BIOLOGICAL CHARACTERIZATION OF MARINE BIOLUMINESCENT BACTERIA UNDER THE STRESS OF METALLIC AND ORGANO-METALLIC ENVIRONMENTAL POLLUTANTS

THESIS SUBMITTED TO THE

GOA UNIVERSITY

FOR THE AWARD OF DEGREE OF

DOCTOR OF PHILOSOPHY

IN

MICROBIOLOGY

BY

VEERA BRAMHA CHARI. P

Department of Microbiology
Goa University
Goa-403206

2006

गोंय विद्यापीठ
तळेगांव पठार
गोंय - ४०३ २०६
फोन : ०८३२ - २४५९३४५ - ४८ / २४५६४८०-८५
: +०९१-८३२-२४५९९८४ / २४५२८८९

(Accredited by NAAC with a rating of Four Stars)

Goa University
Taleigao Plateau, Goa - 403 206
Tel : 0832-2451345-48/2456480-85
Fax : + 091- 832-2451184/2452889
E.mail : registra@unigoa.ac.in
Website: www.goauniversity.org

Certificate

This is to certify that Mr. Veera Bramha Chari. P has worked on the thesis entitled "Biological characterization of marine bioluminescent bacteria under the stress of metallic and organo-metallic environmental pollutants" under my supervision and guidance.

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

15/2/06

Dr. Saroj Bhosle

Head
Department of Microbiology
Goa University
Goa- 403206

Santosh Kumar Dubey

Dr. Santosh Kumar Dubey

Reader & Research Guide
Department of Microbiology
Goa University
Goa- 403206

15/2/06

GOA UNIVERSITY : LIBRARY : GOA

(Prof. P. B. Kavi Kishore)
(External Examiner)
8/7/06

(Dr. Santosh Kumar Dubey)
Co-ordinator of Viva-Voce exam.
&
Res. Guide
8/7/06

STATEMENT

As required under the University Ordinance 0.91.8 (vi), I hereby state that this thesis for the Ph.D. degree entitled "Biological characterization of marine bioluminescent bacteria under the stress of metallic and organo-metallic environmental pollutants" is my original contribution. The thesis and any part of it has not been previously submitted for the award of any degree/ diploma in any University or Institute. To the best of my knowledge, the present study is the first comprehensive work of its kind from this area mentioned.

The literature related to the problem investigated has been cited. Due acknowledgements have been made wherever facilities and suggestions have been availed of.

Veera Bramha Chari. P.

Department of Microbiology
Goa University
Goa-403206

ACKNOWLEDEMENT

The successful completion of thoughtful search endeavor is due to the combined encouragement of numerous individuals who have been constantly inspired and motivated me throughout this study.

I would like to express my gratitude and appreciation for the time and commitment provided by my academic and research advisor, Dr. Santosh Kumar Dubey who impressively performed his role as a mentor. Due to the combination of Dr. Dubey's technical and scientific expertise, ceaseless determination this has been a very exciting and rewarding experience, and I am honored to have had the opportunity to work with him. I also wish to thank him for providing valuable assistance, guidance throughout my first teaching experience in Goa University.

I wish to thank Dr. Saroj Bhosle, subject expert, and the Faculty Research Committee of my Ph.D without whose patience, continuous efforts and guidance, the Ph.D. work would not have progressed. I must also express my gratitude to Emeritus Professor S. Mavinkurve.

I am highly obliged for the zeal, enthusiasm, and encouragement provided by Prof. P. B.K.Kishor, Dr. C.G. Nayak, Prof. G.N. Nayak, Dr. Parameshwaran, Dr.Ramarao, Prof. Mahadevan, Dr. Nazarath, Dr. Sandeep, Dr. Irene Furtado, Dr, Janarthanam, Dr. Srinivasan, Dr. Jogeswar, Dr. Sanjeev, Dr. Chanda, Dr. Shanthi, Dr. Suphala, Dr. Keshav, Dr. Madan, Dr. Shashikumar, Dr.Vikranth, Dr. Raghu, Dr. Meenal, Dr.Upal, for their diligent, constructive discussion and valuable criticism and all of them have been a tremendous asset during each stage of this research endeavor.

I would like to thank Sachin and Paul especially for their endless support and photography with uniform style and quality; Ranadheer, Ravi Chand, Rama, Priya, Sunitha, Harish, Murthy, Naveen, Rasika, Aureen, Lakshangy, Rupiali, Celisa, Nimali, Neelam, for their splendid cooperation, warm and cheerful friendship; and other friends in the laboratory for special thanks. I would like to thank office assistants Ana for her endless support from start to finish, Laboratory Manager, Mr. Shashi, and lab assistants Domnic, Budhaji, and Ladu for their efficient cooperative technical assistance

I should, finally, dedicate this thesis to my beloved parents, particularly for their unending love and encouragement without whom this would not have been possible. All my supporters, well wishers are privileged to share this thesis with a constructive mind.

-- Veera Bramha Chari .P -

DEDICATED TO

ABBREVIATIONS

α	Alpha
Abs	Absorbance
$AgNO_3$	Silver nitrate
APS	Ammonium per sulphate
AOr	Acridine Orange
As (V)	Arsenate
As (III)	Arsenite
β	Beta
5-BU	5-Bromouracil
°C	Degree Celsius
Cd^{2+}	Cadmium ion
CFU	Colony forming unit
DBT	Dibutyltin
D/W	Double distilled water
DTT	Dithiotritol
dm	Diameter
dNTP	Dinucleotide triphosphates
EDTA	Ethylene diamine tetra acetic acid
EtBr	Ethidium Bromide
EPS	Exopolysaccharide
ESI	Electron spray ionization
Fig	Figure
FTIR	Fourier transform infrared spectroscopy
gm	Gram
γ	Gamma
GBMA	Glycerol based marine agar
hrs	Hours
GC	Gas chromatography
Hg^{2+}	Mercuric ion
IR	Infra Red
KI	Potassium Iodide
KDa	Kilo Daltons
Kbps	Kilo base pairs
KNO_3	Potassium nitrate
lt	Litre
LB	Luria Bertani
Lbs	Pounds
λ	Lambda
CH_3OH	Methanol
mA	Milli Amperes
M	Molar
MSM	Mineral salt media
MS	Mass spectrometry
mM	Millimolar
μM	Micromolar
min	Minutes
mL	Milliliter
μg	Micrograms
MIC	Minimal Inhibitory Concentration
nm	Nanometer
NaCl	Sodium Chloride
NH_4NO_3	Ammonium nitrate
NH_4Cl	Ammonium Chloride
NTG	N-methyl-N-nitro-N-Nitrosoguanidine
O.D.	Optical Density
%	Percentage
PAGE	Polyacrylamide gel electrophoresis
PCR	Polymerase chain r eaction
PBS	Phosphate Buffer Saline
Rf	Resolution factor
RLU	Relative light units
rpm	Revolutions per minute
RT	Room temperature
SA	Sodium azide
SDS	Sodium Dodecyl Sulphate
sec	Seconds (s)
Sn^{2+}	Tin
SEM	Scanning Electron Microscopy
sp.	Species
TBT	Tributyltin
Thiol	β-Mercaptoethanol
TE	Tris-EDTA
TEMED	Tetra methyl ethylene diamine
TAE	Tris acetate EDTA
TCBS	Thiosulphate citrate bile sucrose
UV	Ultra violet

Contents

Chapter II

Materials and methods

Sampling sites for collecting environmental samples
Enumeration of luminous bacterial isolates (CFU/ml)

Physicochemical analysis of water samples

Salinity
pH
Alkalinity
Temperature
Dissolved oxygen
Nitrite content (NO_2)
Nitrate content (NO_3)
Phosphate content (PO_4)

Determination of viable counts of luminous bacterial isolates

Growth behavior of the isolates in minimal and rich media.

Utilization of carbon sources

Optimization of environmental parameters for growth
Determination of optimum pH for growth of isolates

Determination of optimum temperature for growth of marine isolates

Determination of optimum salt (NaCl) concentration for the growth of marine isolates

Maintenance of the luminous bacterial strains in heavy metals
Preservation of the luminous bacterial strains:

Utilization of carbon sources:

Microscopy

Phase contrast Microscopy
Scanning electron microscopy:

Identification of the bacterial isolates
Morphological, Physiological and Biochemical characterization.

Effect of Protein synthesis- inhibitors and $HgCl_2$ on Bacterial bioluminescence

Effect of chemical mutagens on Bacterial bioluminescence

Effect of UV light on Bacterial bioluminescence

Effect of n-Decanaldehyde on bacterial bioluminescence

Growth and Bioluminescence by serial transfer in (heavy metals and organo-metals).

3.3 Protein profiles of *Vibrio harveyi* isolate VB23

Induction of stress proteins

Preparation of cellular proteins (Cell lysis and protein extraction)

Estimation of protein concentration

Polyacrylamide gel electrophoresis (SDS-PAGE)

Coomassie brilliant blue staining

Silver staining
Gel photography

3.4 EPS Characterization

EPS Characterization

Screening and culturing of exopolymer-producing luminous bacteria

Correlation of Growth with exopolymer production of VB23

Exopolymer production in different Carbon and Nitrogen sources:

Exopolymer production of Luminous *Vibrio harveyi* in different heavy metal concentrations

Extraction and purification of Exopolysaccharide

Alcian blue staining of the exopolymer

Scanning electron microscopy (SEM)

Emulsifying activity of exopolysaccharide

Quantitative analysis of exopolysaccharide

Chemical analysis of the exopolysaccharide

Analytical gas chromatography of the exopolymer

ESI -Mass spectra of the exopolymer

Fourier transformed infrared spectroscopy of the exopolymer

3.5 Pigment profile of bioluminescent *Vibrio harveyi*

Induction of pigments under heavy metals and organometals stress

List of Tables

List of Figures

Chapter-I

Chapter II

2.15 Optimum Temperature (°C) for growth of luminous bacterial isolates grown in MSM + 2% NaCl +0.2% Glucose.

2.16 Optimum NaCl (%) concentration for luminous bacterial isolates grown in MSM + 2% NaCl +0.2 % Glucose.

Chapter III

3.1. **Growth**

3.1.1. Growth behavior of luminous bacterial strain *Vibrio harveyi* VB23 in Hg (II)

3.1.2. Growth behavior of luminous bacterial strain *Vibrio harveyi* VB23 in Cd (II)

3.1.3. Growth behavior of luminous bacterial strain *Vibrio harveyi* VB23 in Cr (VI)

3.1.4. Growth behavior of luminous bacterial strain *Vibrio harveyi* VB23 in As (III)

3.1.5. Growth behavior of luminous bacterial strain *Vibrio harveyi* VB23 in As (V)

3.1.6. Growth behavior of luminous bacterial strain *Vibrio harveyi* VB23 in Sn (II)

3.1.7. Growth behavior of luminous bacterial strain *Vibrio harveyi* VB23 in TBTC

3.1.8. Growth behavior of luminous bacterial strain *Vibrio harveyi* VB23 in DBTC

3.1.9. Percent survival of *Vibrio harveyi* strain VB23 in various heavy metals

3.1.10. Percent survival of *Vibrio harveyi* strain VB23 in various Organo- metals

3.1.11 A & 3.1.11 B).

Effect of chelating agents and thiol compounds on (Hg^{2+}) toxicity of luminous *Vibrio harveyi* strain VB23

3.1.12 A & 3.1.12 B).

Ameliorative Effect of chelating agents and thiol compounds on (Cd^{2+}) toxicity of luminous *Vibrio harveyi* strain VB23.

3.1. 13. Ameliorative Effect of chelating agents and thiol compounds on (TBT) toxicity of luminous *Vibrio harveyi* strain VB23

3.1.14 A. and 3.1.14 B.

Percent Survival of *Vibrio harveyi* strain VB23 in various Antibiotics

3.2 **Bioluminescence**

3.2.1. % Relative light Intensity of *Vibrio harveyi* VB23 in Hg (II) and Cd (II)

3.2.2 % Relative light Intensity of *Vibrio harveyi* VB23 in Hg (II) and Chelating agent

3.3.3 % Relative light Intensity of *Vibrio harveyi* VB23 in Sn (II), As (III) & As (V).

3.2.4 % Relative light Intensity of *Vibrio harveyi* VB23 in TBT and DBT

3.2.5 % Relative light Intensity of *Vibrio harveyi* VB23 in antibiotics

3.2.6 % Relative light Intensity of *Vibrio harveyi* VB23 in antibiotics and Hg^{2+}

3.2.7 % Relative light Intensity of *Vibrio harveyi* VB23 in chemical mutagens

3.2.8 % Relative light Intensity of *Vibrio harveyi* VB23 in Sodium Azide and 5-BU

3.2.9 % Relative light Intensity of *Vibrio harveyi* VB23 under UV light

3.2.10 % Relative light Intensity of *Vibrio harveyi* VB23 in antibiotics and UV exposure

3.2.11. % Relative light Intensity of *Vibrio harveyi* VB23 in n-Decanal.

3. 3 **Protein profile**

3.3.1 Induction of heavy metal stress proteins As (II), As (V), Cd (II), Hg (II) high range

3.3.2 Induction of heavy metal stress proteins As (II), As (V), Cd (II), Hg (II) low range

3.3.3 Induction of heavy metal stress proteins Hg (II)

3.3.4 Induction of heavy metal stress proteins Cd (II)

3.3.5 Induction of organo- metal stress proteins TBT and DBT

3.3.6 Induction of ethanol stress proteins

3.4. **Exopolysaccharides (EPS)**

3.4.1 Schematic representation for isolation of exopolymer produced by marine luminous bacterial strains

3.4.2 Growth and EPS production of *V. harveyi* VB23

3.4.3. Effect of various Carbon sources on the growth a production of EPS (exopolymer) by marine luminous bacterial strain VB23.

