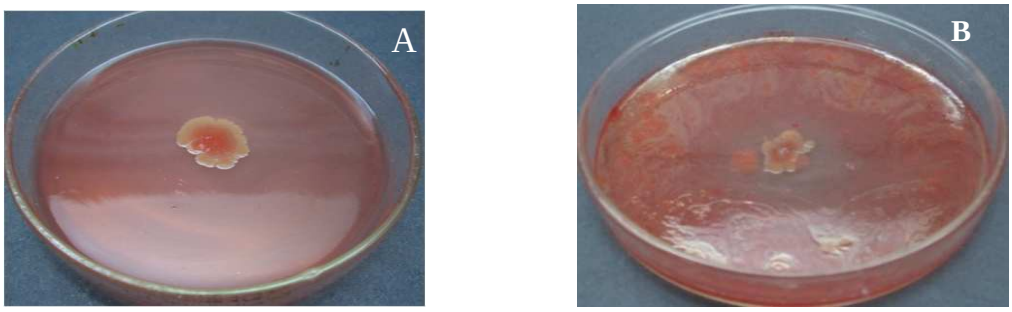
Antibiotic	Resistant (R) / Sensitive (S)		
	Alcaligenes faecalis strain SD5	*Klebsiella pneumoniae* strain SD9	*Pseudomonas stutzeri* strain DP1
Cephalothin 30mcg/disc	R	R	R
Clindamycin 2 mcg/disc	R	R	R
Cloxacillin 5 mcg/disc	R	R	R
Gentamicin 10 mcg/disc	R	R	R
Oxytetracyclin 30 mcg/disc	S	S	S
Penicillin G 10 Units/disc	R	R	R
Co-Trimoxazole 25 mcg/disc	R	S	R
Erythromycin 15 mcg/disc	R	R	R
Amikacin 10 mcg/disc	S	R	R
Carbenicillin 100 mcg/disc	R	R	S
Ciprofloxacin 10 mcg/disc	S	S	R
Co-trimazine 25 mcg/disc	S	S	S
Kanamycin 30 mcg/disc	R	S	R
Nitrofurantoin 300 mcg/disc	R	R	R
Streptomycin 10 mcg/disc	S	S	S
Tetracycline 30 mcg/disc	S	S	S

Table. 3.1 Antibiotic resistance exhibited by TBTCl resistant bacterial strains to commonly used antibiotics.



(a) *Alcaligenes faecalis* strain SD5

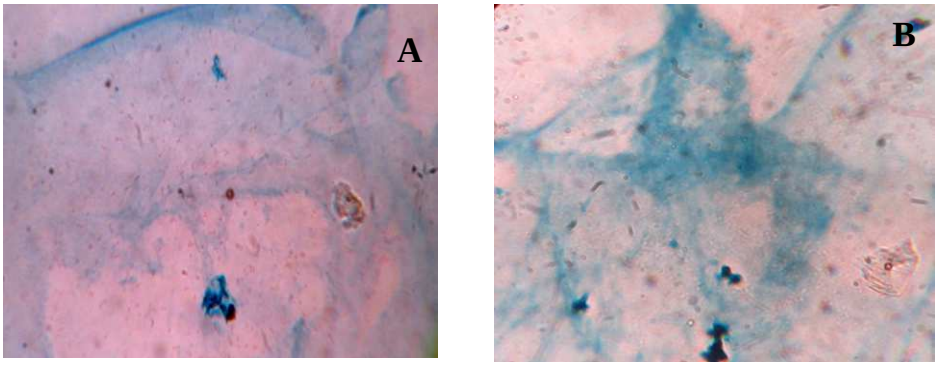
(A. in absence of TBTCl (control) and B. in presence of 3 mM TBTCl)



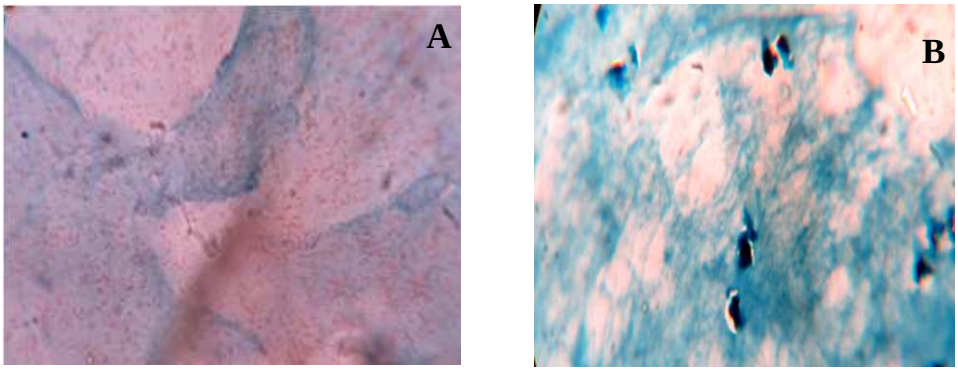
(b) *Klebsiella pneumoniae* strain SD9

(A. in absence of TBTCl (control) and B. in presence of 2mM TBTCl)

Fig. 3.8 (a, b) Detection of EPS production by TBTCl resistance bacterial strains on Congo Red Agar



(a) *Alcaligenes faecalis* strain SD5
(A. EPS production in absence of TBTCl and B. EPS production in presence of 3 mM TBTCl) (magnification 40X)



(b) *Klebsiella pneumoniae* strain SD9
(A. EPS production in absence of TBTCl and B. EPS production in presence of 2 mM TBTCl) (magnification 40X)

Fig. 3.9 (a, b) Detection of EPS production by TBTCl resistance bacterial strains by Alcian Blue Staining Technique.

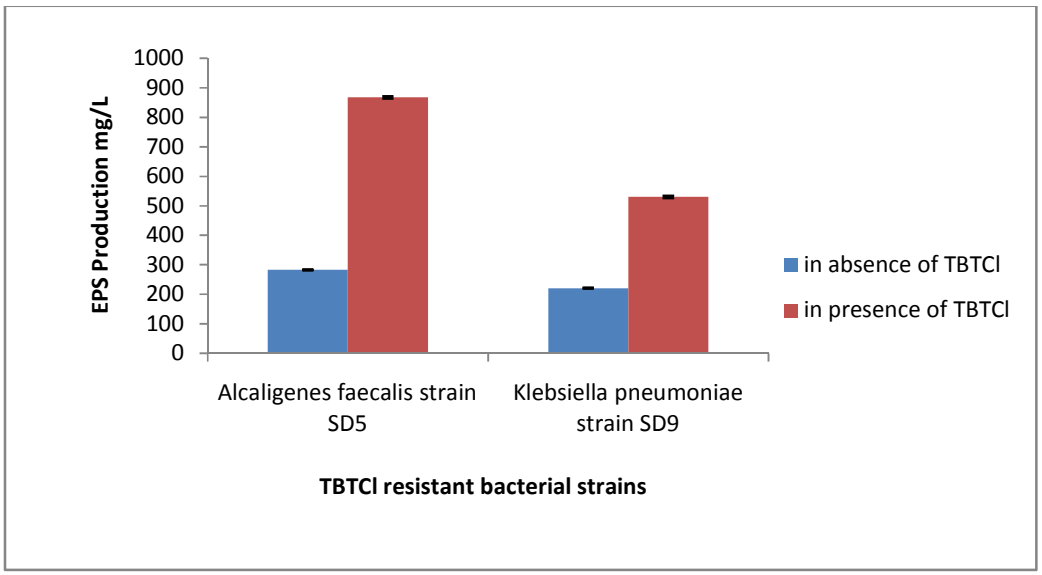


Fig. 3.10 **Quantitative detection of EPS production by *Alcaligenes faecalis* strain SD5 and *Klebsiella pneumoniae* strain SD9**

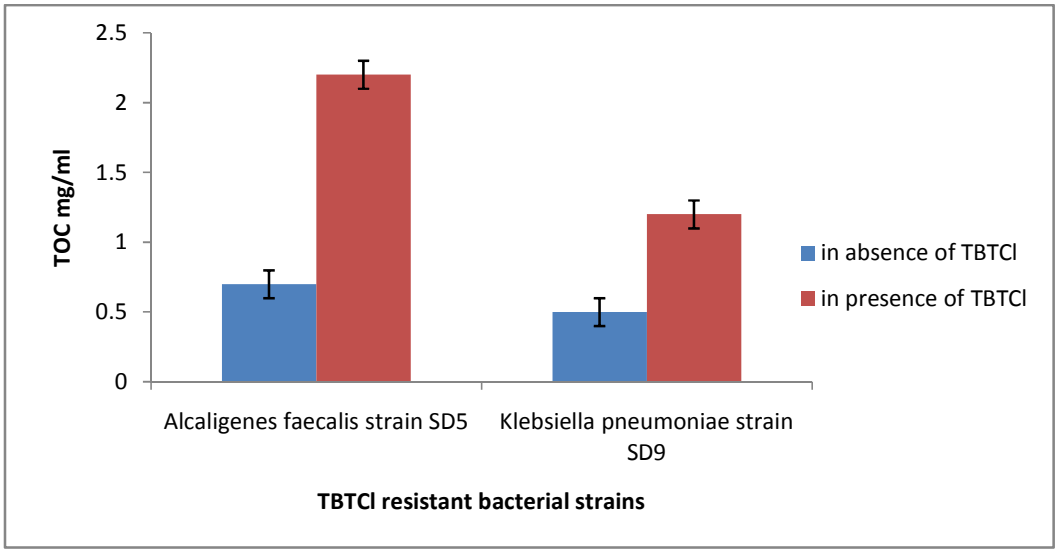
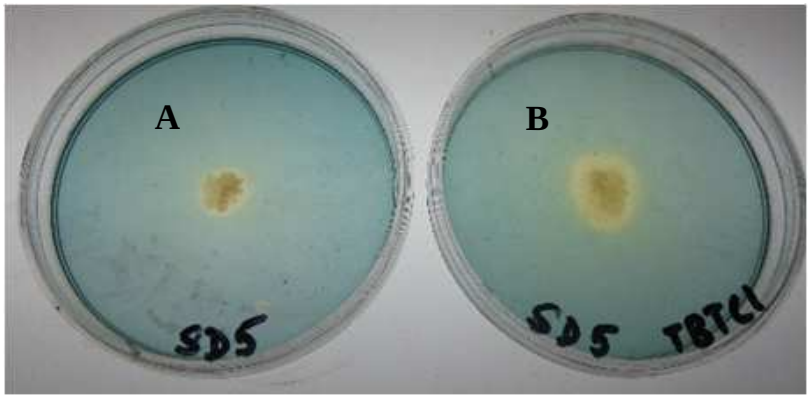
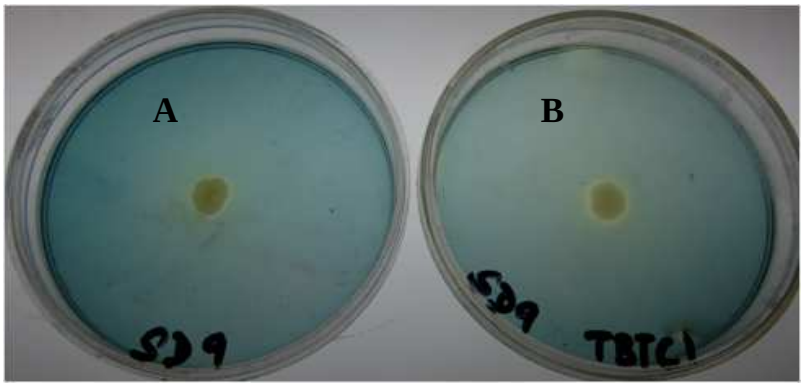


Fig. 3.11 **Quantitative detection of total carbohydrates (TOC) in EPS produced by *Alcaligenes faecalis* strain SD5 and *Klebsiella pneumoniae* strain SD9**



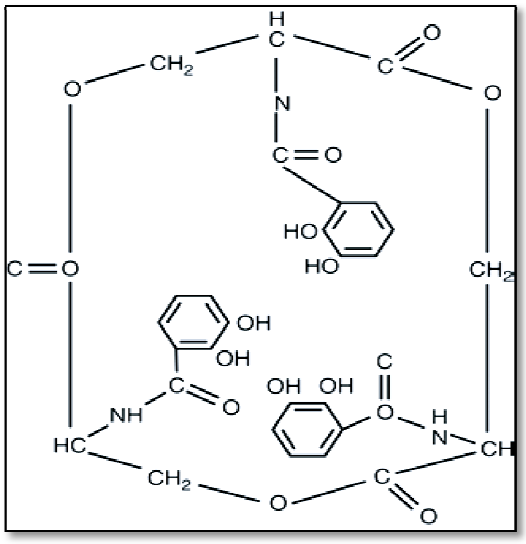
(a) *Alcaligenes faecalis* strain SD5
(A. in MSM + 0.1% glucose and B. in MSM + 3 mM TBTCl)



(B. *Klebsiella pneumoniae* strain SD9
(A. in MSM + 0.1% glucose and B. in MSM + 2 mM TBTCl)

Fig. 3.12 (a, b) Detection of siderophores produced by TBTCl resistant bacterial strains

(a)



(b)

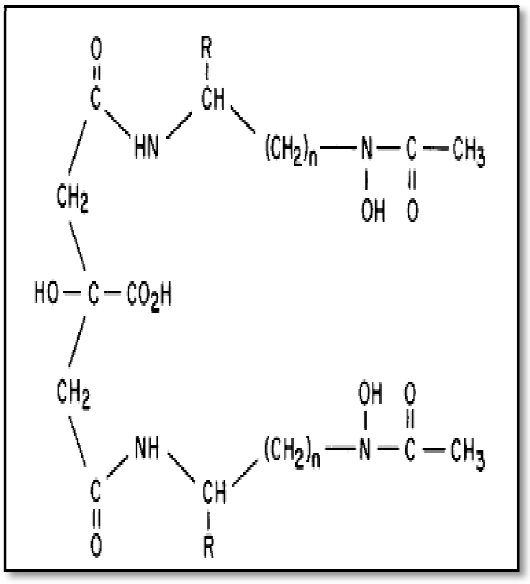


Fig. 3.13 **Chemical structure of siderophores produced by TBTCl resistant bacterial strains**

a. Catecholate type siderophore (produced by *Alcaligenes faecalis* strain SD5)

b. Hydroxamate type siderophore (produced by *Klebsiella pneumoniae* strain SD9)

CHAPTER IV

MOLECULAR BIOLOGICAL CHARACTERIZATION OF TBTCl RESISTANT BACTERIAL ISOLATES

MATERIALS AND METHODS

4.1 Screening of plasmids from TBTCl resistant bacterial strains

TBTCl resistant bacterial isolates were screened for the presence of plasmids in order to confirm plasmid mediated TBTCl resistance. Plasmid minipreps were done using alkaline lysis method and analysed by 1% agarose gel electrophoresis (Birnboim and Doly 1979; Sambrook et al. 1989). A single bacterial colony was transferred into 10 ml of ZMB and incubated overnight (16 hrs) at 28 °C at 120 rpm. 1.5 ml of culture suspension was taken in an Eppendorf microfuge tube and harvested at 11,000 rpm for 5 min at 4°C. The supernatant was discarded leaving the bacterial pellet. The pellet was suspended in 100 µl ice-cold glucose EDTA tris-buffer (solution I**)** (Appendix D.4) by vortexing and microfuge tube was subsequently incubated in ice for 10 min. Freshly prepared solution II (200 µl) (Appendix D.4) was added and the contents of microfuge were mixed by inverting the microfuge tubes rapidly 4-5 times. The microfuge tubes were incubated in ice bucket for 10 min. Ice-cold solution III (150 µl) (Appendix D.4) was added and the microfuge tubes were gently vortexed to disperse solution III through the viscous bacterial lysate. The microfuge tubes were incubate in ice for 3-5 min, followed by harvesting at 11,000 rpm for 5 mins at 4°C. The clean supernatant was transferred into a clean microfuge tube. Plasmid DNA was precipitated by mixing two volumes of cold ethanol to the supernatant and incubation at room temperature. The contents were gently mixed by inversion followed by incubation on ice for 2 min. The sample in microfuge tube was centrifuged at 11000 rpm for 5 min at 4°C. The supernatant was discarded and the pellet (plasmid DNA) was washed with 70% (v/v) cold ethanol. The supernatant was discarded again and the pellet was allowed to dry in air for 10 min by keeping microfuge tubes inverted on tissue paper. The dry pellet containing plasmid DNA was

resuspended in appropriate volume i.e. 20-50 µl of TE buffer (pH 8.0) containing DNase free RNAse-A (20 mg/m1) and stored at -20°C until needed for agarose gel electrophoresis analysis.

4.2 Genomic DNA extraction of TBTCl resistant bacterial strains

Genomic DNA of bacterial isolates was extracted following standard procedure of Jones and Barlet (1990). The bacterial isolates were cultured at 28 °C at 120 rpm, for 16 hrs in Zobell marine broth for estuarine isolates (Appendix A.3, A7). Culture suspension (1.5 ml) was transferred in an eppendorf microfuge tube followed by harvesting for 2 minutes at 10,000 rpm using Eppendorf refrigerated centrifuge (Eppendorf 5417R, Germany). The supernatant was decanted and cell pellet was air dried. The cell pellet was resuspended in TE buffer by repeated pipetting 30 µl of 10% SDS and 3 µl of 20 mg/ml Proteinase-K were added and mixed well and suspension was incubated for 1 hr at 37°C. Equal volume of phenol: chloroform (1:1) was added and mixed well by inverting the tube until the phases are completely mixed. The sample was harvested at 9,000 rpm at 4°C for 10 min. The upper aqueous phase was transferred to a new microfuge tube and an equal volume of phenol: chloroform was added again followed by centrifugation at 9000 rpm at 4°C for 5 minutes. The upper aqueous phase was transferred to a new microfuge tube. 3 M sodium acetate ($1/10^{th}$ volume of aqueous phase) and isopropanol (0.6 volumes of aqueous phase) were also added and mixed gently in order to precipitate the' genomic DNA. This sample was centrifuged at 9000 rpm at 4°C for 10 min to get the pellet genomic DNA. The DNA pellet was washed with 0.5 ml 70% cold ethanol and finally resuspended the air dried pellet in appropriate volume of TE buffer depending on the pellet volume. The genomic DNA of bacterial strains was subsequently used as

template to detect *TbtB* and *BmtA* genes in TBTCl resistant bacterial strains through PCR analysis using gene specific primers.

4.3 Agarose gel electrophoresis of genomic and plasmid DNA

Agarose gel electrophoretic analysis of DNA (Genomic DNA and plasmid DNA) was performed using horizontal slab gels BG-100, 10x6 cms apparatus (Banglore Genei, Bangalore, India) and 0.8 % agarose (with 0.5 µg/ml ethidium bromide) prepared in 1X TAE buffer (Appendix D.5). Electrophoresis was performed using 1X TAE buffer (Appendix E.4) as electrode buffer at constant voltage of 80 V for 90 min in the gel. DNA samples (5 µl) containing genomic DNA/ plasmid DNA were mixed with 2 µl 6X DNA loading buffer (Appendix D.5), briefly spun using eppendorf centrifuge and loaded into the wells of agarose gel flooded with 1X TAE buffer. Large agarose gel electrophoresis units (BG-200) were also used (Bangalore, Genei). (Approximately 10x 15 cm, with capacity of 100 ml of agarose gel solution) for 4 hrs (80 V, IX TAE electrophoresis buffer). The electrophoresis was done usually until the dye front has travelled 2/3 rd of the agarose gel. The agarose gel was visualized using a gel documentation system (Alpha-Innotech, USA) and image was saved in the computer. If required, appropriate DNA markers (viz. 1 kbps and 100 bps) were also loaded on agarose gels in parallel wells along with the DNA samples to determine the size of the DNA fragments (Sambrook et al. 1989).

4.4. Screening and detection of TBTCl resistance genes

4.4.1 Detection of *Tbt B* gene encoding a putative transporter protein in *Pseudomonas mendocina* strain DP4

PCR amplification of *Tbt B* gene (amplicon) was carried out using following primers:

(Forward Primer) *TbtB3* 5'-CGCCGGCGCGTTATCGCTGG-3'

(Reverse Primer) *TbtB4* 5'-GGTGGCGCACAGCGCCGGGG-3'

(Jude et al. 2004)

Genomic DNA of TBTCl resistant *Pseudomonas stutzeri* strain DP1 was used as template (Jude et al. 2004). Geenomic DNA of TBTCl sensitive *E. coli* HB101 was used as a negative control. PCR reaction was performed using 20 pmoles each primer, 25 ng genomic DNA as template, 0.2 mM each dNTPs and 1.0 U *Taq* DNA polymerase. The total reaction volume was 50 µl. The conditions included an initial denaturation step of 94°C for 2 min followed by 30 cycles of denaturation at 94°C for 1 min, annealing at 65°C for 1 min and extention at 72°C for 1.5 min. Final extention reaction was performed at 72 °C for 10 min. PCR product was analysed on 1% agarose gel by agarose gel electrophoresis and result was recorded using gel documentation system (Alpha Innotech, USA). Size of the amplicon was determined by comparison with 1 kbps DNA ladder (Bangalore, Genie).

.

4.4.2 Detection of bacterial metallothionein gene (*BmtA*) in TBTCl resistant bacteria

In order to investigate involvement of bacterial metallothionein (*BmtA*) gene in TBTCl resistance in *Pseudomonas mendocina* strain DP4, PCR amplification of

bacterial metallothionein encoding gene, (*BmtA*) was carried out using genomic DNA as a template with following primers :

Forward Primer: P3 5'-GGTGGATCCCCATGAACAGCGAAACCT-3'

Reverse Primer: P4 5'-GGTGAATTCTCAGGGCGAGATCGGGTCGC-3'

(Blindauer et al. 2002)

PCR was performed using PCR kit (Bangalore Genei, India) using 30 pmole of each primer, 25 ng purified genomic DNA (template), dNTPs (0.2 mM each), 1 unit *Taq* DNA. PCR conditions include 10 min hot start step at 94°C followed by 30 cycles of PCR reactions with 94°C and 1min denaturation step, 62°C, 1min annealing step, 72°C and 1 min extension step. Final extension was also done at 72 ºC for 10 min. PCR product was analysed on 1% agarose gel by agarose gel electrophoresis and result was recorded using gel documentation system (Alpha Innotech, USA). Size of the amplicon (bps) was determined using standard 100 bps DNA marker (Bangalore Genie, India).

4.5 Proteomic studies of TBTCl resistant bacterial strains

Proteomic studies were also conducted in order to confirm involvement of specific bacterial proteins such as membrane proteins and metallothionein like proteins in TBTCl resistance in these bacterial isolates.

4.5.1. SDS-PAGE analysis of whole cell proteins of TBTCl resistant bacterial strains

Whole cell lysates were prepared following method of Hitchock and Brown (1983). Bacterial cells were grown in MSM with 0.5 mM and 1 mM TBTCl and without TBTCl but 0.1% glucose (control) separately for 24 hrs at 28 °C. Bacterial

cells were harvested at 8000 rpm for 5 min at room temperature, washed at least three times with 10 ml PBS and resuspended in 10 ml PBS (Appendix B.2). Bacterial cells resuspended in PBS (1.5 ml) were harvested in a microfuge tube, the cell pellet was resuspended in 100 µl of double strength (2X) sample solubilizing buffer (Appendix D.1) and incubated in boiling water bath (100 ℃) for 10 min.This sample was harvested at 5000 rpm for 10 min at room temperature and 10 µl of supernatant was loaded on SDS-PAGE gel. Standard protein markers (Bangalore Genie, India) were also loaded in a parallel lane in order to determine the molecular mass of specific proteins. Gel electrophoresis was performed using 1X Tris- Glycine electrophoresis buffer (Appendix D.1) at room temperature (28 °C) following standard procedure of Laemmli (1970). Protein samples were initially electrophoresed at 35 mA until tracking dye entered the resolving gel, subsequently current was increased to 70 mA and run was done till the tracking dye reaches bottom of the resolving gel. The gel was silver stained following standard procedures (Sambrook et al. 1989).

4.5.2 SDS-PAGE analysis of periplasmic proteins of TBTCl resistant bacterial strains

Periplasmic proteins of bacterial isolates were extracted by osmotic shock treatment following modified method of Nossal and Heppel (1966). Bacterial cells were grown in 25 ml MSM with 0.5 mM amd 1 mM TBTCl and with 0.1% glucose (control) separately at 28 ℃ for 24 hrs at 120 rpm. Cells were harvested by centrifugation at 8000 rpm for 30 min at 4 ℃ and washed twice with 1x PBS (pH 7.2). The washed cell pellet was resuspended in 0.5 ml fractionation buffer (Appendix) and incubated in ice for 10 min. Cells were harvested at 8000 rpm for 10 min at 4 ℃ and the pellet was re-suspended in 0.5 ml of ice-cold $MgSO_4$ with shaking

at room temperature for 10 min. Supernatant containing periplasmic proteins was collected by centrifugation at 5000 rpm for 10 min at 4 °C. The periplasmic protein fractions (100 µl) from each bacterial isolates were mixed with equal volumes (i.e. 100 µl) of double strength sample solubilizing buffer, incubated in boiling water bath (100 °C) for 5 min and analyzed using SDS-PAGE followed by silver staining (Appendix). Standard molecular weight protein markers were also used to determine the molecular mass of specific periplasmic proteins. Gel electrophoresis was performed using 1X Tris- Glycine electrophoresis buffer (Appendix D.1) at room temperature (28 °C) following standard procedure of Laemmli (1970). Protein samples were initially electrophoresed at 35 mA until tracking dye entered the resolving gel, subsequently current was increased to 70 mA and run was done till the tracking dye reaches bottom of the resolving gel. The gel was silver stained following standard procedures (Sambrook et al. 1989).

4.5.3 SDS-PAGE analysis of extracellular proteins of *Klebsiella pneumoniae* strain SD9

Extra-cellular proteins were also extracted by growing bacterial cells in MSM with 2mM TBTCl and grown overnight at 28 °C, 120 rpm. Cells grown in MSM containing 0.1% glucose without TBTCl served as control. Cells were separated from the supernatant by centrifugation at 8000 rpm for 10 min at 4 °C. The Cell free supernatant was analysed using SDS-PAGE followed by silver staining (Appendix D.2). Standard molecular weight protein markers were used to determine the molecular mass of specific extracellular proteins. Gel electrophoresis was performed using X Tris- Glycine electrophoresis buffer (Appendix) at room temperature (28 °C) following standard procedure of Laemmli (1970). Protein samples were initially

electrophoresed at 35 mA until tracking dye entered the resolving gel, subsequently current was increased to 70 mA and run was done till the tracking dye reaches bottom of the resolving gel. The gel was silver stained following standard procedures (Sambrook et al. 1989).