3.4.4 Effect of various Nitrogen sources on the growth and production of EPS (exopolymer) by marine luminous bacterial strain VB23.

3.4.5 Exopolymer (EPS) production of luminous bacterial strain VB23 in the presence of heavy metals and organo-metals.

3.4.6 Images of Alcian blue staining of luminous bacterial strain VB23 exopolymer Uronic acids (pH 2.5) and Sulphates (pH 0.5)

3.4.7 Scanning electron microscopy image of the VB23 exopolymer

3.4.8 Gas chromatogram of alditol acetate derivative of EPS from *Vibrio harveyi* VB23

Chapter – I

Introduction

Bioluminescence phenomena

Bioluminescence refers to the process of visible light emission in living organisms mediated by an enzyme catalyst. The phenomenon of bioluminescence has been observed in many different organisms such as bacteria, fungi, fish, insects, algae, and squids. The enzymes that catalyze the bioluminescence reaction are called luciferases and the substrates are often referred to as luciferins. Bioluminescent organisms comprise a diverse set of species that are widely distributed, inhabiting terrestrial, freshwater and marine ecosystems. The discoveries of physiology, biochemistry, molecular biology and genetic control of bacterial bioluminescence have revolutionized the area of environmental microbiology and biotechnology, ecology, industrial and medical significance (Zeigler and Baldwin 1981; Hastings et al. 1985; Dunlap, 1991; Meighen, 1991; Baldwin and Zeigler 1992; Meighen,1993, 1994; Tu and Mager. 1995; Wilson and Hastings 1998;). The elucidation of luciferase genes regulation permitted the discovery of intercellular communication among bacteria, which in turn, has led to a better understanding of bacterial pathogenesis and the associations of microorganisms in the environment (Stewart and Williams 1992; Steinberg, 1995; Stevens and Greenberg 1997; Bassler, 2002). With the advent of molecular biology, it has been possible to construct bioluminescent bacteria that were naturally dark, by insertion of *lux* genes (Selifonova et al. 1993; Virta et al.1995).

Biodiversity of Bioluminescent bacteria

Bioluminescent organisms comprise a diverse set of species that are widely distributed, inhabiting terrestrial, freshwater and marine ecosystems occuring in three groups within the proteobacteria (Meighen, 1991). Bioluminescence is exhibited by both prokaryotes and eukaryotes (Meighen, 1988, 1993). The luminescent system of marine bacteria predominates more in *Vibrio* and *Photobacterium* species (e.g., *Vibrio fischeri, Vibrio harveyi,* and *Photobacterium phosphoreum*), There are many non-luminous bacteria of the genus *Vibrio,* and several other non-luminous *Photobacterium angustum, P. damsela, P. histaminum, P. iliopscarum and P. profundum*). Additionally, other luminescent bacteria are of interest including light-emitting *Vibrio cholerae* strains found from brackish or

freshwater (*Vibrio albensis*) and the aerobic *Shewanella* (*Alteromonas*) *hanedai* (Meighen, 1991, 1993; Thompson et al. 2004). In eukaryotes the fireflies (*Photinus pyralis*), and click beetles (*Pyrophorous plagiophtalamus*) exhibit luminescence. Significant differences exist between the bioluminescence mechanism of prokaryotic and eukaryotic luminescent organisms with respect to the structure and properties of the luciferase and substrates. The requirement for molecular oxygen and luciferase enzymes are the only common features in both prokaryotic and eukaryotic luminescence (Bourgois et al. 2001).

Each species of luminous bacteria differs in a number of properties, including the specific growing conditions (nutritional requirements and growth temperature), and the reaction kinetics of the luciferase involved in light generation; however, all luminous bacteria are rod-shaped, gram-negative microorganisms with flagella facilitating motion (Haygood and Allen 2000). Luminous bacteria are also facultative anaerobes capable of growth when the supply of molecular oxygen is limited. Despite the physiological diversity among different species of luminous bacteria, all luminescent microorganisms utilize highly homologous biochemical machineries to produce light. The onset as well as the energy output of this light-producing molecular machinery are tightly regulated under a central signalling pathway (Miyamoto et al. 1990; Stevens and Greenberg 1997).

Ecological Habitats of Bioluminescent bacteria

The distribution of luminous bacterial species in seawater can be predicted largely by their temperature optima for growth. During cooler months when temperatures are below 20°C, *Vibrio fischeri* predominates as symbionts, in warmer months *Vibrio harveyi* predominates in open ocean environments and surface waters, while *Photobacterium phosphoreum*, a psychrophillic species, is found at greater depths of 500-1000 meters (Ruby et al. 1980). The genus *Vibrio* and *Photobacterium* are common members in the enteric habitats of marine animals. *Photobacterium* can be the dominant bacterium in the gut tracts of some fishes such as gut flora of cods (*Gadus morua*) and it is virtually the sole microbial spoilage agent of marine fish (Ruby and Morin 1979; Dalgaard et al. 1997) *Vibrio* spp. are one of the predominant pathogenic microbes, which cause high mortality among economically important species of farmed marine fish, shrimps, oysters, mussels and clams. Vibriosis, especially luminous disease has caused serious loss in prawn hatcheries

(Lavilla-Pitogo et al. 1990). Most fascinating quality of luminous bacteria is their propensity for forming symbiotic associations with fishes. Twenty families of fishes contain species that have symbiotic light organs. *Photobacterium phosphoreum* is a symbiont forming association with 6 families and 4 orders, which are common enteric inhabitants, *Photobacterium leiognathi* occurs in light organs of 3 families, *Vibrio fischeri* is a symbiont of 1 family, the Monocentridea and also squids (Haygood and Allen 2000). The genus *Xenorhabdus luminesecens* is a terrestrial luminous bacteria having symbiotic relationship with nematodes that are inturn pathogenic to insects (Hosseini and Nealson 1995).

Ecological significance of bioluminescent bacteria

The function of light emission in higher organisms usually falls under 3 categories: i) to assist in predation (offense), ii) to aid in avoiding predators (defense) and iii) intraspecies communication such as courtship. Bacterial bioluminescence predominates in marine ecosystems, particularly among fish (Steinberg, 1995), *Euprymna scolopes* (Squid)- *Vibrio fischeri* mutualism (Boettcher and Ruby 1990). Both organisms benefit from this interaction; the fish consume nutrients that otherwise would have been lost to the ocean floor and the bacteria find themselves in the gut, a more nutrient-rich environment, where they can proliferate, get excreted, and continue the cycle. Overall, bioluminescence has helped understand the intricacies of microbial ecology. It has led to significant discoveries on how bacteria interact with higher organisms and among themselves. The ecological benefit for a fish or squid living in a symbiotic association with luminescent bacteria has been established (Nealson and Hastings 1979). The host organism can use the light emitted by bacteria to attract prey, escape from predators or for communication. However, it is not understood what specific benefits symbiotic bacteria derive from producing light. Although one could imagine some advantages for bacteria living in the light organs of animals, it seems unlikely that the establishment of such a symbiosis could have been the main evolutionary drive to develop very complicated light-emitting systems (Boettcher and Ruby 1990). The biological role of luminescence in free-living bacteria remains even more mysterious (Wilson and Hastings 1998).

Natural bioluminescent bacteria as biosensors

Marine luminous bacteria have continued to create interest among microbial ecologists because they are ecologically versatile, utilize several nutrients and occupy several econiches in the marine environment (Hastings and Nealson 1981), their bioluminescence being extremely sensitive to toxicants that has been employed in bioassays for detecting nano or picomolar concentrations of impurities in pharmaceuticals (Hastings, 1982), food industry (Bar and Ulitzur 1994), and water quality testing (Bulich et al. 1981; 1982) commercially available Microtox test is based on the inhibition of bioluminescence of the bacterium, *Photobacterium phosphoreum* (Bulich, 1982) when it is exposed to toxic substances, including solvents and toxic metals (Bulich, 1986; Kamlet et al. 1986). Changes in bioluminescence relative to a control used on the same day indicates the presence of toxicants, where the exact nature of the toxicant cannot be identified, as this test indicates only the presence of some form of toxicants. However, the kinetics of the dose-related decline in bioluminescence can indicate the classes of toxins present in the marine environment (Ribo and Kaiser 1983). The response time for this system ranges from 15 minutes to 1 hour (Tescione and Belfort 1993). Intact freeze-dried cells have been used for testing toxicity in long-term assays with toxic substances in the Mutatox test (Arfsten et al. 1994). The Mutatox test uses a dark variant of *Vibrio fischeri, which* produces bioluminescence after incubation at 27 °C for 16-24 hours in the presence of genotoxic agents.

Genetically modified luminous bacteria as biosensors

Several bioluminescent bacterial sensors for detection of toxic metals and organo-metals have been customized by genetic manipulation of *E. coli.* By using transcriptional fusion of Tn21 mercury resistance encoding (*mer*) operon with *lux* CDABE from *Vibrio fischeri*, three biosensors for Hg (II) have been constructed and tested (Selifonova et al. 1993). This *mer-lux* biosensor demonstrated the semi quantitative detection of inorganic Hg (II) in natural water in the range of 0.1 to 200 ppb levels and was a good system for distinguishing bioavailable from unavailable forms of mercury (Selifonova et al. 1993). Recombinant luminescent bacteria have been constructed and used for general toxicity

testing including heavy metals (Rychert and Mortimer 1991; Lampinen et al. 1990; Virta et al. 1994). Metal-specific recombinant bacterial sensors have been constructed and used for the detection of inorganic mercury (Dubey et al.1993; Selifonova et al. 1993; Virta et al.1995), organomercurials (Ivask et al. 2001), zinc, cadmium, cobalt and lead, cadmium and nickel (Tauriainen et al. 1998). In a metal-specific bacterial sensor the expression of a reporter gene is controlled by a genetic regulatory unit (receptor), which responds to the given heavy metal, i.e. receptor–reporter concept by (Lewis et al. 1998) is used. Most of the regulatory units used in the construction of metal-specific sensor bacteria originate from bacteria that possess natural precisely regulated resistance systems towards heavy metals. Heitzer et al. (1992) developed a bioassay to assess the bioavailability of naphthalene and salicylate in contaminated soils, using genetically engineered *Pseudomonas fluorescens* HK44 carrying the *nah-lux* reporter plasmid capable of degrading both. Applegate et al. (1998) have constructed a *tod-lux* fusion and introduced it into *Pseudomonas putida* F1, which was used as a whole-cell reporter for benzene, toluene, ethylbenzene, and xylene (BTEX) sensing and bioavailability determination. A novel mutagenicity assay for detection of mutagenic pollutants in the marine environment has recently been developed by using genetically modified *Vibrio harveyi* strains (Czyz et al. 2000).

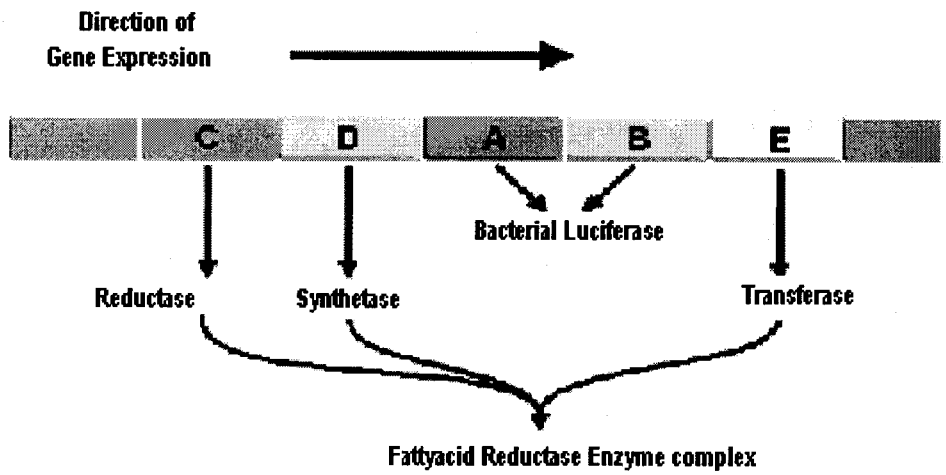
Biochemistry of bacterial bioluminescence

In the bacterial system, aldehydes are essential in the bioluminescence reaction, where the substrate is a long-chain aldehyde (tetradecanal), which is syhthesized from a fatty acid precursor by a fatty acid reductase (Meighen, 1988; 1994; Tu and Mager, 1995). Light emission happens due to the reaction of molecular oxygen with aldehyde and flavinmononucleotide catalyzed by luciferase, to yield the corresponding long chain fatty acid and FMN as shown below.

$$FMNH_2 + RCHO + O_2 \xrightarrow{\text{Luciferase}} FMN + RCOOH + H_2O + \text{Blue green light } (\lambda max = 490 \text{ nm})$$

Bacterial luciferase is the enzyme catalyzing the bioluminescent reaction and it is linked to the respiratory pathway. The luciferase is a heteropolymeric protein with α and β subunits of approximately 40 to 44 kDa and 35 to 40 kDa, respectively, having arisen by gene

duplication (Zeigler and Baldwin 1981; Meighen, 1988). The active site is located primarily on α subunit, but the β subunit is still essential for the light-emitting reaction. Neither α or β subunit alone exhibits luciferase activity, but both the preparations regain activity when combined with the second subunit, indicating that the individual subunits do not possess an active site. The aldehyde binds at or near the interface of the luciferase α and β subunits (Hastings et al. 1985; Baker et al. 1992). In contrast, the firefly luciferase is active as a monomer with a molecular weight of approximately 62 kDa (deWet et al. 1987). It has been proposed that bacterial bioluminescent systems are a branch of the electron transport pathway in which electrons from reduced substrates are shunted to O_2 through two flavin enzymes, flavin mononucleotide reductase and luciferase (Hastings and Nealson 1977). Luciferase may have evolved as a functional terminal oxidase alternative to the cytochrome system (Hastings, 1982), as the growth of cytochrome-deficient bacteria is dependent on luciferase induction and iron. Iron is required for cytochrome synthesis, but represses luciferase synthesis (Hastings et al. 1985). Coupling between respiration and bioluminescence has been indicated by response to the respiratory inhibitors cyanide (Wada et al. 1992) and carbonylcyanide-*m*-chlorophenyl hydrazone (Grogan, 1983).