RESULTS AND DISCUSSION

4.6 Plasmid profile of TBTCl resistant bacterial strains

Agarose gel analysis of samples of TBTCl resistant bacterial strains revealed the absence of plasmid in all the isolated bacterial strains. This clearly indicates that resistance to TBTCl in these bacterial strains is not governed by plasmids and the genes encoding TBTCl resistance are located on chromosomal genome. The genetic determinants conferring metal resistance in bacteria may either be present on chromosomal genome, plasmids or transposons (Silver 1992). Bacterial plasmids possess genetic determinants encoding resistance to several toxic metal ions viz. Cd^{+2}, Co^{+2}, CrO_4^{-2}, Cu^{+2}, Hg^{+2}, Ni^{+2}, Pb^{+2}, Sb^{+3}, TeO_3^{-2}, Ti^{+} and Zn^{+2} (Silver 1992). Among organometals, only organomercurial resistance is known to be plasmid mediated in bacteria (Oehlmann et al. 1991). Although there are reports of presence of plasmids in TBTCl resistant bacterial strains (Miller et al. 1995), but till date no report of plasmid mediated TBTCl resistance has been confirmed. Due to lack of supporting evidence with respect to plasmids involved in TBTCl resistance and availability of data demonstrating specific chromosomal genes involved in it's resistance, it has been suggested that resistance to TBTCl may not be plasmid mediated but chromosomal genome mediated (Fukagawa and Suzuki 1993; Miller et al. 1995; Jude et al. 2004; Fukushima et al. 2009; Cruz et al. 2010; Fukushima et al. 2012).

4.7 Agarose gel analysis of genomic DNA extracted from TBTCl resistant bacterial strains

Agarose gel analysis of genomic DNA samples from TBTCl resistant bacterial strains viz. *Pseudomonas salmonicida* strain DP2, *Pseudomonas stutzeri* strain DP1, *Vibrio* sp. strain DP3, *Pseudomonas mendocina* strain DP4, *Chromohalobacter salexigens* strain DP5, *Klebsiella pneumoniae* strain SD9 and *Alcaligenes faecalis* strain SD5 clearly revealed clean and very prominent bands of genomic DNA (Fig. 4.1).

4.8 Screening and detection of TBTCl resistance genes

TBTCl resistance in bacteria is not well studied and there are very few reports demonstrating involvelment of specific genes that encode TBTCl resistance in bacteria. These genes are mainly known to encode several transport proteins suggesting efflux of TBTCl outside the bacterial cells as a resistance mechanism (Fukagawa and Suzuki 1993; Jude et al. 2004; Cruz et al. 2010; Fukushima et al. 2012). In TBTCl resistant *Aeromonas molluscorum* Av27, *SugE* gene has been reported which encodes *sugE* protein belonging to small MDR family, a lipophillic drug transporter (Cruz et al. 2010). Similarly a chromosomal gene responsible for TBTCl resistance in *Alteromonas* sp. M-1 has been reported to encode proteins which are homologous to known transport proteins (Fukagawa and Suzuki 1993). The *TbtRABM* operon of *Pseudomonas stutzeri* strain 5MP1 associated with TBT resistance is also known to regulate efflux of toxic TBT from the bacterial cells (Jude et al. 2004). Interestingly, a novel TBTCl resistance gene, *PA0320* from *Psuedomonas aeruginosa* 25W which is a *YgiW* homologue of *E. coli* has been reported (Fukushima et al. 2012). This

gene is also known to confer resistance to TBTCl in *Psuedomonas aeruginosa* 25W.

4.8.1 Detection of *TbtB* gene encoding a putative transporter protein in *Pseudomonas stutzeri* strain DP1

It is interesting to mention that *Pseudomonas stutzeri* possessed *TbtRABM* operon belonging to RND family which conferred multi-drug resistance along with TBT resistance through efflux pump (Jude et al. 2004). PCR amplification of *TbtB* gene of this operon using genomic DNA of TBTCl resistant *Pseudomonas stutzeri* strain DP1 as a template and gene specific primers *TbtB3* and *TbtB4* clearly demonstrated presence of 1.4 kbps *Tbt B* gene in *Pseudomonas stutzeri* strain DP1(Fig. 4.2). *TbtRABM* encodes various proteins involved in efflux pump belonging to the RND family of genes. Amoung these *TbtB* encodes a transport protein, *TbtA* encodes membrane fusion protein and *TbtM* encodes outer membrane proteins which function in synergy to extrude TBTCl outside the cell membrane of the Gram-negative bacteria (Jude et al. 2004). Jude et al. 2004 have confirmed that the *TbtB* gene encodes a putative transporter (transmembrane) protein which possessed four characteristic motifs (A, B, C and D) homologous to RND transporters. Since we have also got similar results in *Pseudomonas stutzeri* strain DP1, we may infer that TBTCl resistance is conferred by *TbtRABM* operon which regulates active efflux of this toxic biocide to the cell exterior. Thus we may suggest a *TbRtABM* operon mediated removal of TBTCl outside the cells in *Pseudomonas stutzeri* strain DP1 as a resistance mechanism.

4.8.2 Detection of bacterial metallothionein gene (*BmtA*) in *Pseudomonas mendocina* strain DP4

Metallothioneins (MTs) are small, cysteine and histidine rich proteins which bind and sequester multiple metal ions and are ubiquitous in eukaryotes (Hamer 1986; Vasak and Hasler 2000). Higher eukaryotes have multiple metallothionein genes; a minimum of 4 and 16 in mice and humans respectively (West et al. 1990). SmtA from *Synechococcus* PCC7942 is the only fully characterized prokaryotic metallothionein which is currently known as BmtA (Olafson et al. 1988; Robinson et al. 1990; Huckle et al. 1993). Prokaryotic MTs have been identified in various bacteria and cyanobacteria whichincludes *Stigmatella aurantiaca, Lactobacillus johnsonii, Corynebacterium glutamicum, Mycobacterium smegmatis,* marine *Synechococcus CC9311, Microcystis aeruginosa* and *Pseudomonas fluorescens* (Turner et al. 1996; Pazirandeh et al. 1995; Blindauer et al. 2002).

Intracellular metal bioaccumulation and homeostasis in cell cytosol involves low molecular weight, cystein-rich metallothioneins which range from 3.5 to 14 kDa (Olafson et al. 1979; Hamer 1986). These unique proteins also demonstrate induction in response to specific heavy metals such as Cd, Pb, Zn, and Cu (Gadd 1990; Pazirandeh et al. 1995; Turner et al. 1996; Blindauer et al. 2002; Liu et al. 2003; Naik et al. 2012 a, b). Metallothioneins (MTs) play an important role in immobilization of toxic heavy metals thereby protecting bacterial metabolic processes catalysed by enzymes (Blindauer et al. 2002; Liu et al. 2003).Several cyanobacterial and bacterial strains have been reported to encode metallothioneins for maintaining cytosolic metal homeostasis viz. *Synechococcus* PCC 7942 (*SmtA*), *Anabaena* PCC 7120 (*SmtA*), *Oscillatoria brevis (BmtA), Pseudomonas aeruginosa* (*BmtA*) and *Pseudomonas putida* (*BmtA*) (Robinson et al. 1990; Turner et al. 1996; Blindauer et al. 2002; Liu et

al. 2003; Naik et al. 2012a). *Pseudomonas* sp. S8A when exposed to >10 mg/l cadmium showed induction of unidentified 28 kDa protein responsible for cadmium resistance (Kassab and Roane 2006). It is interesting to note that genetically modified *Escherichia coli* expressing the metal binding peptide was also demonstrated to possess enhanced binding of Cd^{2+} and Hg^{2+} compared to bacterial cells lacking this metal binding peptide (Pazirandeh et al. 1998).

In our experiment, TBTCl resistant *Pseudomonas mendocina* strain DP4 showed PCR amplification of 250 bps amplicon indicating presence of bacterial metallothionein gene, *BmtA* (Fig. 4.3). The involvement of a *BmtA* gene encoded metallothionein protein may thus be suggested in conferring TBTCl resistance in this bacterial strain. Metallothioneins are reported to maintain intracellular homeostasis of essential metal ions viz. Zn^{+2}, Co^{+2} and Cu^{+2} and also sequester toxic metal ions viz. Cd^{+2}, Hg^{+2}, Pb^{+2} etc. (Blindauer et al. 2002; Liu et al. 2003). Although till date no reports on metallothionein governed organotin resistance have been documented, it is imperative to understand the role of bacterial metallothioneins in TBTCl resistance.

4.9 Proteomic studies of TBTCl resistant bacterial strains

4.9.1 SDS-PAGE analysis of whole cell proteins of TBTCl resistant bacterial strains

Microorganisms including bacteria express stress induced proteins in response to stress stimuli viz. heavy metals, oxidative agents, salinity, temperature and organic toxicants (Highgam et al. 1984; Harwood-Sears and Gordon, 1990; Noel-Georis et al. 2004; Sharma et al. 2006; Cheng et al. 2009; Ramachandran and Dubey, 2009; Yildirim et al. 2011; Bernat et al. 2014).One of the common mechanisms of metal resistance in bacteria is induction of specific metal binding proteins facilitating the

sequestration of toxic metals inside the cell. Two copper-inducible supernatant proteins viz. CuBPl and CuBP2 with molecular mass 19 kDa and 21 kDa were identified in marine bacterium, *Vibrio alginolyticus* which were 25-46 times amplified in the supernatant of copper-challenged culture as compared with control.Thus these proteins facilitated copper accumulation and homeostasis (Harwood-Sears and Gordon, 1990). Similarly *Pseudomonas fluorescens* exposed to lead although showed 18 differentially expressed proteins, but only one protein could match significantly to spoVG protein which showed Pb-induced up-regulation (Sharma et al. 2006). These unique proteins also demonstrated induction in response to specific heavy metals such as Cd, Pb, Zn, and Cu (Gadd 1990; Harwood-Sears and Gordon 1990; Turner et al. 1996; Blindauer et al. 2002; Liu et al. 2003; Sharma et al. 2006; Yildirim et al. 2011). Similar response was observed in TBTCl resistant *Vibrio* sp., where up-regulation of two polypeptides of approximately 12 kDa and 30 kDa was observed when cells were grown in the presence of 125 μM TBTCl (Fukagawa et al. 1992).

In our investigation, TBTCl resistant bacterial strains grown in MSM with TBTCl clearly revealed up-regulation of several whole cell proteins. *Aeromonas salmonicida* strain DP2 showed upregulation of two whole cell proteins of molecular mass of 12 kDa and 100 kDa respectively (Fig. 4.4 a). Similarly in *Pseudomonas mendocina* strain DP4 a prominent upregulation of two low molecular mass proteins viz. 14 kDa and 10 kDa (Fig. 4.4 e). While in *Vibrio* sp. DP3 three whole cell proteins of approximate molecular mass viz. 66 kDa, 44 kDa and 42 kDa were upregulated (Fig. 4.4 c). But in *Chromohalobacter salexigens* strain DP5 no such upregulation of any whole cell proteins was observed (Fig. 4.4 b).

In *Pseudomonas stutzeri* strain DP1 two whole cell proteins of approximate molecular mass 112 kDa and 98 kDa respectively were prominently upregulated

while a protein of approximate molecular mass 50 kDa showed slight upregulation in presence of TBTCl (Fig. 4.4 d). Interestingly, studies already carried out on TBTCl resistant *Pseudomonas stutzeri* strain 5MP1 have suggested involvement of a *TbtRABM* operon in conferring TBTCl resistance. It is important to mention that the *TbtB* gene of this operon is known to encode a protein of 113.1 kDa (Jude et al. 2004). In our earlier experiment, we have already confirmed presence of 1.4 kbps amplicon of *TbtB* gene in the estuarine bacterial isolate *Pseudomonas stutzeri* strain DP1. Therefore analysis of the whole cell protein profile of this strain, demonstrating possible presence and up-regulation of a 112 kDa whole cell (transporter) protein further confirms presence of *TbtB* gene and its involvement in TBTCl resistance in this bacterial strain, through efflux mechanism.

4.9.2 SDS-PAGE analysis of periplasmic proteins of TBTCl resistant bacterial strains

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 (Miller et al. 1983; Ramachandran and Dubey 2009). 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 synthesis of two polypeptides of approximately 12 k Da and 30 k Da when cells were grown in presence of 125 µM TBTCl (Fukagawa et al. 1992). Transcriptome analysis of tributyltin resistant bacterium, *Pseudomonas aeruginosa* 25W, exposed to 500 µM TBTCl also revealed up-regulation and down- regulation of six genes respectively. Interestingly, the down-regulated genes were involved in transcription and translation

(Dubey et al. 2006). Similarly expression of TBTCl induced three periplasmic proteins of molecular mass 43, 63 and 68 kDa was also reported in a TBTCl-resistant marine sediment isolate *Alcaligenes* sp. suggesting their possible involvement in resistance as well as degradation of TBTCl (Ramachandran and Dubey 2009). The site of action of organotins may be both at cytoplasmic membrane level as well as intracellular level. Studies on the effect of TBTCl on certain microbial enzymes indicates that in some bacteria TBTCl can interact with cytosolic enzymes (White et al. 1999). TBTCl also acts on mitochondria and chloroplast causing exchange of ions through the membranes, inhibiting phosphorylation and ATPase activity.

Our studies have confirmed up-regulation of various periplasmic proteins in TBTCl resistant bacterial isolates on gradual increase in TBTCl concentrations in the growth medium (Fig. 4.5 a-e). The periplasmic protein profile of TBTCl resistant *Pseudomonas mendocina* strain DP4 revealed upregulation of a protein having molecular mass of approximately 14 kDa in presence of TBTCl (Fig. 4.5 e). This 14 kDa protein may be a metallothionein which may be playing a role in transport of TBTCl in and across the cell. Thus, it may be imperative in understanding the resistance mechanism adopted by this bacterial strain against TBTCl. As mentioned earlier *Pseudomonas mendocina* strain DP4 showed presence of 250 bps amplicon of corresponding to *BmtA* gene (Fig. 4.3) which is known to encode low molecular weight prokaryotic metallothioneins (4 kDa -14 kDa). The presence and up-regulation of 14 kDa protein in *Pseudomonas mendocina* strain DP4 further confirms involvement of metallothioneins in conferring TBTCl resistance in this bacterial strain. This is the first report where a TBT resistant bacterial isolate showed presence and up-regulation of low molecular weight protein encoded by *BmtA* gene. Our investigation has clearly demonstrated that *Pseudomonas mendocina* strain DP4

exhibits bacterial metallothionein mediated resistance by accumulating significantly high levels of TBTCl (i.e. 8 µg/g dry weight) intracellularly which was clearly demonstrated by AAS, PCR amplification and SDS-PAGE analysis. This unique characteristic of this TBTCl accumulating strain may be exploited for bioremediation of environmental sites contaminated with considerably high levels of toxic TBTCl.

In *Aeromonas salmonicida* strain DP2 upregulation of four periplasmic proteins with approximate molecular mass viz. 12 kDa, 18 kDa, 25 kDa and 32 kDa respectively was observed in presence of TBTCl (Fig. 4.5 a). Interestingly, the 12 kDa protein was also found to have been upregulated in presence of TBTCl as observed in the whole cell protein profile of this bacterial strain (Fig. 4.4 a). This data substantiates that TBTCl regulates the up-regulation of 12 kDa protein found in *Aeromonas salmonicida* strain DP2.

Pseudomonas stutzeri strain DP1showed slight up-regulation of approximately 50 kDa protein (Fig. 4.5 d) which was also observed to be up-regulated in the whole cell protein profile in presence of TBTCl (Fig. 4.4 d). This may thus be some transport protein helping the transport of TBTCl across the cell membrane. In our earlier experiment, we have also confirmed presence of 1.4 kbps amplicon of *TbtB* gene in TBTCl resistant *Pseudomonas stutzeri* strain DP1 (Fig. 4.2). This gene is known to encode a 52 kDa membrane transporter protein. Interestingly, these findings viz. presence of *TbtB* gene and up-regulation of 50 kDa protein in *Pseudomonas stutzeri* strain DP1 go hand in hand with earlier reports of *TbtRABM* operon and a 52 kDa outer membrane protein in TBTCl resistant *Pseudomonas stutzeri* strain 5MP1 (Jude et al. 2004). Thus it may be assumed that these two TBTCl resistant strains possess *TbtRABM* operon regulating TBTCl resistance through efflux.

4.9.3 SDS-PAGE analysis of extracellular proteins of *Klebsiella pneumoniae* strain SD9

Extracellular protein fraction basically comprises of proteins secreted by the bacterial strains into the growth medium involving their secretory systems. Several commercially important microbial enzymes viz. amylases, protease, xylanases and ligninases etc. are often secreted outside the cells which aid in breakdown of complex compounds (D'costa et al. 2013). Organotins including tributyltin chlorides are complex and persistent organic pollutants which are broken down slowly into their lesser toxic derivatives viz. DBT and MBT through stepwise removal of it's butyl groups. This may be mediated by certain enzymes which directly or indirectly would aid in the degradation of this toxic biocide. It may also be taken into consideration that several biomolecules such as siderophores are also liberated into the growth medium which help in quenching and sequestration of TBTCl thereby decreasing its bioavailability and toxicity to the growing bacterial cells (Sampath et al. 2012). Several such factors may therefore be responsible for increase in extracellular protein concentration and up-regulation or induction of TBTCl specific proteins.

Our studies have clearly indicated up-regulation of 54 kDa and 66 kDa extracellular proteins and significant induction of a low molecular mass 15 kDa protein in the extracellular protein fraction of *Klebsiella pneumoniae* strain SD9 when cells were exposed to 2 mM TBTCl (Fig.4.6). It may be noted that this strain is a TBTCl degrader bacterial strain also shows enhanced production of siderophores in presence of TBTCl (Fig. 3.13 b). Siderophores are often known to possess low molecular weight protein moieties. The induction of low molecular weight proteins by TBTCl thus may be correlated indirectly to production of siderophores in this bacterial strain. Although up-regulation of whole cell proteins, outer membrane

proteins and periplasmic proteins in presence of TBTCl has been reported earlier (Fukagawa et al. 1992; Dubey et al. 2006; Ramachandran and Dubey 2009), but this is the very first report showing induction and up-regulation of extracellular proteins in resistant bacterial strains due to TBTCl.

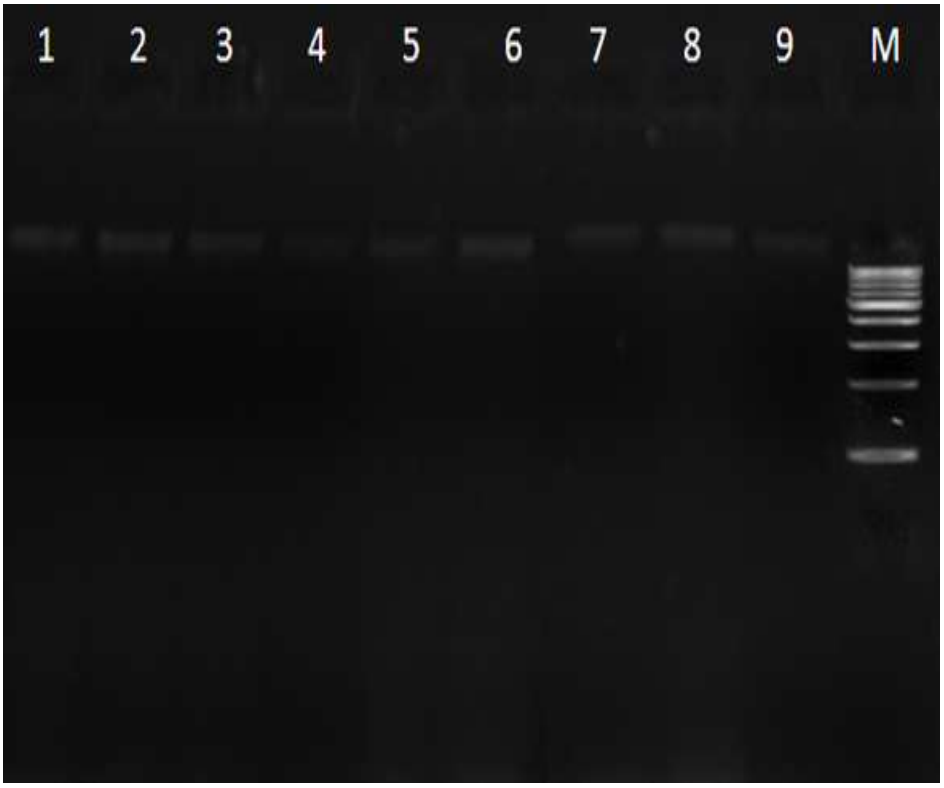


Fig. 4.1 **Genomic DNA profiles of TBTCl resistant bacterial strains**

Lane 1: *Pseudomonas stutzeri* strain DN2

Lane 2: *Klebsiella pneumoniae* strain SD9

Lane 3: *Alcaligenes faecalis* strain SD5

Lane 4: *Pseudomonas stutzeri* strain DP1

Lane 5: *Vibrio* sp. DP3

Lane 6: *Pseudomonas mendocina* strain DP4

Lane 7: *Chromohalobacter salexigens* strain DP5

Lane 8: *Aeromonas salmonicida* strain DP2

Lane 9: *E. coli* HB101

Lane M: DNA ladder 1 kbps

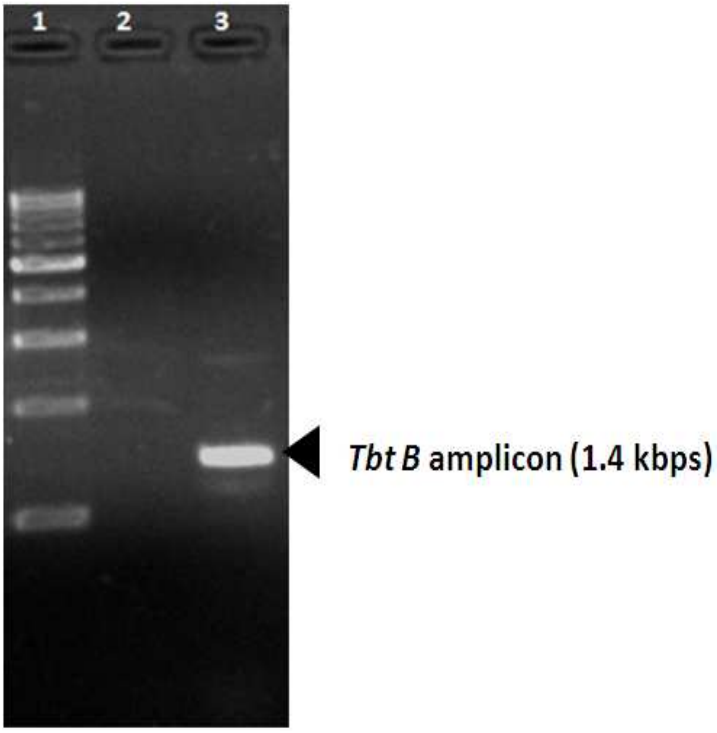
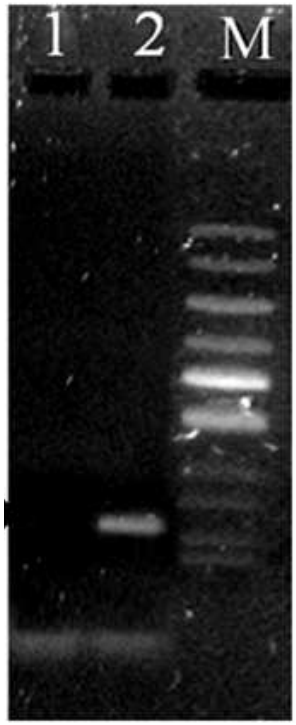


Fig. 4.2 **PCR amplification of *TbtB* amplicon using the *TbtB* specific primer pair**

Lane 1: DNA ladder (1 kbp)

Lane 2: Negative control using genomic DNA of TBTCl-sensitive *E. coli* HB101 as template

Lane 3: *TbtB* amplicon (1.4 kpbs) using genomic DNA of TBTCl resistant *Pseudomonas stutzeri* strain DP1 as template



***BmtA* amplicon**

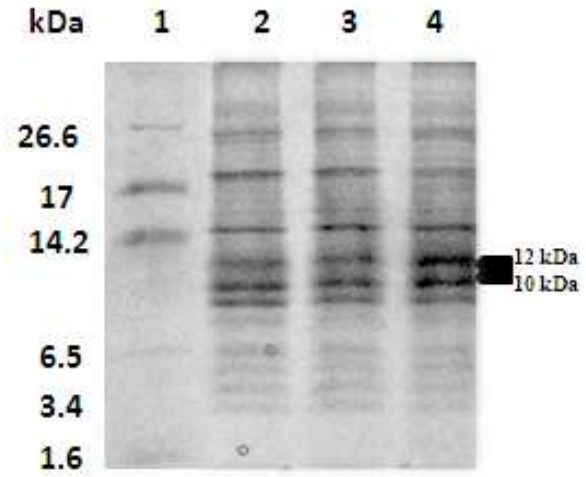
(250 bps)

Fig. 4.3 PCR amplification using *BmtA* specific primer pair

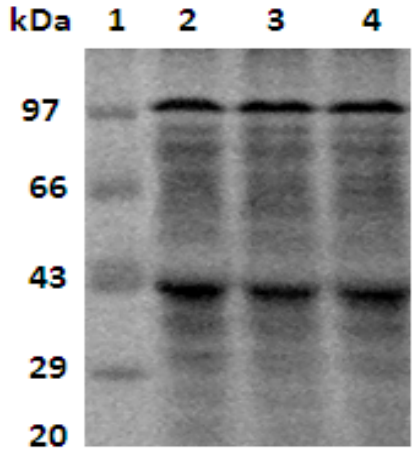
Lane 1: Negative control using genomic DNA of TBTCl-sensitive *E. coli* HB101 as template

Lane 2: *BmtA* amplicon (250 bps) using genomic DNA of TBTCl resistant *Pseudomonas mendocina* strain DP4 as templates

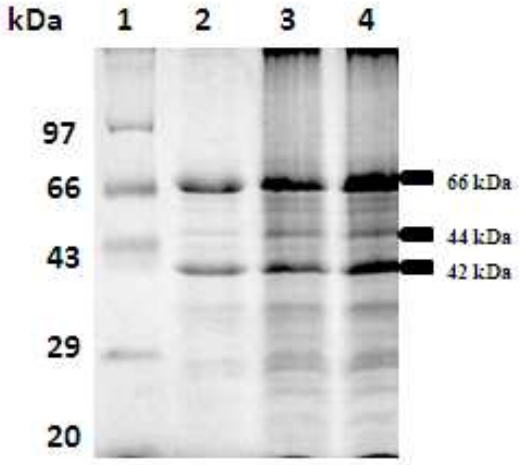
Lane: M DNA ladder (100 bps)