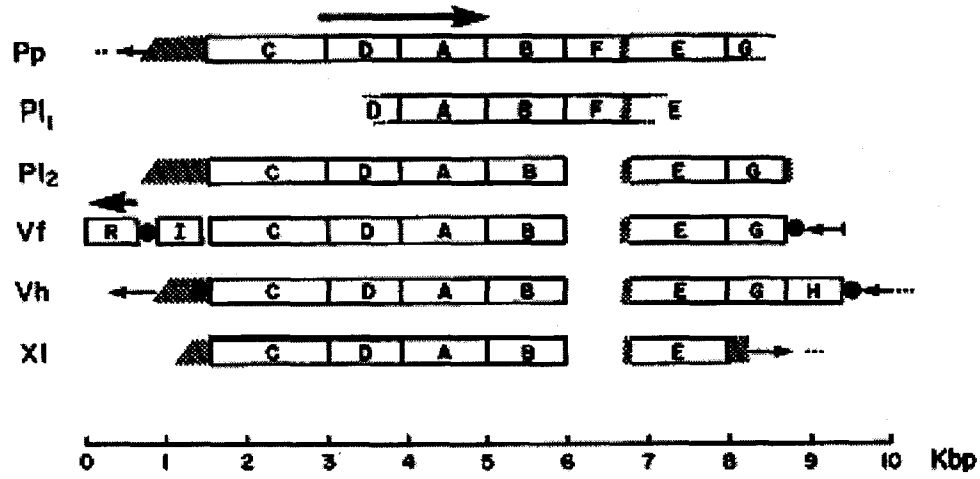


(**Fig.1.1**) The arrangement of the *Lux*CDABE open reading frame (genes in *lux* operon of *Vibrio fischeri*

Molecular Biology of Bioluminescence

Bacterial *lux* genes

Bacterial bioluminescence has been well characterized genetically as well as biochemically (Hastings et al. 1985; Meighen, 1988, 1991 and 1993; Stewart and Williams, 1992; Meighen and Dunlap 1993). Engebrecht et al. (1985) first identified the enzymic and regulatory functions necessary for expression of the bioluminescent phenotype and determined the key aspects of genetic organization (Engebrecht and Silverman, 1984). Genes coding for bacterial luciferase subunits (*lux AB*) and the fatty acid reductase polypeptides (*lux CDE*) responsible for biosynthesis of the aldehyde substrate for the luminescent reaction have been cloned and sequenced from *lux* operons of luminescent bacteria from of least three genera: *Photobacterium*, *Vibrio* and *Photorhabdus* (Fig 1.2). The *lux CDE* genes flank the *lux AB* genes in the different luminescent bacterial species with transcription in the order *lux CDABE*, although an additional gene is located between *lux B* and *lux E* in *Photobacterium phosphoreum* (Meighen, 1991, 1993; Meighen and Dunlap, 1993) (Fig 1.1 & Fig 1.2). A multienzyme fatty acid reductase complex has been characterized from *Photobacterium phosphoreum* (Ferri and Meighen 1991; Soly and Meighen 1991). The structural genes (*lux CDABE*) of *Vibrio harveyi* and *Vibrio fischeri* are highly conserved, indicating that the light emitting systems are very similar in the two bacteria. However, it was also found that the *lux* regulatory systems appear to have diverged. In *Vibrio harveyi*, there was no open reading frame of greater than 40 codons within 600 bp of the start of *luxC*, which is where *lux I* is located in *Vibrio fischeri* (Fig 1.2). The basic mechanism for the induction of luminescence or the location of the regulatory genes in relation to the structural genes differs in the two *Vibrio* systems (Miyamoto et al. 1989). Regulation of light production by *Vibrio fischeri* strains is controlled transcriptionally *via* a mechanism termed autoinduction. The autoinducer accumulates in the growth medium and, as the concentration of the inducer reaches a thresold level contributed by the cell density of 10^7 cells/ml, wherein it acts as a specific inducer for transcription of structural genes, *lux* CDABE.



(**Fig.1.2**) *lux* gene organization for *P. phosphoreum* (Pp), *P. leiognathi* (Pl), *V. fischeri* (Vf), *V. harveyi* (Vh), *and X. luminescens* (Xl). The nucleotide sequences have been determined for all regions represented.

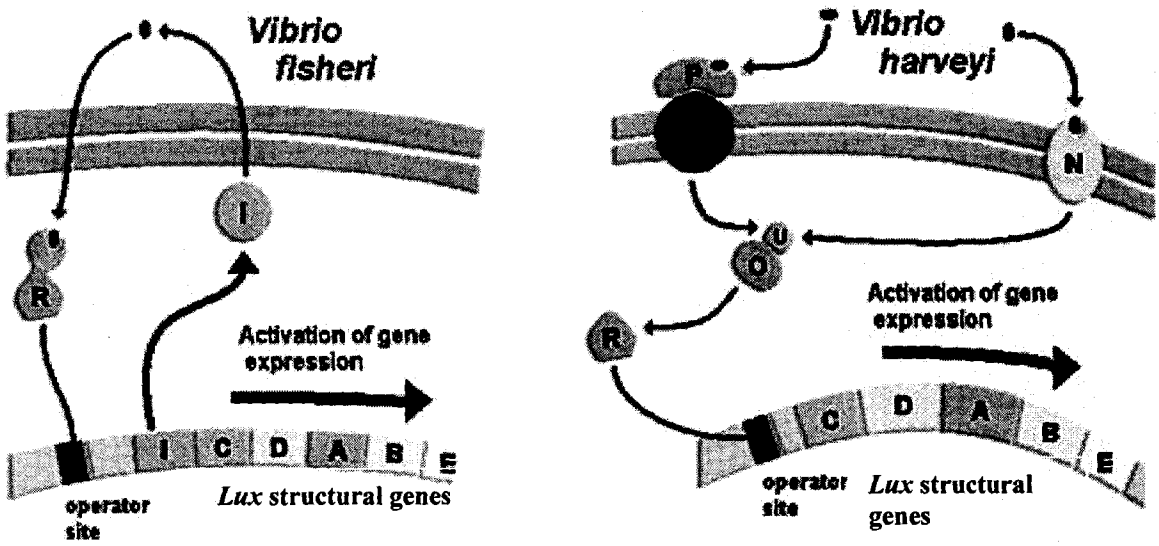
Regulation of *Lux* operon in bioluminescent bacteria

The regulation of luminescence through autoinduction in the *lux* system of *V. fischeri* has been studied in detail (Friedrich and Greenberg, 1983). It is regulated by two genes (*lux R* and *lux I*), which are present in two divergent operons. The *lux I* gene is in the rightward operon together with the *lux CDABE* genes while the *lux R* is in the leftward operon. The *lux I* codes for an autoinducer synthase that is responsible for the production of the autoinducer (AI). The *lux R* gene encodes the LuxR protein, which serves as both a receptor for the autoinducer (AI) and a transcriptional activator of the *lux* operon (Friedrich and Greenberg 1983; deKievit and Iglewski 2000). Binding of the AI to the Lux R protein forms a complex that acts as a transcriptional regulator, activating transcription from the *lux* operon promoter (Stevens and Greenberg 1997). Once induction begins the level of autoinducer (AI) increases rapidly because the gene for AI synthase is part of the *lux* operon. In this way, the autoinducer molecules controls its own synthesis through a positive feedback circuit (Flemming et al. 1994).

Comparison of the Regulation Mechanisms of *V. fischeri* and *V. harveyi*

Although autoinducers and the *luxCDABE* genes (luciferase and fatty acid reductase) are the common structural elements essential for the onset of light emission in most luminous

bacteria, the control mechanism by which the level of the expression of *lux CDABE* is regulated in *Vibrio harveyi* is extremely complex compared to that of *Vibrio fischeri.* (Fig 1.3) In contrast to the *lux I /R* pair of regulatory genes in directing *lux CDABE* expression in *Vibrio fischeri*, a multitude of at least eight regulatory lux genes are involved in signal transduction in controlling the onset of *Vibrio harveyi* luminescence (Fig 1.2). However, the signal transduction in *Vibrio harveyi* from the autoinducer quorum sensor in the extracellular environment to the operon in the cell is functionally homologous to the *luxI /R* of *Vibrio fischeri* in luminescence activation (Miyamoto et al. 1989). The purpose of the partitioning of the integrated function over various regulatory components may be due to the coupling of the luminescence regulation to one or more metabolic pathways and fine-tuning the level of luminescence emission in *Vibrio harveyi* in response to nutritional signals (Miyamoto et al. 1990; Showalter et al.1990; Bassler et al. 1993).



(Fig 1.3). Comparison of the different mechanisms involved in the regulation of expression of the lux structural genes in *V. fischeri* and *V. harveyi*.

Quorum sensing in bioluminescent bacteria

Bioluminescence in bacteria can be regulated through a phenomenon known as a quorum sensing (de Kievit and Iglewski 2000). Autoinduction or quorum sensing was first discovered in *Vibrio fischeri,* which is cell-to-cell communication that ties gene expression to bacterial cell density. Quorum sensing involves the self production of a diffusible pheromone called an autoinducer (AI), which serves as an extra cellular signal molecule

that accumulates in the medium and evokes a characteristic response from cells (Nealson and Hastings 1991; Stevens and Greenberg 1997). In bioluminescence, once the concentration of the autoinducer (AI) reaches a specific threshold at a population density (above 10^7 cell mL^{-1}), it triggers the energetically costly synthesis of luciferase and other enzymes involved in luminescence. Thus, by sensing the level of autoinducer (AI), the cells are able to estimate their density and ensure that the luminescent product will be sufficiently high to cause an impact in the environment (Stevens and Greenberg 1997; Wilson and Hastings, 1998). The autoinducer (AI) for *Vibrio fischeri*, N-acyl homoserine lactone (AHL), was once thought to be species-specific (Hastings et al. 1964). However recent studies have established that AHL can serve as a signaling molecule for more than 16 genera of gram-negative bacteria. This suggests that the AI protein can facilitate interspecies communication (Von Bodman et al.1998) allowing quorum-sensing bacteria to monitor the population of other species as well as their own. Quorum sensing is now known to be a widespread regulatory mechanism in bacteria, particularly among a number of pathogens (Cline and Hastings 1972; Stevens and Greenberg 1997; Bassler, 2002), influencing their ecology and associations with eukaryotic organisms. Once considered exclusive to a few marine vibrios, AHL-mediated quorum sensing has now been demonstrated in diverse Gram-negative genera including *Agrobacterium*, *Aeromonas*, *Burkholderia*, *Chromobacterium*, *Citrobacter*, *Enterobacter*, *Erwinia*, *Hafnia*, *Nitrosomonas*, *Obesumbacterium*, *Pantoea*, *Pseudomonas*, *Rahnella*, *Ralstonia*, *Rhodobacter*, *Rhizobium*, *Serratia* and *Yersinia* (Bassler, 2002).

Sources of heavy metal pollutants and biogeocycling of toxic metals in the marine environment

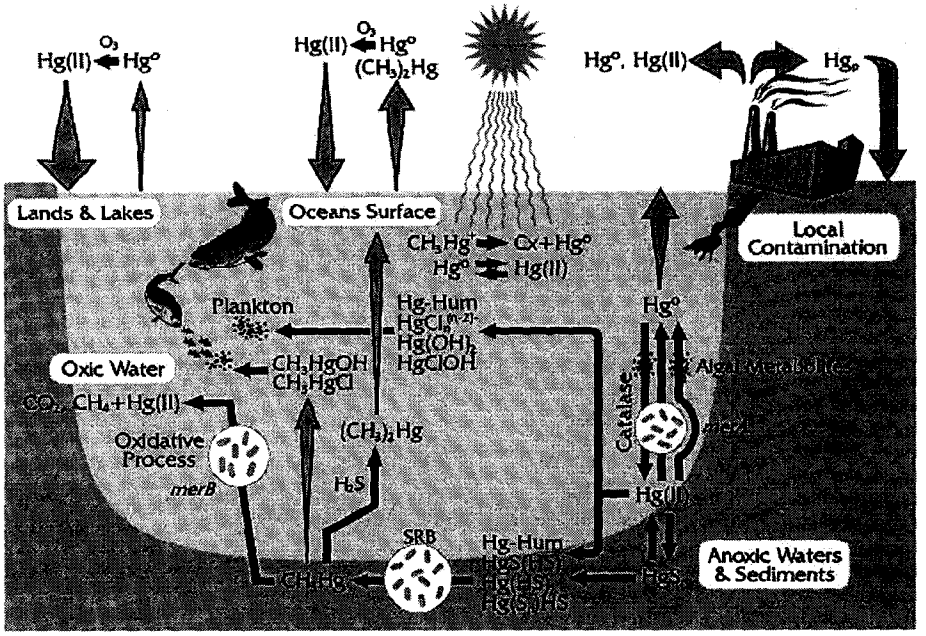
Heavy metals are widespread pollutants of great environmental concern as they are non-degradable and thus persistent. Among the pollutants of serious concern, toxic metals viz. Cd, Hg, As, Pb and Cr are important since they accumulate through the food chain and cause serious environmental hazards (Rani and Mahadevan 1993). Highly toxic heavy metals and organometals are common contaminants of marine and estuarine waters (Forstner and Wittmann 1979). Sources of these substances include industrial and domestic wastewater, atmospheric deposition, erosion, and even direct application, as algicides and antifouling coatings on bottom of ships and hulls (Table 1.1 & Fig 1.4). India has an extensive coastline of nearly 7000 kms, and 75% of river water enters the Bay of

Bengal (East Coast) and 25% to the Arabian Sea (West Coast). About 25% of the 800 million people live in or near coastal areas and are directly or indirectly, dependent on the sea for their living. (Sanzgiry et. al. 1988). The level of mercury in seawater along the west coast of India ranged up to 0.116 µg / lit (Kaladharan et. al.1999). High levels of mercury causes health hazard to humans as well as aquatic life (Naimo, 1995). Such toxic environmental pollutants exert selection pressure for the evolution of metal-resistant organisms (Hada and Sizemore 1981; Malik and Ahmad 1994). These anthropogenic and biogeochemical perturbations are a matter of crucial interest since many heavy metals generated by such activities are potentially toxic for marine and terrestrial life, above certain concentration levels (Nriagu and Pacyna 1988).

About two-third of the total mining activities in Goa are located along the Mandovi and Zuari basin. There are 27 large mines that generate 1500-6000 tons of rejects/day per mine. A substantial portion of which is expected to ultimately end up in the river. Arsenate concentration in the surface water ranged from 0.40 to 0.78 mg/l and from 0.34 to 0.79 mg/l for the bottom waters of the Mandovi estuary. For Zuari estuary, it ranged from 0.45 to 0.79 mg/l at the surface and from 0.42 to 0.78 mg/l at the bottom. Arsenate concentration in the sediments ranged from 9.27 to 9.72 mg/g (dry wt) for sediments of Mandovi; whereas for Zuari it ranged from 7.97 to 9.22 mg/g (dry wt) (Maheswari, 1994). A study by Kaladharan, et al. (1999) indicated that the distribution of mercury in the Arabian Sea had a conspicuous pattern showing very low values ranging from below detection level (BDL) to 0.058 µg/l during the pre-monsoon period, whereas during the post-monsoon Hg ranged from BDL to 0.117 µg/l. Several heavy metal contaminated sites in India have been enlisted in (Table 1.2). Organotins including tributyltin and triphenyltins have been used widely as antifoulants in ship paints, wood preservatives, bactericides, fungicides, molluscicides, and insecticides, and as anthelminthics in poultry feeds and they ultimately reach into aquatic ecosystems, where they can be concentrated up to 10,000-fold in the surface microlayer and up to 4,000 times in oily sediments (Hallas and Cooney 1981; Cooney and Wuertz 1989). The concentrations of monobutyltin (MBT), dibutyltin (DBT) and tributyltin (TBT) in seawater from Tuticorin harbour, Tamilnadu, India varied from 0.64 to 4.97 ng.Sn.g^{-1}, 3.0 to 26.8 ng.Sn.g^{-1} and 0.3 to 30.4 ng.Sn.l^{-1}, respectively. MBT, DBT and TBT in sediments from harbour areas ranged from 1.6 to 393

ng.Sn.g^{-1}, 1.3 to 394 ng.Sn.g^{-1} and 1280 ng.Sn.g^{-1} (dry weight), respectively (Rajendran et al. 2001).

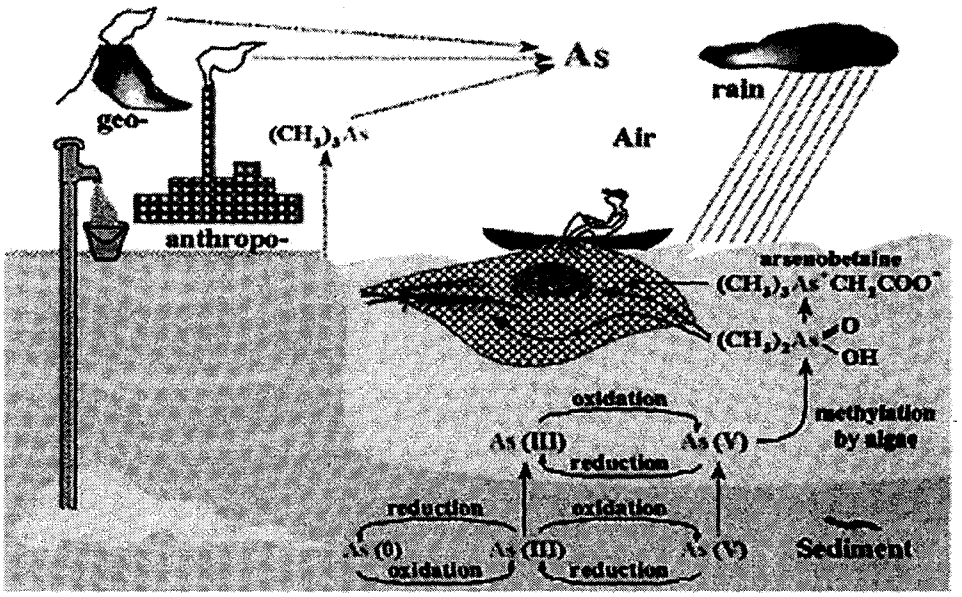
There are several sources of mercury exposure and contamination, such as thermal power plants release, oil combustion release, smelting, chlor-alkali plants, batteries, paints, dental amalgam fillings, household products, fluorescent light bulbs, broken thermometers, and industrial settings (Morel et al. 1998). Mercury is a persistent pollutant and its total annual global input to the atmosphere from all sources including natural, anthropogenic, and oceanic emissions is 5,500 tons (Sanzgiri et al. 1988). Cadmium is also a serious lethal occupational and environmental toxic metal, known for its high toxicity, which may affect living systems in various ways.



(Fig 1.4). The biogeochemical cycle of Mercury in the environment

Cadmium is primarily used in plating iron and steel to prevent corrosion, and manufacturing of nickel-cadmium batteries, plastics, ceramics, paints and various solder and brazing alloys, solar cells, television tubes, lasers, and cadmium telluride devices prepared by semiconductor manufacturers. Anthropogenic point sources contributing to arsenic in the marine environment include smelter slag, coal combustion, runoff from mine

tailings, hide tanning waste, pigment production for paints and dyes, volcanic activity, coal burning, arsenical pesticides and the processing of pressure-treated wood (e.g., copper chromated arsenate) acid mine drainage, organoarsenic compounds and wood preservatives (Fig. 1.5 and Table 1.1) (Jain and Ali 2000; Smedley and Kinniburgh 2002).



(Fig. 1.5) The biogeochemical cycle of Arsenic in the environment

Heavy-metals, Chemical characteristics and Toxicology

Heavy metals have a density of more than 5 g cm^{-3}, and are transition elements with incompletely filled d-orbitals. These 'd' orbitals give heavy metal ions their unique ability to form complexes that are (a) redox active, (b) Lewis acids, (c) or both (Weast, 1984). Heavy metal cations with high atomic masses tend to bind strongly to sulfide groups. Solubility of heavy metal pollutants in seawater is controlled by several factors such as pH, temperature, salinity, nature of different anions etc. The toxic effect of heavy metals in the environment depends on their bioavailability, valency state, organic matter, redox potential and pH of aquatic environment (Nies, 1992).

Mercury is an inorganic compound that can exist in three forms, metallic (Hg^0), mercurous (Hg^{2+}), and mercuric (Hg^{2+}). All three of these oxidation states of the inorganic

mercury are hazardous. Mercury is a toxic element, which binds to sulfhydryl groups of enzymes and proteins and can halt the cell functions within an organism (Clarkson, 1997). Mercury is harmful to many living species. Toxicity of mercury is due to the ability of both organomercurial compounds and inorganic forms of mercury and their high affinity to bind to membranes and tends to be lipid soluble (Mason et al. 1994; Rouch et al. 1995). Epidemics of mercury poisoning following high level of exposure to mercury in Minamata, Japan, and in Iraq demonstrated that neuro-toxicity is the health effect of the greatest concern when mercury exposure occurs. Chronic health effects include central nervous system effects, kidney damage and birth defects; genetic damage is also suspected (Watras et al.1998; Boening, 2000). Whereas Cadmium exposure at the cellular level, leads to protein denaturation, DNA strand breaks, and formation of reactive oxygen species and lipid peroxidation (Pazirandeh et al.1998). Cadmium interacts with thiol groups of proteins and can substitute zinc in certain proteins (Vallee and Ulmer 1972). Consequently, cellular proteins are abnormally denatured, possibly, through weakening of polar bonds and exposure of hydrophobic residues (Wedler, 1987).

Arsenic appears in group V of the periodic table and appears as semi metallic contaminant. It exists in four oxidation states -3, 0, +3, +5. In an aerobic environment, As (V) is dominant. Arsenate (AsO_4^{3-}) and its various protonation states are as follows; H_3AsO_4, H_2AsO_4 -, $HAsO_4^{2-}$ and AsO_4^{3-}. (Nicholas et al. 2003). The metallic gray form is the stable form of arsenic. Arsenic in the environment can be divided into two categories: i) inorganic arsenic and ii) organic arsenic. The more common inorganic arsenic species, arsenate (As V) and arsenite (As III) are more toxic than the many organic species such as monomethylarsonic acid (MMA) and dimethylarsinic acid (DMA). The drinking water standard (as stated by WHO & EPA) for arsenic is 10 $\mu g.L^{-1}$ (Smedley and Kinniburgh 2002). Elevated arsenic concentrations can be toxic to humans, causing adverse health effects such as skin lesions, carcinoma, keratosis and black foot disease (Morton and Dunnette 1994; Lin et al. 1998). The mode of toxicity depends on the chemical form of arsenic. Arsenate, with its structural similarity to phosphate, enters microbial cells readily through phosphate uptake proteins. Its primary mode of toxicity is to displace phosphate in the production of ATP, the primary energy currency of the cell and inhibits oxidative phosphorylation, short-circuiting life's main energy-generation system. The resulting

molecules hydrolyze spontaneously, causing the cell to deplete its energy stores rapidly (Winship, 1984). Arsenite, in contrast, is uncharged at neutral pH and appears to gain access to the cytoplasm by less specific mechanisms, possibly including diffusion across the membrane. Once inside, it crosslinks sulfhydryl groups on enzymes, forming stable adducts that permanently disable the enzyme and proteins. This mechanism is even more destructive to the cell than that of arsenate (Winship, 1984). The best studied arsenic detoxification is the microbial reduction of arsenate to arsenite by the *Ars* operon encoded enzymatic process in which energy is actually consumed to drive the reduction. The *Ars* resistance encoding system is borne on plasmids that are easily transferred among both Gram-positive and Gram-negative bacteria, and it is induced at low concentrations of arsenite and arsenate (Ji and Silver 1995).

The biological effects of organotin ecotoxicants depend mainly on the number and nature of the alkyl and aryl groups bound to tin. The most pronounced effects have been observed in the marine environment because of the intensive use of tributyl and triphenyltin containing antifouling paint compositions for ships, boats and hulls (Alzieu et al. 1989). In the aquatic or terrestrial environment organotins can be toxic to non-target organisms. The ecotoxicological effects of tributyltins results in morphological and reproductive aberrations that include imposex wherein female gastropods (*Nucella lapillus* develop male sex organs and the population cannot reproduce (Gibbs and Bryan 1994), balling (shell weakening) in oysters (Alzieu et al. 1989) and death of mollusk larvae (Horiguchi et al. 1998). In addition it may also act as endocrinal disruptor (Mathiessen and Gibbs 1998). A variety of microorganisms are sensitive to TBT (Cooney and Wuertz 1989). TBT can accumulate in the flesh of shellfish (Horiguchi et al. 1998) and in finfish held on hulls treated with paint containing TBT (Maguire et al. 1986), thus enters the food chain. Several TBT contaminated sites worldwide has been enlisted in (Table 1.2 and Fig 1.6).