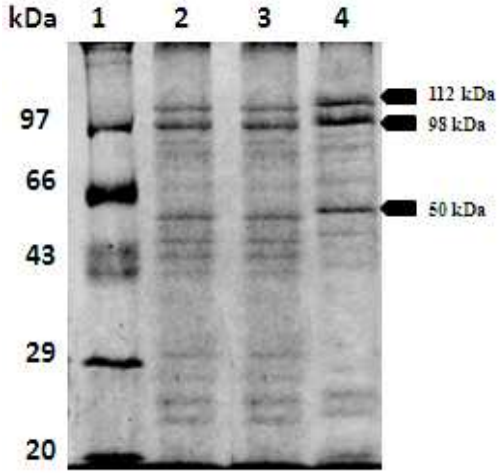
(a) ***Aeromonas salmonicida*** **strain DP2**



(b) ***Chromohalobacter salexigens*** **strain DP5**



(c) ***Vibrio*** **sp. DP3**



(d) ***Pseudomonas stutzeri*** **strain DP1**

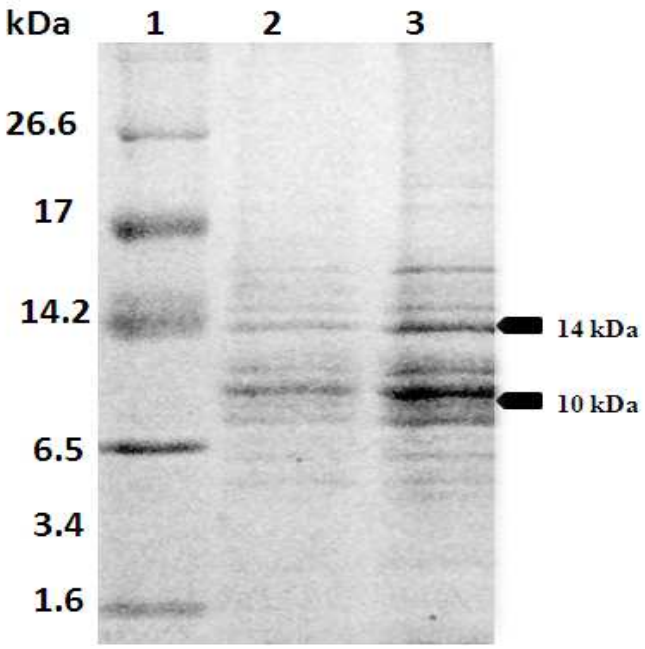
Fig. 4.4 (a-e) SDS-PAGE analysis of whole cell proteins of TBTCl resistant bacterial strains

Lane 1: SDS-PAGE marker

Lane 2: MSM + 0.1% glucoe (Control)

Lane 3: MSM + 0.5 mM TBTCl

Lane 4: MSM + 1 mM TBTCl

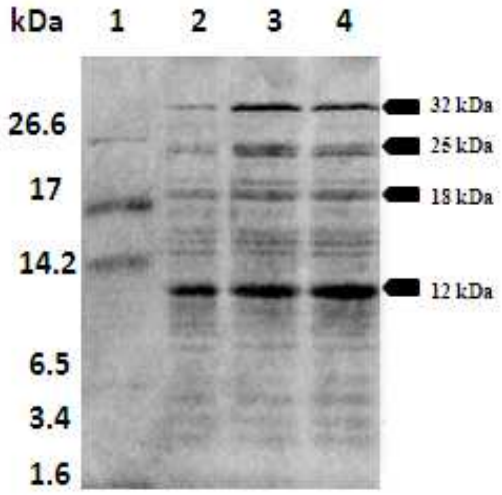


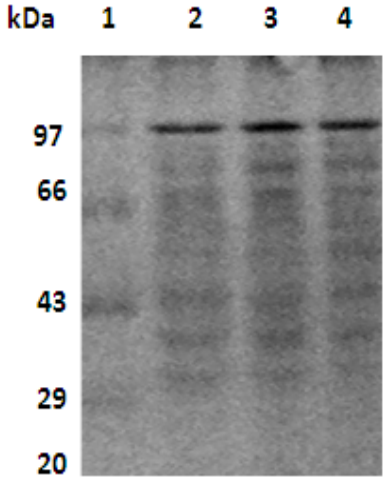
(e) *Pseudomonas mendocina* strain DP4

Lane 1: SDS-PAGE marker

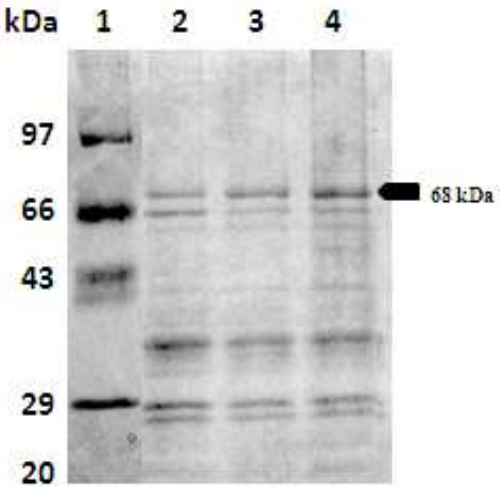
Lane 2: MSM + 0.1% glucoe (Control)

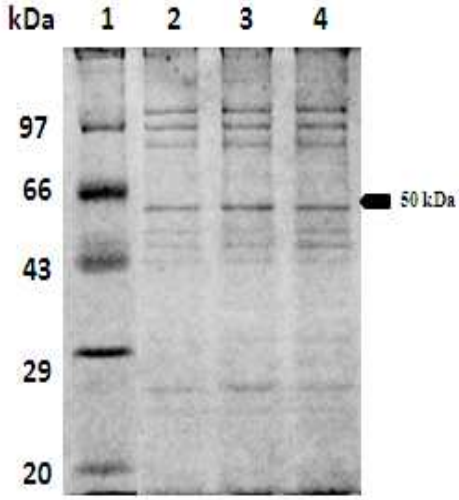
Lane 3: MSM + 1 mM TBTCl





(a) ***Aeromonas salmonicida*** **strain DP2** **(b)** ***Chromohalobacter salexigens*** **strain DP5**





. **(c)** ***Vibrio*** **sp. DP3** **(d)** ***Pseudomonas stutzeri*** **strain DP1**

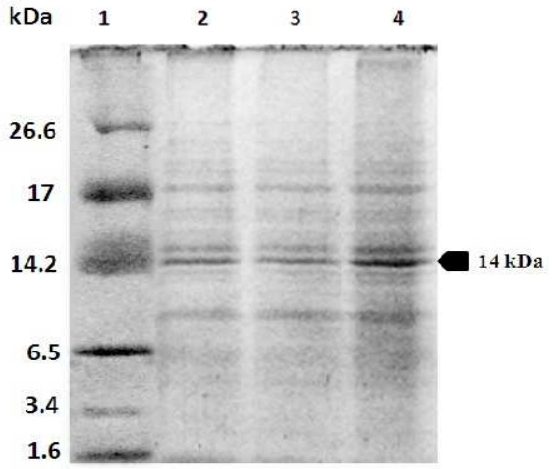
Fig. 4.5 (a-e) SDS-PAGE analysis of periplasmic proteins of TBTCl resistant bacterial strains

Lane 1: SDS-PAGE marker

Lane 2: MSM + 0.1% glucoe (Control)

Lane 3: MSM + 0.5 mM TBTCl

Lane 4: MSM + 1 mM TBTCl



(e) ***Pseudomonas mendocina*** **strain DP4**

Lane 1: SDS-PAGE marker

Lane 2: MSM + 0.1% glucoe (Control)

Lane 3: MSM + 0.5 mM TBTCl

Lane 4: MSM + 1 mM TBTCl

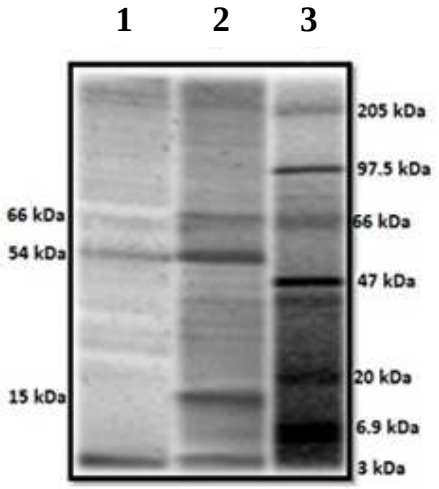


Fig. 4.6 SDS-PAGE analysis of extracellular proteins of TBTCl resistant ***Klebsiella pneumoniae*** **strain SD9**

Lane 1: MSM + 0.1% glucose (control)

Lane 2: MSM + 2 mM TBTCl

Lane 3: SDS-PAGE marker

CONCLUSION

Ship building sites release appreciably high levels of TBTCl, which poses a serious threat to both, target and non-target aquatic biota of Goa including bacteria. Microorganisms possess variety of resistance mechanisms to withstand TBTCl.

The present study deals with isolation and biological characterization of bacteria from TBTCl contaminated Zuari estuary of Goa, India. Biochemical and molecular biological studies carried out on these bacterial isolates, revealed several resistance mechanisms and their role in resistance to TBTCl. These mechanisms include:

(i) Biodegradation of TBTCl and its utilization as a sole source of carbon.

(ii) Biotransformation of TBTCl into its lesser toxic derivatives viz. $DBTCl_2$ and $MBTCl_3$.

(iii) Morphological alterations in the cells to reduce the area of exposure to TBTCl.

(iv) Production of siderophores and EPS as a protective mechanism of sequestration of TBTCl.

(v) Induction and up-regulation of specific whole cell, periplasmic and extra-cellular transport proteins in presence of TBTCl playing a significant role to resist TBTCl stress.

(vi) Presence of *BmtA* gene encoding one bacterial metallothionein protein involved in possible intracellular sequestration of TBTCl.

(vii) Presence of transport protein (50 kDa) encoded by *TbtB* gene which is known for efflux of TBTCl to the cell exterior.

These interesting findings on TBTCl resistant bacteria will definitely facilitate in understanding the various cellular responses against TBTCl stress as well as in designing strategies for bioremediation of TBTCl contaminated environmental sites.

SUMMARY

Tributyltin is a potent biocide used in marine antifouling paints to paint submerged surfaces of ships, boats and marina platforms to prevent attachment and growth of barnacles, algae, mussels, tube worms and other marine organisms. Although an effective anti foulant, it poses threat to a variety of non-target marine organisms causing several lethal and carcinogenic effects and as well gets biomagnified in the food chain. Due to this, the IMO banned the usage of this biocide in marine anti fouling paints. Despite this, it is still found to be persistant at toxic levels in marine sediments and water columns. Interestingly, some natural microbial strains employing a variety of protective mechanisms can survive even toxic levels of this anti fouling agent. Various strategies through which they resist high levels of tributyltin include utilization as sole source of carbon, degradation or transformation into less toxic derivatives, efflux, extracellular sequestration and intracellular bioaccumulation It is interesting to note that they possess certain proteins and genetic determinants (genes) conferring organotin resistance on chromosomal genome. This unique characteristic of tributyltin resistant microbes including bacteria makes them an ideal tool for bioremediation of environmental sites contaminated with this biocide.

- In the present study we have screened several TBTCl resistant bacterial strains from ship building sites of Zuari estuary of Goa which is known to be heavily contaminated with TBTCl due to extensive ship building activity.
- Thirty six potential TBTCl resistant bacteria were selected from these estuarine ship building sites for further characterization.
- Based on growth in liquid media (ZMB and MSM) amended with 1 mM TBTCl, eight bacterial isolates showing good growth were selected for further characterization.

- Based on morphological, biochemical characteristics, Fatty acid methyl esters profile and molecular characteristics (16S rDNA sequencing) these TBTCl resistant bacterial strains were identified. These include: *Pseudomonas mendocina* strain DP4, *Pseudomonas stutzeri* strain DP1, *Klebsiella pneumoniae* strain SD9, *Alcaligenes faecalis* strain SD5, *Chromohalobacter salexigens* strain DP5, *Vibrio* sp. DP3, *Aeromonas salmonicida* strain DP2 and *Pseudomonas stutzeri* strain DN2.
- All the identified TBTCl resistant bacterial strains are capable of utilizing TBTCl as a sole source of carbon.
- *Klebsiella pneumoniae* strain SD9, *Alcaligenes faecalis* strain SD5 and *Pseudomonas stutzeri* strain DN2 resist TBTCl upto concentration of 2 mM by utilizing it as a sole source of carbon and transforming it into less toxic derivatives viz. $DBTCl_2$ and $MBTCl_3$ as revealed by TLC profiles, IR, NMR and Mass spectrometric analysis of degradation products present in their cell free supernatants.
- *Klebsiella pneumoniae* strain SD9 and *Alcaligenes faecalis* strain SD5 showed enhanced production of siderophores and EPS in presence of TBTCl as demonstrated by CAS agar and Alcian blue staining respectively.
- Interestingly, *Klebsiella pneumoniae* strain SD9 also revealed induction of 15 kDa protein and up-regulation of 66 kDa and 54 kDa proteins which were secreted extracellularly in the growth medium.
- *Pseudomonas stutzeri* strain DN2 and *Klebsiella pneumoniae* strain SD9 showed morphological alterations in presence of 2 mM TBTCl as wrinkling and roughening of cell surface and reduction in cell size to tolerate TBTCl which was clearly revealed by SEM analysis.

- *Klebsiella pneumoniae* strain SD9, *Alcaligenes faecalis* strain SD5 and *Pseudomonas stutzeri* strain DP1 showed resistance to various commonly used antibiotics which suggested a similar mode of resistance adopted to counteract TBTCl stress.
- *Pseudomonas mendocina* strain DP4 resists 1 mM TBTCl by intracellular bioaccumulation of 8 µg/l of TBTCl and produced a bacterial metallothionein (bmtA) which was evidently demonstrated by AAS, SDS-PAGE and PCR amplification.
- *Pseudomonas stutzeri* strain DP1 resists 1 mM TBTCl by efflux of TBTCl as it clearly possessed transport protein of molecular mass, 50 kDa which is probably encoded by *TbtB* gene as confirmed by SDS PAGE and PCR analysis.