(Table 1.1). Most Heavy Metal Polluted sites in India

S. N	Location	Source	Heavy metal	Source
1	Chittagong, Bangladesh	Geochemical and anthropogenic activities	Hg, Cd, As, & Tributyltin	***Down to Earth*, Aug 31, 1999**
2	Vapi, Machua, Lali, Chiri, Gujarat, India	Industrial effluents & Chlor-alkali Plants	Cd, Cr, Hg,	September 2001, **www.indiatogether.com**
3	Kanpur, Uttar Pradesh, India	Industrial effluents & Chlor-alkali Plants:	Cr, Hg, As, Cd & Pb	***Down to Earth*, Aug 31, 1999**
4	Singrauli, India	Thermal power effluents, Smelting	Hg, As, Cd, Cr, and Pb	31st August, 999 ***Down to Earth***
5	Nandesari, Sarangpur, Bapunagar, Ankaleswar, Gujarat, India	dyes, paints, pigments, pharmaceuticals,chemical & pesticides	Cd, Hg, Cr, ,	***Down to Earth*, Aug 31, 1999**
6	Eloor, Kerala, India	dyes, paints, pigments, pharmaceuticals,chemical & pesticides	Hg, Cd, Cr,	14th December, 1997 **The Week**
7	Mormugao, Goa	Mining, shipping, Chlor-alkali Plants, Industrial effluents	As, Cd & Hg	Kaladharan et al.1999
8	Mumbai	Shipping, Chlor-alkali Plants and industrial effluents	As & Cd	**Aug 31, 1999 Down to Earth.**
9	Tuticorin, Kodaikanal, Tamilnadu	Shipping and industrial effluents, thermometers	Cd, Hg and Tributyltins	15th July, 2001 ***Down to Earth***
10	Panipat, Harayana	Chlor-alkali Plants Industrial effluents	Hg, Pb, Cd and Pb	*Down to Earth,* September 15, 2000.
11	Delhi	caustic-chlorine industry	Hg	September, 2002 www.infochageindia.org
12	Patancheru, Andhra Pradesh	Industrial effluents Chlor-alkali Plants	Hg, Cd, Pb, Cr and As	*Down to Earth,* November 30, 1995.

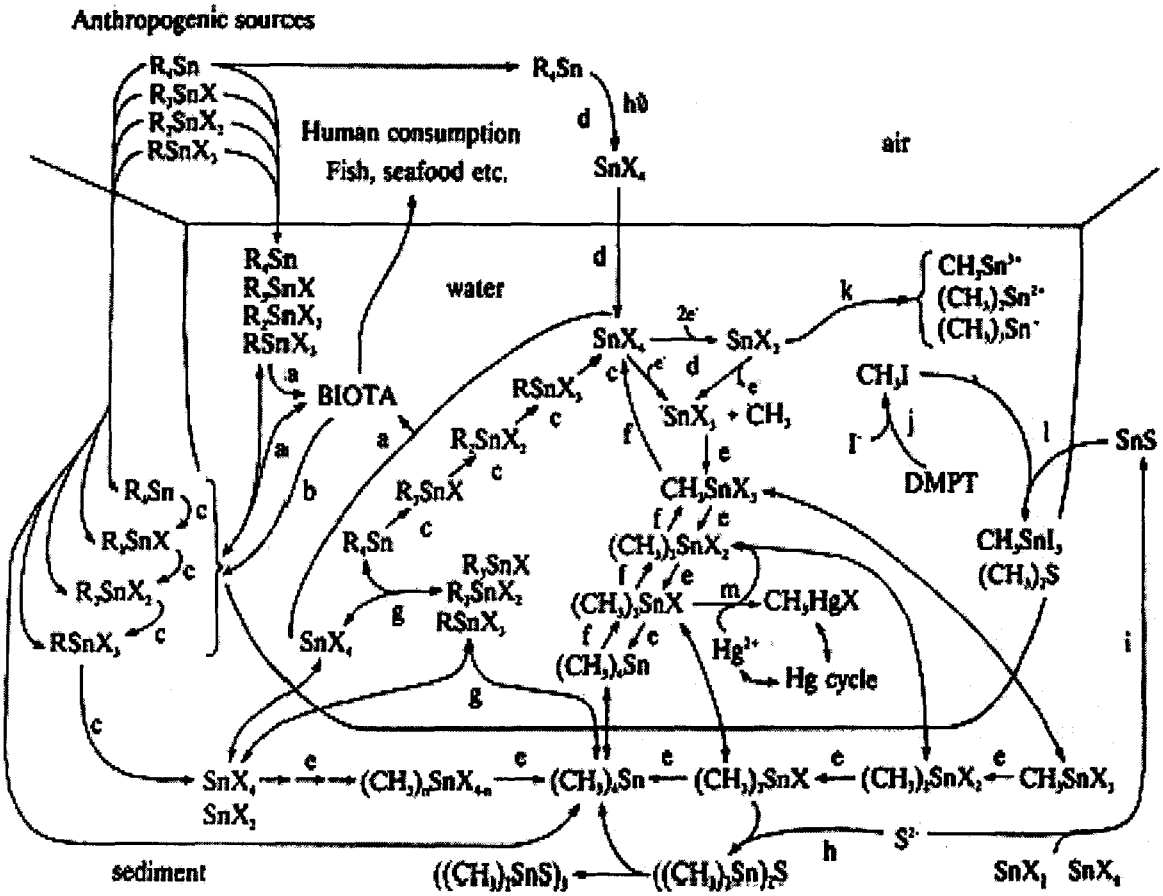
level of mercury(mg/l) in industrial effluents

Permissible limit (industrial effluent)..... 0.001
Industrial Area, Panipat (Haryana)..... ..0.268
Barsai Road, Panipat (haryana)..... .....0.074
Machua Village, Vatva (Gujarat)..... ..... 0.115
Lali Village, Vatva (Gujarat) 0.211
Chiri Village.Vapi (Gujarat)..... ..... 0.096
Sarangpur Village,Ankleshwar (Gujarat)..... .... 0.118
Bapunagar, Ankleshwar (Gujarat)..... ..... 0.176
Pocharam Village,Patancheru (Andhra Pradesh)..... ..... 0.058

Source : *Down to Earth*, Aug 31, 1999

(Table 1.2). Marked Tributyltin effected areas in the world

S.No	Location	Yr of detection	Amount of TBT	Reference
1	Ontario lakes and rivers, Canada	1982	N.D	Maguire et al. 1986
2	Vancouver harbor, Canada	1982-85	11,000 ng/g. dry wt	Maguire et al. 1986
3	Arcachan Bay, France	1982-85	N.D	Alzieu, 1989
4	South West England	1986	N.D	de Mora et al. 1995
5	SanDeigo Bay, USA	1986	N.D	Seligman et al. 1986
6	SanDeigo Bay, USA	1986	0.005 mg/lt	Seligman et al. 1986
7	Pool Harbor, USA	1985-87	520 ng/g dry wt	Langston et al. 1987
8	Atlantic Coastal waters	1988	N.D	Alzieu et al. 1989
9	Boston Harbor, USA	1988	518 ng/g dry wt	Krone et al.1996
10	Mediterranean Sea French, Italy.Turkey, Egypt Coast	1986-91	N.D	Gabriellides et al 1990
11	East Gulf &Pacific Coast of USA	1988-89	770 ng/g dry wt	Krone et al.1996
1	Mariana, Hong Kong	1990	1160 ng/g dry wt	Lau, 1991
13	Auckland, New Zealand	1990	N.D	de Mora et al. 1995
14	Boston Harbor, USA	1990	N.D	Wuertz et al. 1991
15	Funk Bay, Hokkaido, Japan	1991	N.D	Fukagawa et al. 1992
16	Hakodate Bay, Hokkaido, Japan	1991	N.D	Fukagawa et al. 1994
17	Bohemia river, Chesapeake Bay USA	1990-91	590 ng/g dry wt	McGee et al. 1995
18	Sewage and sludge in 5 cities of Canada	1992	N.D	Chau et al. 1997
19	Cadiz in SW-Spain	1990-92	N.D	Gomez Ariza et al. 1999
20	Portland and Boot Bay Harbor, USA	1994	12400 ng/g dry wt	Krone et al.1996
21	Mariana, Hong Kong	1995	3200 ng/g dry wt	Ko et al. 1995
22	Kanpur-Unna Ind.Region. India	1995	32.6 ng/ Sn/lt	Ansari et al.1998
23	Coast of Thailand	1995	4500 ng/g dry wt	Atirekalp et al.1997
24	Suva Peninsula, Fiji	1996	N.D	Davis et al. 1999
25	Strait of Malacca & Tokyo Bay	1993-96	N.D	Hashimoto et al. 1998
26	Killeybegs Harbor, Ireland	1997	N.D	Wuertz et al. 1991
27	Harbors of western Mediterranean Sea	2000	244 ng/g dry wt	Diez et al. 2000
28	Coastal Environment of China	2001	N.D	Gui-bin et al .2001
29	Alang Ship Building, India	2001	N.D	Kanthak et al. 2001
30	Shipping Strait between Denmark and Sweden	2003	19 ng/g dry wt	Strand et al. 2003



(Fig 1.6) A model for the biogeochemical cycling of organotins in the marine environment.

Biochemical basis of metal resistance mechanism in bacteria

Many metals are essential components of the cells at low levels, but they exert toxic effects at high concentrations such as those encountered in polluted environments. In response to toxic concentrations of heavy metals, microorganisms are able to develop tolerance (Kawai et al. 1990). Toxic metals viz. Cd, Hg, As, interact with the essential cellular components through covalent and ionic bonding. At high levels, toxic metals can decrease overall metabolic activity, diversity and population density in microorganims (Roane and Kellogg 1996). Both essential viz. (copper, cobalt, zinc, nickel) and non-essential metals and organo-metals viz. (Cd, Hg, As, Cr, Pb TBT and DBT) can damage cell membrane permeability, alter enzyme specificity, disrupt cellular metabolism and

damage the structural integrity of DNA, inhibiting enzymatic activities (Nies and Silver 1995). The microorganisms have adapted to the metal stress environments by develping a variety of resistance mechanisms such as a) exclusion by permeability barrier b). intracellular sequestration with low molecular weight, cysteine-rich proteins, c) extracellular sequestration by exoplymers d). energy-dependent active transport efflux pumps e). enzyme mediated detoxification f). reduction in the sensitivity of cellular targets to metal ions. g). Precipitation of metals as phosphates, carbonates, and sulfides; h) metal volatilization via methyl or ethyl group addition (Foster, 1983; Beveridge and Doyle 1989; Hughes and Poole 1989; Gadd 1990; 1992; Silver, 1998; Bruins et al. 2000). The bacteria may also carry out transformations of heavy metals by (oxidation, reduction, methylation and demethylation). The mechasnism of mercury and organomercury compounds is mediated by the enzymatic detoxification (mercuric reductase) of the mercurial to volatile compounds. (Silver and Misra 1988). The oxyanions As (V) can readily be differentiated by a chemical reaction: arsenate As (V) is reduced to arsenite As (III), catalyzed by the *ArsC* arsenate reductase. The cofactor for this reaction is glutaredoxin in Gram-negative or thioredoxin in Gram-positive bacteria (Gladysheva et al. 1994; Ji and Silver 1995). However, arsenic and cadmium ions are effluxed from the cells by a specific membrane mediated ATP ases encoded by resistance genes on plasmids. (Cervantes et al.1994).

Microbial metal stress responsive proteins

The microbial stress response is a set of unique biochemical mechanisms that an organism activates or induces under adverse conditions, specifically for the protection of cellular components or the repair of damaged macromolecules. Many of these responses are quite rapid (constitutively expressed, or activated quickly), as would be necessary to prevent lethal effects from highly toxic stressors. Despite the clear importance of these mechanisms to ecological development in complex microbial communities, the environmental microbiology has focused little on this topic.

Microorganisms have evolved several mechanisms that respond to the toxic effects of heavy metals and organometals. One of the common mechanisms is the induction of specific metal binding proteins following the uptake of toxic metals into the cells. A well-studied class of metal binding proteins is referred as metallothioneins (MTs) or low

molecular weight cysteine-rich proteins. A unique property of this class of proteins is their inducibility in response to specific heavy metals such as Cd, Hg, Zn, and Cu (Gadd, 1988; 1992). Metallothioneins have been isolated from diverse organisms including mammals, yeast, algae and fungi. Their presence has been implicated in metal homeostasis and detoxification (Butt and Ecker, 1987; Lupi et al. 1995). The isolation of bacterial cadmium binding proteins from *Pseudomonas putida* and cysteine-rich proteins from *Pseudomonas cepacia* grown in Au (I) thiolate may have a common role in detoxification (Higham et al. 1984). Metallothioneins have been well characterized in cyanobacteria. These small proteins bind cations by means of cysteine thiolate groups and may serve similar functions to the copper binding proteins (CuBP) (Silver and Phung 1996). In addition, studies have shown that many proteins are rapidly generated in pure cultures of *Escherichia coli* (Blom et al.1992) and *Pseudomonas putida* (Lupi et al.1995) after exposure to sub lethal concentrations of toxic organic or inorganic compounds and some unique proteins were found to get induced in response to different classes of chemical stressors (Blom et al.1992). Unique salt stress or osmotic stress-specific proteins were found to be induced in the cyanobacterial strain *Anabaena,* indicating differential regulation of protein synthesis by the two stresses. (Apte and Bhagwat 1989) Exposure to salinity, or osmotic stress resulted in alterations in cyanobacterial protein synthesis (Bhagwat and Apte 1989). Three prominent types of modifications occur under stress conditions: (i) synthesis of several proteins declines, under lethal doses (ii) synthesis of certain specific proteins was selectively enhanced, and (iii) synthesis of a new set of proteins are induced *de novo* (VanBogelen et al. 1987; Jakob, et al. 1993; Lupi et al. 1995; Segal et al. 1996). But there are hardly any reports in heavy metal stress induced metallothionein like proteins of marine bioluminescent bacteria

Exopolysaccharides in marine bacteria and its importance in metal sequestration.

Exopolysaccharides (EPS) are high molecular weight carbohydrate polymers that make up a large component of extracellular polymeric material surrounding microbial cells in the marine environment (Sutherland, 1985; 1998). In the dissolved or particulate form, they comprise a large fraction of the reduced carbon reservoir in the ocean. EPS enhance the survival of marine bacteria by influencing the physico- chemical environment around the cell. Many bacteria also produce extracellular polysaccharides that bind toxic metal ions

and may be involved in detoxifying the microbes immediate environment. For example, extracellular polysaccharides from the various microorganims have been shown to bind a range of metals from solution with relatively strong stability constants (Ford and Mitchell, 1992). Many bacterial polymers have been shown to bind heavy metals with varying degrees of specificity and affinity (Table 1.3). One such approach for heavy metal remediation involves the formation of stable complexes between heavy metals and nucleides with microbial biomass (Kratochvil and Volesky 1998; Volesky and Holan 1995). These complexes are generally the result of electrostatic interactions, binding of the divalent metal cations to bacterial biopolymers generally occurs through interaction with negatively charged functional groups such as 1) uronic acids, teichouronic acids, emulsan, or LPS from various sources 2) Phosphoryl groups associated with the membrane components or 3) Carboxyl groups of aminoacids, or coordination with hydroxyl groups (Beveridge and Murray 1980).

Metal	**Strain**	**Biopolymer**	**Bound metal mg/gm polymer**	**Reference**
Cd^{2+}	*K.aerogenes*	Exopolysaccharides	11	Britton and Freinhofer 1978
	Z.ramigera	Zooglan	1.9	Park et al. 1999.
	Arthrobacter viscosus	Exopolysaccharides	0.9	Scott and Palmer 1988.
	Arthrobacter lwoffi	Apoemulsan	14.1	Scott andPalmer 1988
Hg^{2+}	*E.coli K-12*	EPS, peptidoglycan LPS, protein	12.8	Beveridge and Koval 1981
Pb^{2+}	*E.coli K-12*	EPS, peptidoglycan LPS, protein	31.5	Beveridge and Koval 1981
Zn2+	*E.coli K-12*	Peptidoglycan LPS, protein	25.5	Beveridge and Koval 1981
	B.liqueniformis	g-gelatinyl exopolysaccharides	9.7	Mc Lean et al.1990
Cu2+	*E.coli K-12*	Peptidoglycan,	5.7	Beveridge and Koval 1981
	K.aerogenes	EPS	13.2	Britton and Freinhofer 1978
	P. aeruginosa tye A- B+	LPS	4	Langely and Beveridge 1999
	X. campestris	Xanthan	7.8	Mittleman and Geeesey 1985
	Z. ramigera	Zooglan	32.3	Norberg and Person 1989
	B.liqueniformis	EPS, γ -Glutamyl capsular polymer	56.5	Mc Lean et al. 1990
Ni2+	*B.liqueniformis*	γ-Glutamyl capsular polymer	4.7	Mc Lean et al. 1990
	Bacillus subtilis	Peptidoglycan	37.5	Doyle et al. 1980

(Table 1.3). Heavy metal binding microbial exopolysaccharides

These polymeric molecules participate in EPS/metal interactions by formation of salt bridges with carboxyl groups on acidic polymers, i.e. polysaccharides containing uronic acids, and by forming weak electrostatic bonds with hydroxyl groups on polymers containing neutral carbohydrates. A large number of metals have been reported to cross-link polysaccharides (Geesey and Jang, 1990). The interactions between bacterial EPS and metal ions are well documented by (Ford and Mitchell 1992).

Genetic and Molecular biological basis of metal resistant mechanism in bacteria

It is interesting to mention that, heavy metal and organo-metal resistance encoding genes are located either on plasmids or transposons or chromosomal genome (Rouch et al. 1995; Silver 1998;). Resistance systems have been shown for Ag^{+}, AsO^{2}, AsO_3^{-4}, Cd^{2+}, Co^{2+}, CrO_4^{-2}, Cu^{2-}, Hg^{2+}, Ni^{2+}, Pb^{2+}, TeO_3^{-2}, Tl^{+}, and Zn^{2+} (Silver andPhung, 1996). These systems are primarily energy dependent efflux systems, although a few involve enzymatic transformations. The most prominent example is Hg (II) resistance encoded by *merA* gene of mer operon. Energy dependent efflux systems appear to function either as ATPases (i.e., Cd^{2+}, and Zn^{2+} efflux systems in Gram positive bacteria) or as chemiosmotic ion/proton exchangers (i.e., Cd^{2+}, Zn^{2+}, Co^{2} and Ni^{2+}, in Gram negative bacteria) (Nies and Silver, 1995). Plasmid–encoded systems are mediated by toxic ion efflux mechanism and they can be quickly mobilized to other organism (Silver and Waldenhaug, 1992). Cadmium resistance is encoded by *CadA* and *CadC* genes located on the plasmids. CadA protein has six major domains, which forms a pump to remove Cd (II), (Nies, 1992). Resistance to arsenic can either be governed by an ATPase or by a chemiosmotic transporter (Silver and Waldenhaug, 1992). In some bacteria, resistance to arsenite is conferred by enzymatic oxidation to the less toxic arsenate (Cervantes et al. 1994). The gene products not only detoxify Hg (II) but are also involved in transport and self-regulation (Nies and Silver 1995). The presence of metal ions can up-regulate genes to initiate metal resistance by enzymatic detoxification (Silver and Waldenhaug, 1992).

Genetic studies on TBT resistance from aquatic environments are extremely limited with few reports demonstrating the presence of plasmids (Miller et al. 1995). In most cases it has been demonstrated that the resistance conferring genes are located on chromosomal

genome (Suzuki and Fukagawa 1995). Jude et al. (2004) have demonstrated the involvement of MDR efflux pumps in *Pseudomonas stutzeri* for TBT resistance. Gene for TBT resistance in *Alteromonas* spp. M1 has been successfully cloned which is found to be encoding membrane transport protein (Fukagawa and Suzuki 1993).

Antibiotic Resistance in bacteria and its Correlation with Metal Tolerance

Bacterial resistance to several broad range antibiotics and other antimicrobial agents is a serious problem, which is prevalent. The increased use of antibiotics in health care, disinfectants, sterilants, and heavy metals used in industry as well as in agriculture and animal husbandry, creates a selective pressure in the environment that leads to mutations in microorganisms that will allow them to survive and multiply (Calormiris et al.1984). Bacteria can resist antibiotics as a result of chromosomal mutation or by exchange of genetic materials, which carry resistance genes, through transformation, transduction or conjugation by plasmids. The mechanism of resistance to antimicrobial agents can be due to (Rice and Bonomo 1996) (i) Impermeability of the drug (ii) alteration in target molecules (iii) enzymatic drug modifications (iv) efflux of antibiotics to the cell exterior. Metal resistance is always associated with antibiotic resistance (Belliveau et al. 1987). Metal tolerance and resistance of bacteria have been shown to increase proportionally along industrial contamination gradients. Genes that code for antibiotic resistance traits and genes that code for metal resistance are often carried on the same plasmids or mobile genetic elements or transposons (Roane and Kellogg 1996; Wireman et al. 1997). While a correlation exists between the occurrence of antibiotic resistance and the occurrence of metal tolerance (McArthur and Tuckfield 2000).

Mutagenic assay studies

Mutagenic pollution of the environment is a serious and general problem. It concerns both the artificial urban environment of humans and the natural environment, contaminated with mutagenic pollutants appearing mostly as a result of industrial processes (Goldman and Shields 2003; Jha, 2004). Mutagenic chemicals occurring in various habitats can induce serious diseases, including cancer. The germ line of higher organisms may be also damaged by these compounds, which may lead to fertility problems (Mortelmans and Zeiger 2000). There are thousands of known mutagens and

they have mutagenic effects usually at very low concentrations. The mechanism of action mutagens on the nucleic acids is comparatively quick and more effective as compared to the toxic heavy metals. Acridine orange and ethidium bromide are flouroquinolone drugs, which have a large flat hydrophobic structure that tends to insert between the base pairs of the DNA double helix (i.e. intercalating), which promote additions and subtractions, thus usually cause frameshift mutations. Base analogue viz. 5- Bromouracil (BU) and alkylating agent viz. nitrosogunidine (NTG) usually cause two-way transitions (AT to GC or GC to AT), but alkylating agent can also cause transversions, Base-analogue mutagens are compounds that mimic the structure of true bases and can be incorporated into a DNA strand during replication viz. which is a structural analogue of the pyrimidine base thymine and thus normally base pairs with adenine (Miller, 1992).

Various biological assays for detection of mutagenic agents and a novel mutagenicity assay for detection of mutagenic pollution of the marine environment have been developed by Czyz et al. (2000). Bacterial luminescence systems are used in various mutagenicity and toxicity assays (Bulich and Isenberg 1981, Bar and Ulitzur 1994; Thomulka and Lange, 1995, 1996; Ben-israel et al. 1998). The light emissions of bioluminescent bacteria are easy to quantify and measure with compared to the conventional assay techniques for detection of toxic chemicals (Chatterjee and Meighen 1995). Induction of bacterial luminescence after treatment of cultures with DNA-damaging or DNA synthesis-inhibiting agents or UV-mediated induction was demonstrated previously (Ulitzur, 1989; Weiser et al. 1981; Czyz et al. 2000). Ulitzur, (1989) has demonstrated that, mutagenic agents through their interactions with DNA may cause configuration changes of the double helix resulting in derepression of transcription of the *lux* operon (Ulitzur and Weiser 1981). Repression of the *lux* operons by *LexA* (Ulitzur, 1989, Czyz et al. 2000) indicates that bacterial luminescence can be induced or enhanced under stress conditions that cause DNA damage (Ulitzur and Weiser 1981). There are very few reports on the effect of mutagenic studies of bioluminescent bacteria and it characterization (growth bioluminescence, exopolysaccharides (EPS) and their impact on heavy metals) of wild type to the mutants

AIMS AND OBJECTIVES OF THE PRESENT WORK

Bioluminescent bacteria are the most abundant and widely distributed light emitting microbes in the marine environment of Arabian Sea in the west coast of India (Goa). These microorganisms have been studied extensively with reference to ecology, species diversity, abundance, and distribution in India (Ramaiah and Chandramohan 1993). Whereas their biodiversity, molecular biology and biochemistry with regard to bioluminescence have been studied comprehensively by several biologists from U.K, U.S.A and Europe (Zeigler and Baldwin 1981; Hastings et al. 1985; Dunlap, 1991; Meighen, 1991; Meighen, 1994; Tu and Mager. 1995; Wilson and Hastings 1998).

In spite of the fact that wide ranges of the habitats are occupied by luminous bacteria, very little information concerning their ecology, distribution, occurrence and species composition is available for the Indian Ocean. Earlier reports have documented their occurrence in near shore waters distribution pattern in coastal and oceanic waters and their association with different ecological niches (Ramaiah and Chandramohan 1993)

These ubiquitous microorganisms present in marine and estuarine environment of west coast of India (Goa), serve as a unique bio-reporter of serious alterations in the quality of marine water contaminated by toxic heavy metals (Cd, As, Hg, Sn and Cr) and oragno-metals (TBTC and DBTC), which are released in the marine environment due to extensive anthropogenic and natural geochemical activities. Bioluminescence provides a powerful tool and very fast reporter system to study the impact of toxic environmental pollutants with reference to bacterial adaptation and survival under the stress of metals and organometals. Because bioluminescent bacteria respond rapidly to stimuli (including changes in the inorganic nutrient supply and presence of toxic heavy metals and organometals), it was postulated that the physiological, biochemical and genetic changes of these bacteria in presence of these toxic pollutants might provide a biological system to predict early warning signals of impending ecological perturbations in the aquatic environment specifically marine and estuarine ecosystems.

Marine ecosystems are characterized by complex dynamics and encompass coastal areas from river basins and estuaries to the seaward boundaries of continental shelves. Within these margins, most of the global ocean pollution, overexploitation and coastal habitat alteration occur. The evaluation of response of the native species inhabiting marine ecosystems and their interaction with pollutants is recorded extensively for eubacterial communities mostly in the marine environments of the North and South Americas, Australia, Africa and Europe. There is no information along these lines with regard to microbial communities pertaining to Marine bioluminescent bacteria in the Indian subcontinent.

Majority of the natural bacterial strains possess inherent capability to transform (detoxify) toxic metallic and organo-metallic pollutants of environmental concern into less toxic or nontoxic forms (Ji and Silver 1995; Gadd, 1992; Cervantes et al.1994; Silver, 1996; Nies, 1992; Roane and Kellogg 1996; Bruins et al. 2000). It is interesting to note that they possess genetic determinants (genes) either on chromosomal genome or on plasmids/transposons (Suzuki and Fukagawa 1995; Silver and Phung, 1996; Dubey and Roy, 2003), which confer resistance in these microbial strains against specific toxic inorganic and organo-metallic pollutants of environmental concern. Although biochemistry and molecular biology of bioluminescence mechanism is very well explored in bioluminescent bacteria (Hastings and Nealson, 1985; Meighen, 1991), it is surprising to note that no scientific report is available so far on their metal/organo-metal resistant bioluminescent strains and their characterization.

Several approaches were used to evaluate how this organism responds to metal and organometals pollutants, including: determining the impacts of heavy metals viz. Hg, Cd, As and Sn, organometals viz. TBT and DBT on growth, Exopolymer production, protein profiles, pigments and bioluminescence, determining whether stress proteins were synthesized in response to heavy metal and organo-metal pollution stresses, molecular biological studies and mutagenic studies on luminous bacteria. The three approaches were selected to gauge whether selected pollutants affected the bacteria at a genetic and/or physiological level and indeed, if the pollutants were biologically available to this bacterium (i.e., reached a target site at the surface or intracellular to alter traits). The

literature review therefore covers several areas relevant to this theme, including a brief overview of ecology, biodiversity biochemistry and molecular biology of bacterial bioluminescence, the use of bioluminescence in biological monitoring and biosensor development.