These studies carried out on TBTCl resistant bacteria revealed a variety of interesting findings with respect to their TBTCl resistance mechanisms. Therefore these bacterial isolates may prove to be potential biological tools to bioremediate TBTCl contaminated sites.

FUTURE

PROSPECTS

I have planned to extend my future studies on the following aspects:

(i) Identification and characterization of TBTCl induced transport proteins along with bacterial metallothioneins *(*bmtA) using 1D, 2D-gel electrophoretic analysis followed by MALDI-TOF-MS/ LC-MS.

(ii) Identification and characterization of TBTCl resistance genes: molecular cloning, gene expression and regulation.

(iii) Development of TBTCl sensors using transcriptional fusion of TBTCl induced gene promoters with *lux* genes.

(iv) Molecular characterization of TBTCl resistant bacterial strains will be carried out in depth to explore various other mechanisms of TBTCl resistance viz. siderophore production, changes in cellular lipids EPS synthesis.

BIBLIOGRAPHY

- Adelaja OA, Keenan HE (2012) Tolerance of TBT-resistant bacteria isolates to Methylmercury. Res J Environ Sci 6 (1): 1-13
- Adelman D, Hinga KR, Pilson MEQ (1989) Fractionation of butyltin species during sample extraction and preparation for analysis. Env Monit Ass 16: 117-125
- Alagarsamy R (2006) Distribution and seasonal variation of trace metals in surface sediments of the Mandovi estuary, west coast of India. Estuarine Coastal Shelf Sci 67: 333-339
- Alldredge A L, Passow U, Logan BE (1993) The abundance and significance of a class of transparent organic particles in the ocean. Deep-Sea Res Part I 40: 1131-1140
- Altschul SF, Gish W, Miller W, Myers EW, Lipman DJ (1990) Basic local alignment search tool. J Mol Biol 215: 403-410
- Antizar-Ladislao B (2008) Environmental levels, toxicity and human exposure to tributyltin (TBT) - contaminated marine environment. A review. Environ Int 34: 292-308
- Arnold CG, Ciani A, Müller SR, Amirbahman A, Schwarzenbach RP (1998) Association of Triorganotin Compounds with Dissolved Humic Acids. Environmental Science & Technology, 32: 2976–2983
- Arnow LE (1937) Colorimetric determination of the components of 3,4-dihydroxyphenylalanine tyrosine mixtures. J Biol Chem 118: 531-537
- Arp HPH, Eak E, Nybakk AW, Glette T, Moskeland T, Pettersen A (2014) When will the TBT go away? Integrating, monitoring and modeling to address

TBT's delayed disappearance in the Drammensfjord, Norway. Water Research 65: 213-223

- Attri K, Kerkar S (2011) Seasonal assessment of heavy metal pollution in tropical mangro diments (Goa, India). J Ecobiotechnol 3: 9-15
- Ayanda OA, Fatoki OS, Adekola FA, Ximba BJ (2012) Fate and Remediation of Organotin Compounds in Seawaters and Soils. Chem Sci Trans 1(3) 470-481
- Bangkedphol S, Keenan HE, Davidson C, Sakultantimetha A, Songsasen A (2009) The partition behavior of tributyltin and prediction of environmental fate, persistence and toxicity in aquatic environments. Chem 77: 1326–1332
- Baucheron S, Tyler S, Boyd D, Mulvey MR, Chaslus-Dancla E, Cloeckaert A (2004) AcrAB-ToIC directs efflux mediated multidrug resistance in *Salmonella enteric* serovar typhimurium DTI 04. Antimicrob Agents Chemother 48: 3729-3735
- Bauer AW, Kirby WMM, Sherris JC, Turck M (1966) Antibiotic susceptibility testing by a standardized single disc method. Am J Clin Pathol 45:493-496
- Barnes JM, Stoner HB (1959) The toxicology of tin compounds. Pharmacol Rev 11: 211
- Barug D (1981) Microbial degradation of bis(tributyltin) oxide. Chemosphere 10: 1145-1154
- Bennett RF (1996) Industrial manufacture and applications of tributyltin compounds. In: Tributyltin: case study of an environmental contaminant. S.J. de Mora (Ed.). Cambridge University Press UK. pp. 21-61

- Bernat P, Siewiera P, Sobon A, Dlugonski J (2014) Phospholipids and protein adaptation of Pseudomonas sp. to the xenoestrogen tributyltin chloride (TBT). World J Microbiol Biotechnol 30: 2343-2350
- Bhaskar PV, Bhosle NB (2005) Microbial extracellular polymeric substances in marine biogeochemical processes. Curr Sci 88: 45-53
- Bhaskar PV, Bhosle NB (2006) Bacterial extracellular polymeric substances (EPS): a carrier of heavy metals in the marine food-chain. Environ Int 32**:** 191-198
- Bhosle N, Garg A, Jadhav S, Harjee R, Sawant S, Venkat K, Anil A (2004) Butyltins in water, biofilm, animals and sediments of the west coast of India. Chemosphere 57: 897–907
- Bhosle NB (2007) Distribution of tributyltin (TBT) in the Mandovi estuary. NIO, Goa.10:105-113
- Birnboim HC, Doly J (1979) A rapid alkaline extraction procedure for screening recombinant plasmid DNA. Nucleic Acids Res. 7: 1513-1523
- Blair WR, Olson GJ, Brinkman FE, Iverson WP (1982) Accumulation and fate of tri-butyltin cation in estuarine bacteria. Microbiol Ecol 8: 241-251
- Blindauer CA, Harrison, MD, Robinson AK, Parkinson JA, Bowness PW, Sadler PJ, Robinson NJ (2002) Multiple bacteria encode metallothioneins and SmtA-like fingers. Mol Microbiol 45: 1421–1432
- Braissant O, Decho AW, Dupraz C, Glunk C, Przekop KM, Visscher PT (2007) Exopolymeric substances of sulfate-reducing bacteria: Interactions with calcium at alkaline pH and implication for formation of carbonate minerals. Geobiology 5: 401-411

- Bramhachari P, Dubey SK (2006) Isolation and characterization of exopolysaccharide produced by *Vibrio harveyi* VB23. Lett Appl Microbiol. 43: 571-577
- Bramhachari PV, Kavi Kishor PB, Ramadevi R, Kumar R, Rao BR, Dubey SK (2007) Isolation and characterization of mucous exopolysaccharide produced by *Vibrio furnissii* VB0S3. J Microbiol Biotechnol 17: 44-51
- Bryan GW, Gibbs PE, Burt RG (1988) Comparison of the effectiveness of tri-n-butyltin chloride and five other organotin compounds in promoting the development of imposex in dog-whelk *Nucella lapillus*. J Mar Biolog Assoc UK 68: 733-744
- Buffle J, Wilkinson KJ, Stoll S, Fillela M, Zhang J (1998) A generalized description of aquatic colloidal interactions: The three colloidal component approach. Environ Sci Technol 32: 2887-2899
- Centineo G, Rodriguez-Gonzalez P, Gonzalez EB, Alonso JIC, Sanz-Medel A (2004) Simultaneous determination of mono-,di- and tributyltin in environmental samples using isotope dilution gas chromatography mass spectrometry. J Mass Spectrom 39: 485 – 494
- Champ MA, PF Seligman (1996) An introduction to organotin compounds and their use in antifouling coatings. In: Organotin: Environmental Fate and Effects. Chapman and Hall, London. pp. 1-25
- Cheng Z, Wei Y-YC, Sung WW, Glick WR, McConkey BJ (2009) Proteomic analysis of the response of the plant growth-promoting bacterium *Pseudomonas putida* UW4 to nickel stress. Proteome Science. doi:10.1186/1477-5956-7-18

- Clark EA, Sterritt RM, Lester JN (1988) The fate of tributyltin in aquatic environment. Environ Sci Technol 22: 600-604
- Clarke SE, Stuart J, Sanders-Loehr J (1987) Induction of siderophore activity in *Anabaena* sp. and its moderation of copper toxicity. Appl Environ Microbiol 53: 917-922
- Comte S, Guibaud G, Baudu M (2006) Relations between extraction protocols for activated sludge extracellular polymeric substances (EPS) and EPS complexation properties Part I. Comparison of the efficiency of eight EPS extraction methods. Enz Microbial Technol 38: 237-245
- Cooney JJ (1988) Microbial transformations of tin and tin compounds. J Ind Microbiol 3: 195-204
- Cooney JJ, de Rome L, Laurence OS, Gadd GM (1989) Effect of organotin and organolead compounds on yeast. J Ind Microbiol 4: 279-288
- Corbin HB (1976) The solubilities of bis(tributyltin) oxide (TBTO), tributyltin fluoride (TBTF), triphenyltin hydroxide (TPTH), triphenyltin fluoride (TPTF), and tricyclohexyltin hydroxide (TCTH) in water as functions of temperature and pH value. Research and Development Technical Memorandum R-1145-M. M&T Chemicals Inc., Rahway, NJ.
- Cruz A, Caetano T, Suzuki S, Mendo S (2007) *Aeromonas veronii*, a tributyltin (TBT) degrading bacterium isolated from an estuarine environment, Ria de Aveiro in Portugal. Mar Environ Res 64: 639-650
- Cruz A, Henreques I, Correia A, Suzuki S, Mendo S (2010) *Aeromonas molluscorum* Av27: A potential natural tool for TBT decontamination. Interdidciplinary studies on environmental chemistry- Biological response to contaminants 37- 46

- D'Costa B, Khanolkar D, Dubey SK (2013) Partial purification and characterization of metalloprotease of halotolerant alkaliphilic bacterium *Bacillus cereus* from coastal sediment of Goa, India. Afr J of Biotechnol 12 (31): 4905 – 4914
- De J, Ramaiah N, Vardanyan L (2008) Detoxification of toxic heavy metals by marine bacteria highly resistant to mercury. Mar Biotechnol 10: 471-477
- De Souza M-J, Nair S, Loka Bharati PA, Chandramohan D (2006) Metal and antibiotic-resistance in psychrotrophic bacteria from Antarctic marine waters. Ecotoxicol 15: 379-384
- Decho AW (1990) Microbial exopolymer secretion in ocean environment, their roles in food webs and marine processes. Oceanogr Mar Biol Annu Rev 28: 73-153
- Dessai DVG, Nayak GN (2009) Distribution and speciation of selected metals in surface sediments, from the tropical Zuari estuary, central west coast of India. Environ Monit Assess 158:117-137
- Devi P, Wahidullah S, Rodrigues C, D'Souza L (2010) The Sponge-associated Bacterium *Bacillus licheniformis* SAB1: A Source of Antimicrobial Compounds. Mar Drugs 8 (4) : 1203 -1212
- Dowson PH, Bubb JM, Lester JN (1996) Persistence and degradation pathways of tributyltin in freshwater and estuarine sediments. Estuar Coast Shelf Sci 42: 551-562
- Dubey SK, Roy U (2003) Biodegradation of tributyltins (organotins) by marine bacteria. Appl Organomet Chem 17: 3-8

- Dubey SK, Tokashiki T, Suzuki S (2006) Microarray mediated transcriptome analysis of the TBT resistant *Pseudomonas aeroginosa* 25W in the presence of TBT. J Microbiol 44: 200-205
- Dubois M, Gilles KA, Hamilton JK, Roberts PA, Smith F (1956) Colorimetric method for determination of sugars and related substances. Anal Chem 26: 350 – 356
- Eggleton J, Thomas K (2004) A review of factors affecting the release and bioavailability of contaminants during sediment disturbance events. Environ Int 30: 973–980
- Fent K (1996) Ecotoxicology of organotin compounds. Critical Reviews in Toxicology 26: 3–117
- Florea A-M (2005) Toxicity of Alkylated Derivatives of Arsenic, Antimony and Tin: Cellular Uptake, Cytotoxicity, Genotoxic Effects, Perturbation of Ca2+ Homeostasis and Cell Death. Aachen: Shaker Verlag
- Florea A, Busselbergh D (2006) Occurrence, use and potential toxic effects of metals and metal compounds. Biometals 19: 419-427
- Friedman L, Kolter R (2004) Genes involved in matrix formation in *Pseudomonas aeruginosa* PA14 biofilms. Mol Microbiol 51 (3): 675-690
- Fukagawa T, Suzuki S, Fukagawa K, Suzuki T, Takama K (1992) Isolation and characterization of tributyltin chloride resistant marine *Vibrio*. FEMS Microbiol Lett 93: 83-86
- Fukagawa T, Suzuki S (1993) Cloning of gene responsible for tributyltin chloride (TBTCl) resistance in TBTCl-resistant marine bacterium, *Alteromonas* sp. M-1. Biochem Biophys Res Commun 194: 733-740