Although there are reports employing Microtox, Mutatox, etc. for detecting environmental toxic pollutants (Kamlet et al.1986; Tescione and Belfort 1993; Selifonova et al. 1993; Arfsten et al. 1994; Virta et al.1995). From the available literature so far, it is clear that there are hardly any attempts to enumerate these bacteria from coastal environments receiving industrial and domestic wastes containing viz Hg, Cd, As, Sn, TBT and DBT. Such data would be practically useful to understand the mechanisms for predominance of these luminous bacteria in such ecologically disturbed coastal environments.

Fascinated with these reports, I have tried to explore the possible biochemical and genetic mechanisms for metal and organo-metal resistance in natural bioluminescent bacterial strains abundantly present in several metals and organo-metal pollutant contaminated marine sites of west coast of India (specifically Goa region). Moreover, the responses of bioluminescent bacteria from polluted environments (marine and estuarine systems) are not well documented. These studies would also help to evaluate whether the impact of heavy metals and organometals on luminous bacteria and their stress responses could be one of the useful indicators of ecological perturbation. The findings will definitely culminate in possible use of these metal and organo-metal resistant natural and genetically modified bioluminescent bacterial strains as bio-reporters (bioindicators) of metal and organo-metal contaminated marine environment.

This thesis was concerned with understanding how natural bioluminescent bacteria responded to heavy metal and organometal pollutants, the context of developing bioindicators based on these stress responses for detecting specific toxic metal and organometals. Bioluminescent bacteria are ubiquitous in aquatic environments of west coast of India (Goa), and these environmental natural isolates were selected to provide a knowledge base for evaluating whether their responses would have utility in predictive tool for pending environmental perturbation. So we have studied the marine bioluminescent

bacteria with reference to biodiversity, heavy metal and organometals impact assessment studies, biloluminescence, protein profiles, exopolysaccharide production, pigment characterization detection and and localization of metal resistant determinants, comparision of induced and wild type with reference to growth (MIC), Exopolysaccharide production (EPS) and Protein profile.

Specific Objectives of the thesis:

1. Ecological studies with reference to biodiversity of bioluminescent bacterial strains from marine and estuarine habitats of West Coast of India (Goa region).

2. Physico-chemical characterization of environmental samples viz. pH, Salinity, Alkalinity, Dissolved oxygen, Organic and Inorganic contents (nitrites, nitrates and phosphates).

3. Physiological and biochemical characterization of selected bioluminescent bacterial isolates with reference to growth behavior, exopolysaccharide (EPS) production, pigment production, protein profile and bioluminescence.

4. Impact assessment studies with reference to growth and bioluminescence of selected bioluminescent bacterial isolates under the stress of metallic pollutants viz. Cd, Hg, As, Sn and organo-metallic pollutants viz. TBTC and DBTC.

5. Molecular biological and genetic characterization of selected bioluminescent bacterial isolates with reference to their resistance mechanism against metallic and organo-metallic environmental pollutants, identification and localization of metal resistance genes.

6. Mutagenesis studies to develop metal and organo-metal tolerant bioluminescent bacteria and their characterization.

References

Alzieu, C., Sanjuan, J. Michel, P., Borel, M. and Dreno, J. P. 1989. Monitoring and assessment of butyltins in Atlantic coastal waters. Mar.Poll. Bulletin. 17:494-498.

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

Applegate, B. M., Kehrmeyer, S. R. and Sayler, G.S. 1998. A chromosomally based *tod-luxCDABE* whole-cell reporter for benzyne, toluene, ethybenzene, and xylene (BTEX) sensing. Appl. Environ. Microbiol. 64: 2730-2735.

Apte, S. K. and Bhagwat, A. A. 1989. Salinity stress induced proteins in two nitrogen-fixing *Anabaena* strains differentially tolerant to salt. J. Bacteriol. 171: 909-915.

Arfsten, D.P., Davenpori, R. and Schaeffer, D.J. 1994. Reversion of bioluminescent bacteria (Mutatox) to their luminescent state upon exposure to organic compounds, munitions and metal salts. Biochem. Environ. Sci. 7: 144-149.

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

Baker, J.M., Griffith, M. W. and Collins-Thompson, D.L. 1992. Bacterial bioluminescence: applications in food microbiology. J. Food Prot. 55: 62-70.

Baldwin, T. O. and Ziegler, M.M. 1992. The biochemistry and molecular biology of bacterial bioluminescence. Chem. Biochem. Flavoenzymes. 3: 467-524.

Bar, R. and Ulitzur, S. 1994. Bacterial toxicity of cyclodextrins: luminous *Escherichia coli* as a model. Appl. Microbiol. Biotechnol. 41: 574-577.

Bassler, B. L., Wright, M. and Silverman, M. R. 1993. Intercellular signalling in *Vibrio harveyi*: sequence and function of genes regulating expression of bioluminescence. Mol Microbiol. 9: 773-786.

Bassler, B.L. 2002. Small talk. Cell-to-cell communication in bacteria. Proc. Natl. Acad. Sci. U.S.A. 99: 3129-3134.

Bhagwat, A. A. and Apte, S. K. 1989. Comparative analysis of proteins induced by heat shock, salinity and osmotic stress in the nitrogen-fixing cyanobacterium *Anabaena* sp. strain L-31. J. Bacteriol. 171:5187-5189.

Bulich, A. A. and Isenberg, D.L. 1981. Use of luminescent bacterial system for the rapid assessment of aquatic toxicity. ISA Trans. 20: 29-33.

Bulich, A.A. 1982. A practical and reliable method for monitoring the toxicity of aquatic samples. Process Biochem. 17: 45-47.

Bulich, A. A. 1986. Bioluminescence assays. p.57-74, In : Bitton, G., and Dutka, G.J. (eds), Toxicity testing using microorganisms, Vol I. CRC Press, Boca Raton, Florida.

Belliveau, B.H., Wong, P.T.S. and Trevors, J.T. 1987. Lead, tin and multiple antibiotic resistance *Pseudomonas* spp. isolated from polluted sediment. Toxicity Assessment 2: 377-386.

Ben-Israel, O., Ben-Israel, H. and Ulitzur, S. 1998. Identification and quantification of toxic chemicals by use of *Escherichia coli* carrying *lux* genes fused to stress promoters. Appl. Environ. Microbiol. 64: 4346-4352.

Beveridge, T.J. and Murray, R.G. 1980. Sites of metal deposition in the cell wall of *Bacillus subtilis*. J. Bacteriol 141: 876-887.

Beveridge, T.J. and Koval, S. F. 1981. Binding of heavy metals to cell envelopes of *Escherichia coli* K-12. Appl. Environ.Microbiol. 42: 325-335.

Beveridge, T.J and Doyle, R.J. 1989. Metal Ions and Bacteria. John Wiley and Sons, Inc, New York.

Blom, A., Harder, W. and Matin. A. 1992. Unique and Overlapping Pollutant Stress Proteins of *Escherichia coli*. Appl. Environ. Microbiol. 58 (1): 331-334.

Boettcher, K.J. and Ruby, E.G. 1990. Depressed light emission by symbiotic *Vibrio fischeri* of the Sepiolid Squid *Euprymna scolopes*. J. Bacteriol. 172: 3701-3706.

Boening, D. W. 2000. Ecological effects, transport and fate of mercury: a general review. Chemosphere. 40: 1335, 2000.

Bourgois, J.J., Sluse, F. E., Baguet, F. and Mallefet, J. 2001. Kinetics of light emission and oxygen consumption by bioluminescent bacteria. Journal of Bioenergetics and Biomembranes. 33: (4), 234-240.

Bitton, G. and Freinhofer, V. 1978. Influence of extracellular polysaccharides on the toxicity of copper and cadmium towards *Klebsiella aerogenes*. Microb. Ecol. 4: 119-125.

Bruins, M. R., Kapil, S. and Oehme, F.W. 2000. Microbial resistance to heavy metals in the environment. Ecotoxicol. Environ. Saf. 45:198-207

Butt, T. R. and Ecker, D J. 1987. Yeast metallothionein and applications in biotechnology. Microbiol Rev. 51(3): 351–364.

Calormiris, J., Armstrong, J. L., and Seidler, R.J. 1984. Association of metal tolerance with multiple antibiotic resistance of bacteria isolated from drinking water. Appl Environ Microbiol 47(6): 1238-1242.

Cervantes, C., Ji, G., Ramirez, J. L. and Silver, S. 1994. Resistance to arsenic compounds in microorganisms. FEMS Microbiol Rev 15:355–367.

Chatterjee, J. and Meighen, E. A. 1995. Biotechnological applications of bacterial bioluminescence (*lux*) genes. Photochem. Photobiol. 62: 641-650.

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

Clarkson, T.W. 1997. The toxicology of mercury. Crit. Rev. Clin. Lab. Sci. 34, 369-403.

Cline, T.W. and Hastings, J.W. 1972. Mutationally altered bacterial luciferase: implications for subunit functions. Biochem. 11: 3359-3370.

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

Czyz, A., Jasiecki, J., Bogdan A., Szpilewska H. and Wegrzyn G. 2000. Genetically modified *Vibrio harveyi* strains as potential bioindicators of mutagenic pollution of marine environments. Appl. Environ. Microbiol 66(2): 599-605.

Dalgaard, P., Manfio, G.P. and Goodfellow, M. 1997. Classification of *Photobacteria* associated with spoilage of fish products by numerical taxonomy and pyrolysis mass spectrometry. Zbl. Bakt. 285: 157-168.

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

de Kievit, T.R. and Iglewski, B. H. 2000. Bacterial quorum sensing in pathogenic relationships. Infec. Immun. 68: 4839-4849.

de Wet, J.R., Wood, K.V., Helinski, D.R. and Subramani, S. 1987. Firefly luciferase gene: structure and expression in mammalian cells. Mol. Cell Biol. 7: 725-737

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

Diaz-Ravinfa, M. and Bath, E. 1996. Development of metal tolerance in soil bacterial communities exposed to experimentally increased metal levels. Appl. Environ. Microbiol 62: 2970-2977.

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

Doyle, R.J., Mathews, T.H. and Streips, U.N. 1980. Chemical basis for selectivity of metal ions by the *Bacillus subtilis* cell wall. J. Bact.143: 471- 480.

Dubey, S.K., Gangolli, S. S. and Holmes, D.S. 1993. Development of biosensors to measure metal ion bioavailability in environmental samples. J. North East. Geol. 15: 88-94.

Dubey, S. K and Roy, U. 2003. Biodegradation of Tributyltins (organotins) by marine bacteria. Appl. Organomet.Chem 17: 1-6.

Dunlap, P.V. 1991. Organization and regulation of bacterial luminescence genes. Photochem Photobiol. 54: 1157-1170.

Engebrecht, J. and Silverman, M. 1984. Identification of genes and gene products necessary for bacterial bioluminescence. Proc. Natl. Acad. Sci. U.S.A. 81: 4154-4158.

Engebrecht, J., Simon, M. and Silverman, M. 1985. Measuring gene expression with light. Science. 227: 1345-1347.

Ferri, S.R. and Meighen, E.A. 1991. A *lux*-specific myristoyl transferase in luminescent bacteria related to eukaryotic serine esterases. J. Biol. Chem. 266: 12852-12857.

Flemming, C. A., Lee, H. and Trevors, J. T. 1994. Bioluminescent most probable- number method to enumerate *lux*-marked *Pseudomonas aeruginosa* UG2Lr in soil. Appl. Environ. Microbiol. 60: 3458-3461.

Ford, T. E. and Mitchell, R. 1992. Microbial transport of toxic metals. pp. 83–101, In: R. Mitchell (ed.) Environmental Microbiology. Wiley, New York.

Forstner, U. and Wittmann, G.T.W. 1979. Metal Pollution in the Aquatic Environment. pp 486. Springer Verlag, Berlin.

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

Friedrich, W.F. and Greenberg, E.P. 1983. Glucose repression of luminescence and luciferase in *Vibrio fischeri.* Arch. Microbiol. 134: 87-91.

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

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

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

Gabreilides, G. P., Alzieu, C., Readman, J. W., Bacci, E., Abouldahab, O. and Salihoglu, I. 1990. MED POL survey of organotins in the Mediterranean Sea. Mar.Poll.Bull. 21:233-332.

Gadd, G. M. 1988. "Special Processes" Biotechnology, Weinheim, R. Ed.: VCH Verlagsgesellschaft. 401-433.

Gadd, G.M. 1990. Heavy metal accumulation by bacteria and other microorganisms. Experientia. 46: 834–840.

Gadd, G.M. 1992. Microbial control of heavy metal pollution. pp 59–88 In: Fry JC, Gadd, G.M, Herbert, R.A, Jones, C.W, Watson-Craik IA (eds) Microbial Control of Pollution. Cambridge Press, Cambridge.

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

Geesey, G.G. and Jang, L. 1990. Extracellular polymers for metal binding. pp. 223–247.In: Ehrlich, H.L., Brierley, C.L. (Eds.). Microb. Miner. Recovery. McGraw-Hill, New York.

Gibbs, P. E. and Bryan, G.W. 1994. Biomonitoring of tributyltin (TBT) pollution using the imposex response of neogastropod molluscs. pp. 205-226 In: *Biomonitoring of Coastal Waters and Estuaries* (J. Kess & M. Kramer, ed.). Boca Raton: CRC Press.

Gladysheva, T. B., Oden, K. L. and Rosen, B. P. 1994. Properties of the arsenate reductase of plasmid R773. Biochemistry 33: 7288-7293.