- Fukagawa T, Konno S, Takama K, Suzuki S (1994) Occurrence of tributyltin and methyl mercury tolerant bacteria in natural sea water to which TBT was added. J Mar Biotechnol 1: 211-214
- Fukushima K, Dubey SK, Suzuki S (2009) Quantitative analysis of expression of TBT regulated genes in TBT resistant *Pseudomonas aeroginosa* 25W. Interdisciplinary studies on environmental chemistry - environmental research in Asia 163-166
- Fukushima K, Dubey SK, Suzuki S (2012) *YgiW* homologous gene from *Pseudomonas aeruginosa* 25W is responsible for tributyltin resistance. J Gen Appl Microbiol 58 (4): 283-289
- Gadd GM (1990) Heavy metal accumulation by bacteria and other microorganisms. Experientia 46: 834-840
- Gadd GM (2000) Microbial interaction with TBT compounds, detoxification and environmental fate. Sci Total Environ 258: 119-127
- Garg A, Meena RM, Bhosle NB (2010) Distribution of butyltins in waters and sediments of the Mandovi and Zuari estuaries,west coast of India. Environ Monit Assess 165: 643-651
- Gehrke T, Telegdi J, Thierry D, Sand W (1998) Importance of extracellular polymeric substances from *Thiobacillus ferroxidans* for bioleaching. Appl Environ Microbiol 64: 2743-2747
- Gibbs PE, Spencer BE, Pascoe PL (1991) The American oyster drill, *Urosalpinx cinerea* (Gastropoda): Evidence of decline in an imposex-affected population (R. Blackwater, Essex). J Mar Biol Ass 71: 827-838
- Gillan AH, Lewis AG, Andersen RS (1981) Quantitative determination of hydroxamic acids. Anal Chem 53: 841-844

- Gilis A, Corbisier P, Baeyens W, Taghavi S, Mergeay M, van der Lelie D (1998) Effect of the siderophore alcaligin E on the bioavailability of Cd to *Alcaligenes eutrophus* CH34. J Ind Microbiol Biotechnol 20: 61-68
- Gokarn K, Pal RB (2010) Siderophores and Pathogenecity of Microorganisms. J Biosci Tech 1(3): 127-134
- Gutnick DL, Bach H (2000) Engineering bacterial biopolymers for the biosorption of heavy metals; new products and novel formulations. Appl Microbiol Biotechnol 54: 451-460
- Hamer DH (1986) Metallothioneins. Ann Rev Biochem 55: 913-951
- Harino H, Fukushima M, Kurokawa Y, Kawai S (1997) Degradation of the tributyltin compounds by the microorganisms in water and sediment collected from the harbour area of Osaka City, Japan. Environ Pollut 98: 163–167
- Harwood-Sears V, Gordon A S (1990) Copper-induced production of copper-binding supernatant proteins by the marine bacterium *Vibrio alginolyticus.* Appl Environ Microbiol 56: 1327-1332
- Hepinstall SE, Turner BF, Maurice PA (2005) Effect of siderophores on Pb and Cd adsorption to kaolinite. Clays Clay Miner 53: 557-563
- Higham DP, Saddler PJ, Scawen MD (1984) Cadmium resistant *Pseudomonas putida* synthesizes novel cadmium proteins. Science 225: 1043-1046
- Hitchcock PJ, Brown TM (1983) Morphological heterogenecity among Salmonella lipopolysccharide chemotypes in silver stained polyacrylamide gels. J Bacteriol 154: 269-277
- Hoch M (2001) Organotin compounds in the environment – an overview. Appl Geochem 16: 719-743

- Hua H, Wu Z, Zhang H, Lu D, Wang M, Liu Y, Liu Z (2010) Degradation of hexadecane by Enterobacter cloacae strain TU that secretes an exopolysaccharide as a bioemulsifier. Chemosphere 80 (8): 951 - 956
- Huckle JW, Morby AP, Turner JS, Robinson NJ (1993) Isolation of prokaryotic metallothionein locus and analysis of transcriptional control by trace metal ions. Mol Microbiol 7: 177-187
- Inoue H, Takimura O, Fuse H, Murakami K, Kamimura K, Yamaoka Y (2000) Degradation of triphenyltin by a fluorescent Pseudomonad. Appl Environ Microbiol 66 (8): 3492-3498
- Inoue H, Takimura O, Kawaguchi K, Nitoda T, Fuse H, Murakami K, Yamaoka Y. (2003)a Tin-carbon cleavage of organotin compounds by pyoverdine from *Pseudomonas chlororaphis.* Appl Environ Microbiol 69 (2): 878 – 883
- Inoue H, Takimura O,Yamaoka Y (2003)b Degradation of Organotin Compounds by *Burkholderia cepacia* CNR22. J Jpn Soc Water Environ 29: 775-778
- Iyer A, Modi K, Jha B (2004) Accumulation of hexavalent chromium by an exopolysaccharide producing marine *Enterobacter cloaceae*. Mar Pollut Bull 49: 974-977
- Jadhav S, Bhosle NB, Massanisso P, Morabito R (2009) Organotins in the sediments of the Zuari estuary, west coast of India. Environ Manage 90: 4-7
- Jonas RB, Gilmour, CC, Stoner DI, Weir MM, Tuttle JH (1984) Comparison of methods to measure acute metal and organometal toxicity to natural aquatic microbial communities. Appl Environ Microbiol 47:1005–1011

- Jones and Barlet (1990) "Preparation of Genomic DNA from Bacteria". Modified from Experimental Techniques in Bacterial Genetics. pp 20-23
- Joshi DM, Patel JS, Flora SJS, Kalia K (2008) Arsenic accumulation by *Pseudomonas stutzeri* and its response to some thiol chelators. Environ Health Prey Med 13: 257-263
- Jude F, Arpin C, Castang C, Capdepuy M, Caumette P, Quentin C (2004) TbtABM, a multidrug efflux pump associated with tributyltin resistance in *Pseudomonas stutzeri*. FEMS Microbiol Lett 232: 7-14
- Kassab DM, Roane TM (2006) Differential responses of a mine tailings *Pseudomonas* isolate to cadmium and lead exposures. Biodegradation. 17*:* 379-387
- Kawai S, Kurokawa Y, Harino H, Fukushima M (1998) Degradation of tributyltin by a bacterial strain isolated from polluted river water. Environ Pollut 102: 259-263
- Khetan SK, Collins TJ (2007) Human pharmaceuticals in the aquatic environment: A challenge to green chemistry. Chem Rev 107: 2319-2364
- Kotrikla A (2009) Environmental management aspects for TBT antifouling wastes from the shipyards. Journal of Environmental Management 90: 577–585
- Krieg NR, Holt JG (1984) Bergey's manual of systemic bacteriology, vol 1. The Williams and Wilkins, Baltimore, USA. 140-309
- Krishnamurthy R, Cabral L, Vidya R, Dubey SK (2007) Isolation and biological characterization of a TBTCl degrading marine bacterium, *Vibrio* sp. From Bombay High Oil Field, India. Curr Sci 93: 1073-1074

- Laemelli UK (1970) Cleavage of structural proteins during the assembly of the head of the bacteriophage T4. Nature 227: 680–685
- Lee SE, Chung JW, Won HS, Lee DC, Lee YW (2012) Removal of methylmercury and tributyltin (TBT) using marine microorganisms. Bull Environ Contam Toxicol 88:239–244
- Lemos MFL, Esteves AC, Pestana JLT (2011) Fungicides as Endocrine Disrupters in Non-Target Organisms, Fungicides - Beneficial and Harmful Aspects, InTech, Rijeka, Croatia. 179 – 196
- Liu T, Nakashima S, Hirose K, Uemura Y, Shibasaka M, Katsuhara M, Kasamo K (2003) A metallothionein and CPx-ATPase handle heavy-metal tolerance in the filamentous cyanobacteria *Oscillatoria brevis.* FEBS Letters. 542: 159-163
- Maguire RJ, Carey JH, Hale EJ (1983) Degradation of the tri-n-butyltin species in water. J. Agric. Food Chem. 31: 1060-1065
- Maguire RJ, Tkacz RJ (1985) Degradation of the tri-n-butyltin species in water and sediment from Toronto harbor. J. Agric. Food Chem. 33: 947-953
- Maguire RJ (2000) Review of the persistence, bioaccumulation and toxicity of tributyltin (TBT) in aquatic environments in relation to Canada's Toxic Substances Management Policy. Wat Qual Res J Can 35: 633-679
- Martinez JL (2008) Antibiotics and antibiotic resistance genes in natural environments. Science 321: 365-367
- Matyar F, Akkan T, Ucak Y, Eraslan B (2010) *Aeromonas* and *Pseudomonas:* antibiotic and heavy metal resistance species from Iskenderun Bay, Turkey (northeast Mediterranean Sea). Environ Monit Assess 167: 309-320

- McArthur JV, Tuckfield RC (2000) Spacial pattern in antibiotic resistance among stream bacteria: Effects of industrial pollution. Appl Environ Microbiol 66: 3722-3726
- Meena RM, Garg A, Jadhav S (2009) Seasonal variation in organotins in the waters of the Dona Paula bay, west coast of india. Bull Environ Contam Toxicol 82: 586-58
- Mendo SA, Nogueira PR, Ferreira SCN, Silva RG (2003) Tributyltin and triphenyltin toxicity on benthic estuarine bacteria. Fresenius Environ Bull 12 (11): 1361-1367
- Mereghetti L, Quentin R, Marquet-van der mee N, Audurier A (2000) Low sensitivity of *Listeria monocytogens* to quarternary ammonium compounds. Appl. Environ. Microbiol. 66: 5083-5086
- Miller DM, Olson JS, Pflugrath JW, Quiocho FA (1983) Rates of legand binding to periplasmic proteins involved in bacterial transport and chemotaxis. J Biol Chem 258: 13665-13672
- Miller ME, Cooney JJ (1994) Effects of tri-, di- and monobutyltin on heterotrophic nitrifying bacteria from surficial estuarine sediments. Arch Environ Contam Toxicol. 27: 501-506
- Miller CE, Wuertz S, Cooney JJ, Pfister RM (1995) Plasmids in tributyltin-resistant bacteria from fresh and estuarine waters. J Indus Microbiol 14: 337-342
- Mimura H, Sato R, Sasaki Y, Furuyama, Y, Taniike A, Yoshida K, Kitamura A (2008) Accelerator analysis of tributyltin adsorbed onto the surface of a tributyltin resistant marine *Pseudoalteromonas* sp. Cell Int J Mol Sci 9: 1989-2002

- Morillo JA, Aguilera M, Ramoz-Cormenzana A, Monteoliva SM (2006) Production of metal-binding exopolysaccharide by *Paenibacillus jamilae* using two-phase olive-mill waste as fermentation substrate. Curr Microbiol 53: 189-193
- Morillo JA, Garcia-Ribera R, Quesada T, Aguilera M, Ramos-Cormenzana A, Monteoliva-Sanchez M (2008) Biosorption of heavy metals by the exopolysaccharide produced by *Paenibacillus jamilae*. World J Microbiol Biotechnol 24: 2699-2704
- Naik MM, Dubey SK (2011) Lead – enhanced siderophore production and alteration in cell morphology in *Pseudomonas aeruginosa* strain 4 EA. Curr Microbiol 62: 409-414
- Naik MM, Shamim K, Dubey SK (2012)a Biological characterization of lead resistant bacteria to explore role of bacterial metallothionein in lead resistance. Curr Sci 103(4): 1-3
- Naik MM, Pandey A, Dubey SK (2012)b *Pseudomonas aeruginosa* strain WI-1 from Mandovi estuary possesses metallothionein to alleviate lead toxicity and promotes plant growth. Ecotoxicology and Environmenatal Safety 79: 129-133
- Naik MM, Dubey SK, Khanolkar D, D'costa B (2013) P-type ATPase and MdrL efflux pump-mediated lead and multidrug resistance in estuarine bacterial isolates. Curr Sci 105 (10): 1366-1372
- Namiranian S, Richardson DJ, Russell DA, Sodeau JR (1997) Excited state properties of siderophore pyochelin and its complex with zinc ions. Photochem Photobiol 65: 777-782

- Nies DH, Silver S (1995) Ion efflux systems involved in bacterial metal resistance. J.Ind. Microbiol. 14: 186-199
- Noel-Georis I, Vallaeys T, Chauvaux R, Monchy S, Falmagne P, Mergeay M, Wattiez, R (2004) Global analysis of the *Ralstonia metallidurans* proteome: Prelude for the large-scale study of heavy metal response. Proteomics 4: 151-179
- Nossal NG, Heppel LA 919996) The release of enzymes by osmotic shock from *E. coli* in exponential phase. J Biol Chem 241: 3055 – 3062
- Oehlmann J, Stroben E, Fioroni P (1991) the morphological expression of imposex in *Nucella lapillus* (Linnaeus) (Gastropoda: Muricidae). J Mollusc Stud 57: 375-390
- Okeke IN, Fayinki ST, Lamikarna A (2000) Antibiotic resistance in *Escherichia coli* from Nigerian students, 1986-1998. Emerg Infect Dis 6: 393-396
- Olafson RW, Abel K, Sim RG (1979) Prokaryotic metallothionein: Priliminary characterization of blue green algal heavy metal binding protein. Biochem Biophys Res Commun 89: 36-43
- Olafson RW, McCubbin WD, Kay CM (1988) Primary- and secondary-structural analysis of unique prokaryotic matallothionein from a *Synechococcus* sp. cyanobacterium. Biochem J 251: 691-699
- Pain A, Cooney JJ (1998) Characterization of organotin-resistant bacteria from Boston Harbor sediments. Arch Environ Contam Toxicol 35: 416-421
- Page SD (1989) An analytical method for butyltin species in selfish. Mar Pollu Bull 20: 129-133

- Page DS, Ozbal CC, Lanphear ME (1996) Concentration of butyltin species in sediments associated with shipyard activity. Environ Poll 91: 237-243
- Pagliarani A, Nesci S, Ventrella V (2013) Toxicity of organotin compounds: shared and unshared biochemical targets and mechanisms in animal cells. Toxicol In Vitro 27(2): 978–990
- Pal A, Paul AK (2008) Microbial extracellular polymeric substances: central elements in heavy metal bioremediation. Indian J Microbiol 48: 49-64
- Pazirandeh M, Chrisey LA, Mauro JM, Campbell JR, Gaber BP (1995) Expression of *Neurospora crassa* metallothionein gene in *E. coli* and its effect on heavy metal uptake. Appl Microbiol Biotechnol 43: 1112-1117
- Pazirandeh M, Wells BM, Ryan RL (1998) Development of bacterium-based heavy metal biosorbents: Enhanced uptake of cadmium and mercury by *Escherichia coli* expressing a metal binding motif. Appl Environ Microbiol 64: 4068-4072
- Poerschmann J, Kopinke F-D, Pawliszyn J (1997) Solid phase microextraction to study the sorption of organotin compounds onto particulate and dissolved humic organic matter. Environmental Science and Technology 31: 3629–3636
- Quigley MS, Santschi PH, Hung CC, Guo L, Honeyman BD (2002) Importance of acid polysaccharides for ^{234}Th complexation to marine organic matter. Limnology and Oceanography 47 (2): 367–377
- Rajendran BR, Tao H, Miyazaki A, Ramesh R, Ramachandran S (2001) Determination of butyl-, phenyl-, octyl- and tributylmonomethyltin compounds in a marine environment (Bay of Bengal, India) using gas chromoatography-inductively coupled plasma mass spectrometry. J Environ Monit 3: 627-634

- Ramachandran **V**, Dubey SK (2009) Expression of TBTCl-induced periplasmic proteins in a tributyltin chloride resistant marine sediment bacterium *Alcaligenes* sp. Curr Sci 97: 1717-1718
- Rantala M (2010) Orgaaniset tinayhdisteet sedimenteissä ja kaloissa - Diploma work. 1–95
- Rathnayake IVN, Megharaj M, Krishnamurti GSR, Bolan NS (2013). Heavy metal toxicity to bacteria – Are the existing growth media accurate enough to determine heavy metal toxicity? Chemosphere 90:1195-1200
- Rensing C, Ghosh M, Rosen BP (1999) Families of Soft-metal-ion-transporting ATPases. J. Bacteriol. 181: 5891-5897
- Roane TM (1999) Lead resistance in two bacterial isolates from heavy metal-contaminated soils. Microb. Ecol. 37: 218-224
- Robinson NJA, Gupta A, Fordham-Skelton P, Croy RRD, Whitton BA, Huckle JW (1990) Prokaryotic metallothionein gene characterization and expression: chromosome crawling by ligation mediated PCR. Proc R Lond B 242: 241-247
- Robinson NJ, Whitehall SK and Cavet JS (2001) Microbial metallothioneins. Adv Microb Physiol 44: 183-213
- Rossbach S, Wilson TL, Kukuk ML, Carty HA (2000) Elevated zinc induces siderophore biosynthesis genes and a zntA-like gene in *Pseudomonas fluorescens.* FEMS Microbiol Lett 191: 61-70
- Roy U, Dubey SK, Bhosle SN (2004) Tributyltin chloride-utilizing bacteria from marine ecosystem of west coast of India. Curr Sci 86 (5): 702-705
- Rudel H (2003) Case study: bioavailability of tin and tin compounds. Ecotoxicology and Environmental Safety 56: 180–189

- Sakultantimetha A, Keenan HE, Dyer M, Beattie TK, Bangkedphol S, Songsasen A (2009) Isolation of tributyltin-degrading bacteria *Citrobacter braakii* and *Enterobacter cloacae* from butyltin-polluted sediment. Journal of ASTM International 6 (6): 1-6
- Saitou N, Nei M (1987) The neighbor-joining method: a new method for reconstructing phylogenetic trees. Mol Biol Evol 4:406-425
- Sakultantimetha A, Keenan HE, Dyer M, Beattie TK, Bangkedphol S, Songsasen A (2010) Acceleration of tributyltin biodegradation by sediment microorganisms under optimized environmental conditions. Int Biodeter Biodegr 64: 467-473
- Sambrook J, Fritsch EF, Maniatis T (1989) Molecular cloning: A laboratory manual. Cold Spring Harbour, New York.
- Sampath R, Venkatakrishnan H, Ravichandran V, Chaudhury RR (2012) Biochemistry of TBT-degrading marine Pseudomonads isolated from Indian coastal waters .Water Air Soil Pollut 223: 99 – 106
- Sasser M (1990). Identification of bacteria by gas chromatography of cellular fatty acids. Newark, DE, USA: Microbial ID
- Sayyed RZ, Chincholkar SB (2009) Siderophore-producing *Alcaligenes faecalis* exhibited more biocontrol potential vis-a-vis chemical fungicides. Cur. Microbiol. 58: 47-51
- Schwyn B, Neilands JB (1987) Universal chemical assay for the detection and determination of siderophores. Annal Biochem 160 (1): 47 – 56
- Shamim K, Naik MM, Pandey A, Dubey SK (2012) Isolation and identification of *Aeromonas caviae* strain KS-1 as TBTC and Lead resistant estuarine bacteria. Environ Monit Assess 185 (6): 5243 - 5249

- Seligman PF, Valkirs AO, Lee RF (1986) Degradation of tributyltin in San Diego Bay, CA waters. Draft Report. Naval Ocean Systems Center, San Diego, CA. p. 7
- Seligman P, Grovhoug J, Stang P, Valkirs A, Stallard M, Lee R (1988) Distribution and fate of tributyltin in marine-environment. Abstracts of Papers of the American Chemical Society, 195: 70-74
- Seligman PF, Grovhoug JG, Valkirs AO, Stang PM, Fransham R, Stalland MO, Davidson B, Lee RF (1989) Distribution and fate of tributyltin in the United States marine environment. Appl Organo Chem 3:31-47
- Sharma S, Sundaram CS, Luthra PM, Singh Y, Sirdeshmukh R, Gade WN (2006) Role of proteins in resistance mechanism of *Pseudomonas fluorescens* against heavy metal induced stress with proteomics approach. J Biotechnol 126: 374-382
- Silver S (1992) Plasmid-encoded metal resistance mechanisms: range and overview. Plasmid 27: 1-3
- Sinha S, Mukherjee SK (2008) Cadmium-induced siderophore production by a highly Cd-resistant bacterial strain relieved Cd toxicity in plants through root colonization. Curr Microbiol 56: 55-60
- Snoeij NJ, Penninks AH, Seinen W (1987) Biological activity of organotin compounds–an overview. Environ Res 44: 335–353
- Sousa ACA, Barroso CM, Tanabe S, Horiguchi T (2010) Involvement of Retinoid X Receptor in Imposex Development in *Nucella lapillus* and *Nassarius reticulates* - Preliminary Results. Interdisciplinary Studies on Environmental Chemistry — Biological Responses to Contaminants, Terrapub. 189–196

- Spectral database for organic compounds SDBS

  Available at : http://sdbs.riodb.aist.go.jp/sdbs/cgi-bin/direct_frame_top.cgi
- Stab JA, Frenay M, Freriks IL, Brinkman UA, Cofino WP (1995) Survey of nine organotin compounds in The Netherlands using the zebra mussel *(Dreissena polymorpha*) as biomonitor. Environ Toxicol Chem 14:2023-2032
- Stang PM, Seligman PF (1986) Distribution and fate of butyltin compounds in the sediment of San Diego Bay. In: Oceans 86 (4) Proceedings International Organotin Symposium. Washington, DC. pp. 1256-1261
- Stang PM, Lee RF, Seligman PF (1992) Evidence for rapid non-biological degradation of tributyltin in fine-grained sediments. Environ Sci Technol 26: 1382-1387
- Stoner HB, Barnes JM, Duff JI (1955) Studies on the toxicity of alkyltin compounds. Brit J Pharmacol 10: 16-25
- Suehiro F, Kobayashi T, Nonaka L, Tuyen BC, Suzuki S (2006) Degradation of Tributyltin in microcosm using Mekong river sediment. Microb Ecol 52: 19-25
- Sun G-X, Zhou W-Q, Zhong J-J (2006) Organotin Decomposition by Pyochelin, Secreted by *Pseudomonas aeruginosa* even in an iron-sufficient environment. Appl Environ Microbiol 72: 6411-6413
- Sutherland IW (2001) Biofilm exopolysaccharides: a strong and sticky framework. Microbiology 147:3-9
- Suzuki S, Fukagawa T, Takama K (1992) Occurrence of TBT-Tolerant Bacteria in TBT or Cd containing Seawater. Appl Environ Microbiol 58: 3410-3412

- Suzuki S, Tsukamoto KK, Fukagawa T (1994) The 16S rRNA sequence and genome sizing of tributyltin resistant marine bacterium, strain M-1. Microbiol 101: 101 -109
- Suzuki S, Fukagawa T (1995) TBT Resistant Marine Bacteria: A Summary of Recent Work. J Indust Microbiol 14: 154-158
- Tay JH, Liu QS, Liu Y (2001) The role of cellular polysaccharides in the formation and stability of aerobic granules. Lett Appl Microbiol 33: 222-226
- Tamura K, Dudley J, Nei M, Kumar S (2007) MEGA4: molecular evolutionary genetics analysis (MEGA) software version 4.0 Mol Biol Evol 24:1596-1599
- Thouvenin M, Peron J, Charreteur C, Guerin P, Langlois J, Vallee-Rehel K (2002) A study of the biocide release from antifouling paints. Progress in Organic Coatings 44: 75–83
- Tobin JM, Copper DG, Neufeld RJ (1984) Uptake of metal ions by *Rhizopus arrhizus*. Appl Environ Microbiol 47: 821-824
- Turner JS, Glands PD, Samson ACR, Robinson NJ (1996) Zn+2-sensing by the cyanobacterial metallothionein repressor SmtB: different motifs mediate metal-induced protein-DNA dissociation. Nucleic acids Res 24: 3714-3721
- Turner A (2010) Marine pollution from antifouling paint particles. Mar Poll Bullet 60: 159-171
- Valkirs A, Seligman P, Lee R (1986) Butyltin Partitioning in Marine Waters and Sediments. OCEANS 86: 1165–1170
- Vasak M, Hasler DW (2000) Metallothioneins: new structural and functional insights. Curr Opin Chem Biol 4: 177-183

- West AK, Stallings R, Hildebrand CE, Chiu R, Karin M, Richards RI (1990) Human metallothionein genes: structure of the functional locus at 16q13. Genomics 8: 513-518
- Wingender J, Neu TR, Flemming HC (1999) What are bacterial extracellular polymeric substances? In: Microbial extracellular polymeric substances (Wingender J, Neu TR, Flemming HC, eds). Springer, Berlin. pp 1-20
- White JS, Tobin JM, Cooney JJ (1999) Organotin compounds and their interactions with microorganisms. Can J Miceobiol 45 (7): 541-554
- White JS, Tobin JM (2004) Inorganic tin and organotin interactions with *Candida maltosa*. Appl Microbiol Biotechnol 63: 445-51
- Wuertz S, Miller CE, Pfister RM, Cooney JJ (1991) Tributyltin-Resistant bacteria from estuarine and fresh water sediments. Appl Environ Microbiol 57: 2783-2789
- Yildirim V, Ozcan S, Becher D, Buttner K, Hecker M, Ozcengiz G (2011) Characterization of proteome alterations in *Phanerochaete chrysosporium* in response to lead exposure. Proteome Science 9:12
- Yonezawa Y, Fukui M, Yoshida T, Ochi A, Tanaka T, Noguti Y, Kowata T, Sato Y, Masunaga S, Urushigawa Y (1994) Degradation of tri-n-butyltin in Ise Bay sediment. Chemosphere 29: 1349-1356
- Zobell CE (1941) Studies on marine bacteria. I. The cultural requirements of heterotrophic aerobes. J Mar Res 4: 42-75

PUBLICATIONS

1. Naik MM, **Khanolkar DS**, Dubey SK (2012) Lead resistant *Providencia alcalifaciens* strain 2EA bioprecipitates Pb(+2) as lead phosphate. ***Letters in Applied Microbiology***. 56: 99 – 104
2. Naik MM, Dubey SK, **Khanolkar D**, D'Costa B (2013) P-type ATPase and MdrL efflux pump-mediated lead and multi-drug resistance in estuarine bacterial isolates. ***Current Science***. 105 (10): 1366 – 1372
3. D'Costa B, **Khanolkar D**, Dubey SK (2013) Partial purification and characterization of metalloprotease of halotolerant alkaliphilic bacterium *Bacillus cereus* from coastal sediment of Goa, India. ***African Journal of Biotechnology***. 12 (31): 4905 – 4914
4. **Khanolkar D**, Naik M, Dubey SK (2014) Biotransformation of Tributyltin chloride by *Pseudomonas stutzeri* strain DN2. ***Brazilian Journal of Microbiology*** (**in press**)
5. **Khanolkar D**, Dubey SK, Naik MM (2014) TBTCl - enhanced EPS and siderophore production in TBTCl degrading *Alcaligenes faecalis* strain SD5. ***Archives of Environmental Contamination and Toxicology*****. (under revision)**
6. **Khanolkar D**, Dubey SK, Naik MM (2014) Biotransformation of Tributyltin chloride to less toxic Dibutyltin dichloride and Monobutyltin trichloride by *Klebsiella pneumoniae*. ***International Biodeterioration and Biodegradation.*** **(under review)**
7. **Khanolkar D**, Pandey A, Naik M, Dubey SK (2014) *Pseudomonas mendocina* strain DP4 resists TBTCl by metallothionein mediated bioaccumulation. ***Bulletin of Environmental Contamination and Toxicology.*** **(communicated)**