Goldman, R. and Shields, P.G. 2003. Food mutagens. J. Nutr .133 (Suppl 3): 965–973.

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

Grogan, D.W. 1983. The response of bacterial luminescence to an inhibitor of fatty acid biosynthesis. Arch.Microbiol. 136: 137-139.

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

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

Hada, H. S. and Sizemore, R. K. 1981. Incidence of plasmids in marine *Vibrio* spp. isolated from an oil field in the northwestern Gulf of Mexico. Appl. Environ. Microbiol. 41:199-202.

Hastings, J. W., Gibson, Q.H. and Greenwood, C. 1964. On the molecular mechanism of bioluminescence. The role of long-chain aldehyde. Proc. Nat. Acad. Sci., 52: 1529- 1535.

Hastings, J. W. and Nealson, K.M. 1977. Bacterial bioluminescence. Ann. Rev. Mirobiol. 31: 549-595.

Hastings, J. W. and Nealson, K. H. 1981. The symbiotic luminous bacteria. p. 1332–1345. In: M. P. Starr, H. Stolp, H. G. Trüper, A. Balows, and H. G. Schlegel (ed.), The prokaryotes. Springer-Verlag Berlin.

Hastings, J. W. 1982. Biological diversity, chemical mechanisms, and the evolutionary origins of the bioluminescent system. J. Mol. Evol. 19: 309-321.

Hastings, J. W., Potrikus, C.J., Gupta, S.C., Kurfurst, M., and Makemson, C.J. 1985. Biochemistry and physiology of bioluminescent bacteria. Adv. Microbiol. Physiol. 26: 236-291.

Haygood, M.G. and Allen, S.W. 2000. Luminous Bacteria. In: Journey to Diverse Microbial Worlds: Adaptation to Exotic Environments, Seckbach, J. ed. Kluwer Publishers. pp. 269-285.

Heitzer, A., Webb, O. F. Thonnard, J. E. and Slayler, G. S. 1992. Specific and quantitative assessment of naphthalene and salicylate bioavailability by using a bioluminescent catabolic reporter bacterium. Appl. Environ. Microbiol. 58: 1839-1846.

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

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

Horiguchi, T. Imai, T., Cho, H. S., Shiraishl, H., Shibata, Y., Morita, M. and Shimizu, M. 1998. Acute toxicity of organotin compounds to the larvae of the rock shell, *Thais clavigera*, the disk abalone, *Haliotis discus* and the giant abalone, *Haliotis madaka*. Marine Environ. Research. 46: 469-473.

Hosseini, P.K. and Nealson, K.H. 1995. Symbiotic luminous soil bacteria: unusual regulation for an unusual niche. Photochem. Photobiol. 62:633–40.

Hughes, M.N. and Poole, R.K. 1991. Metal speciation and microbial growth, the hard and soft facts. J. Gen. Microbiol. 137: 725-734.

Ivask, A., Hakkila, K., Virta, M. 2001. Detection of organomercurials with whole- cell bacterial sensors. Anal. Chem. 73 (21): 5168–5171.

Jakob, U., Gaestel, M., Engel, K., and Buchner, J. 1993. Small heat shock proteins are molecular chaperones. J. Biol. Chem. 268:1517-1520.

Jain, C. K. and Ali, I. 2000. Arsenic: occurrence, toxicity and speciation techniques. Water Resources 34:4304-4312.

Jha, A. N. 2004. Genotoxicological studies in aquatic organisms: an overview. Mutat Res 552: 1–17.

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

Jude, F. C., Arpin, C., Brache, C., Capdepuy, M., Caumette, P. and Quentin, C. 2004. TbtABM, a multidrug effluxpump associated with tributyltin resistance in *Pseudomonas stutzeri*. FEMS Microbiol. Lett. 232: 7- 14.

Kamlet, M. J., Doherty, R. M., Velth, G. D., Taft, R. W. and Abraham, M. H. 1986. Solubility properties in polymer and biological media 7. Analysis of toxicant properties that influence inhibition of bioluminescence in *Photobacterium phosphoreum* (The Microtox test). Environ. Sci. Technol. 20: 690-695.

Kaladharan, P., Pillai, V. K., Nandakumar, A. and Krishnakumar, P.K. 1999. Mercury in seawater along the west coast of India. Ind. J. Marine Scien 28: 338–340.

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

Kawai, K., Horitsu, H., Hamada, K. and Watanabe, M. 1990 Induction of cadmium resistance of *Pseudomonas putida*. GAM-1. Agri. Biol. Chem. 54: 1553-1555.

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

Kratochvil, D. and Volesky, B. 1998. Advances in the biosorption of heavy metals Trends Biotechnol 16:291-300.

Krishnakumar, P.K. and Bhat, G.S. 1998. Heavy metal distribution in the biotic and abiotic matrices along Karnataka coast, west coast of India. Ind. J. Marine Scien. 27: 201–205.

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

Lampinen, J., Korpela, M., Saviranta, P., Kroneld, R. and Karp, M. 1990. Use of *Escherichia coli* cloned with genes encoding bacterial luciferase for evaluation of chemical toxicity. Tox. Assess. 5: 337-350.

Langley, S. and Beveridge, T.J. 1999. Effect of O-side chain-lipopolysaccharide chemistry on metal binding. Appl.Environ.Microbiol. 65: 489-498.

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

Lavilla-Pitogo, C.R, Baticados, M. C. L, Cruz-Larcierda, E.R, dela Pena, L.D. 1990. Occurrence of luminous bacterial disease of *Penaeus monodon* larvae in the Philippines. Aquaculture. 91: 1-13.

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

Lin, T.H., Huang, Y. L. and Wang, M. Y. 1998. Arsenic species in drinking water, hair, fingernails and urine of patients with blackfoot disease. J. Toxicol and Environ Health. 53: 85-93.

Lewis, J. C., Feltus, A., Ensor, C. M., Ramanathan, S. and Daunert, S. 1998. Applications of reporter genes. Anal. Chem. 70 (17), 579–585.

Lupi, C.G., T. Colangelo, and C.A. Mason. 1995. Two-dimensional gel electrophoresis analysis of the response of *Pseudomonas putida* KT2442 to 2-chlorophenol. Appl. Environ. Microbiol. 61(8): 2863-2872.

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

Maheswari, N. 1994. Study of arsenic and related parameters along the central west coast of India. Ph.D. Thesis. Goa University, India.

McArthur, J.V. and Tuckfield, R.C. 2000. Spatial patterns in antibiotic resistance among stream bacteria: effects of industrial pollution. Appl.Environ.Microbiol. 66(9): 3722-3726.

Mc Lean, R.J., Beauchemin, D., Claham, C and Beverridge, T. J. 1990. Metal –binding characteristics of the gamma-glutamyl-capsular polymer of *Bacillus lecheniformis* ATCC 9945. Appl. Environ.Microbiol. 56: 3671-3677.

Mc Gee, B.L., Schlekat, C.E., Boward, D.M. and Wade, T. L. 1995. Sediments contamination and biological effects in a Chesapeake Bay marine. Ecotoxicol.4: 39-59.

Malik, A. and Ahmad, M. 1994. Incidence of drug and metal resistance in *E. coli* strains from sewage water and soil. Chem. Environ. Res. 3: 3-11.

Mathissen, P. and Gibbs, P.E. 1998. Critical appraisal of the evidence for tributyltin-mediated endocrine disruption in mollusks. Environ Toxicol and Chemistry. 17:37-43.

Mason, R. P., Fitzgerald, W. F. and Morel, F. M. M. 1994. The biogeochemical cycling of elemental mercury - anthropogenic influences. Geochim. Cosmochim. Acta. 58: 3191.

Meighen, E. A. 1988. Enzymes and genes from the *lux* operons of the bioluminescent bacteria. Annu. Rev. Microbiol. 42:151-176.

Meighen, E. A. 1991. Molecular biology of bacterial bioluminescence. Microbiol. Rev. 55: 123-142.

Meighen, E. A. 1993. Bacterial bioluminescence: organization, regulation, and application of the *lux* genes. FASEB. J. 7:1016-1022.

Meighen, E. A., and Dunlap, P. 1993. Physiological, biochemical, and genetic control of bacterial bioluminescence. Adv. Microbiol. Physiol. 34;1-6

Meighen, E. A. 1994. Genetics of bacterial bioluminescence. Annu. Rev. Genet. 28: 117-139.

Mergeay, M. 1994. Towards an understanding of the genetics of bacterial metal resistance. Trends in Biotechnology. 9: 17-24.

Miller, J. H. 1992. A Short Course in Bacterial Genetics. Alaboratory Manual and Handbook for *Escherichia coli* and Related Bacteria. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NewYork.

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

Miller, M. B. and Bassler, B. L. 2001. Quorum sensing in bacteria. Annu Rev Microbiol. 55: 165-199.

Mittleman, M. W. and G.G. Geesy. 1985. Copper-binding characteristics of exopolymers from a fresh water sediment bacterium. Appl. Environ.Microbiol. 49: 846-851.

Miyamoto, C.M, Boylan, M., Cragg, L., and Meighen, E. A. (1989). Comparison of the *lux* systems in *Vibrio harveyi* and *Vibrio fischeri.* J. Biolum. Chemilum. 3: 193-199.

Miyamoto, C., Meighen, E. A. and Graham. A. F. 1990. Transcriptional regulation of lux genes transferred into *Vibrio harveyi.* J. Bacteriol. 172: 2046-2054.

Morel, F. M. M., Kraepiel, A.M.L. and Amyot, M. 1998. The chemical cycle and bioaccumulation of mercury. Annu. Rev. Ecol. Syst. 29: 543-566.

Mortelmans, K. and Zeiger, E, 2000. The Ames *Salmonella* microsome mutagenicity assay. Mutat. Res 455: 29–60.

Morton, W. E and Dunnette, D. A. 1994. Health effects of environmental arsenic. In: Nriagu JO (Ed) Arsenic in the Environment. Part II: Human Health and Ecosystem Effects (pp 17–34). John Wiley and Sons Inc, New York.

Naimo, T. J. 1995. A review of the effects of heavy metals on fresh water mussels. Ecotoxicology. 4: 341- 362.

Nealson K. H. and Hastings J. W. 1979. Bacterial bioluminescence: its control and ecological significance. Microbiol. Rev. 43: 496–518

Nealson, K. H. and Hastings, J. W. 1991. The luminous bacteria. In: The Prokaryotes, vol 1, edn 2: 625-639. Edited by Balows, A., Trueper, H.G., Dworkin, M., Harder, W., Schleifer, K.H. Springer-Verlag, New York.

Nicholas, D. R., Ramamoorthy, S., Palace, V., Spring, S., Moore, J. N. and Rosenzweig, R. F. 2003. Biogeochemical transformations of arsenic in circumneutral freshwater sediments. Biodegradation 14:123-137.

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

Nies, D. H., and Silver, S. 1995. Ion efflux systems involved in bacterial metal resistances. J. Ind. Microbiol. 14: 186-199.

Norberg, A. B. and Person, H. 1984. Accumulation of Heavy metal ions by *Zooglea ramigera,* Biotechnol. Bioengg. 26: 239-246.

Nriagu, J. O. and Pacyna, J. M. 1988. 'Quantitative assesment of worldwide contamination of air, water and soils by trace metals'. Nature 333: 134–139.

Park, J. K., Jin, Y. B., Chen, J. H. 1999. Resuable biosorbents in capsules from *Zooglea ramigera* cells for cadmium removal. Biotechnol. Bioengg. 63: 116-121.

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

Rajendran, R. B., H. Tao, A., Miyazaki, R. Ramesh and S. Ramachandran. 2001. Determination of butyl-, phenyl-, octyl- and tributylmonomethyltin compounds in a marine environment (Bay of Bengal, India) using gas chromatography-inductively coupled plasma mass spectrometry. J. Environ. Monit. 3: 627–634.

Ramaiah, N. and Chandramohan, D. 1993. Ecological and laboratory studies on the role of luminous bacteria and their luminescence in coastal pollution surveillance. Mar Poll Bull 26:190–201.

Rani, D.B.R. and Mahadevan, A. 1993. Patterns of heavy metal resistance in marine *Pseudomonas* MRI. Ind. J. Experi Biol 31: 682-686.

Ribo, J. M. and Kaiser, K. L. E. 1987. *Photobacterium phosphoreum* toxicity bioassay. I. Test procedures and applications. Tox. Ass. Internat. Quart. 2: 305-323.

Rice, L. B. and Bonomo, R. A. 1998. Genetic and biochemical mechanisms of bacterial resistance to antimicrobial agents. p. 453-501, In: Lorian V, editor. Antibiotics in laboratory medicine. 4th ed: Willims & Wilkins; Baltimore, U.S.A.

Roane, T. M. and Kellogg, S.T. 1996. Characterization of bacterial communities in metal-contaminated soils. Can. J. Microbiol. 42:593–603

Rouch, D.A., Lee, B.T.O., and Morby, A.P. 1995. Understanding cellular responses to toxic agents: a model for mechanism-choice in bacterial metal resistance. J. Indus. Microbiol. 14:132-141.

Ruby, E. G. Greenberg, E. P. and Hastings, J. W. 1980. Planktonic marine luminous bacteria: species distribution in the water column. Appl. Environ. Microbiol. 39: 302–306.

Sanzgiri, S., Mesquita, A. M. and Kureishy, T. W. 1988. Total mercury in water, sediments and organisms along the Indian coast. Mar. Pollut. Bull. 19, 339-343.

Scott, J. H. and Palmer, S. J. 1988. Cadmium biosorption by bacterial exopolysaccharides. Biotechnol.Lett.10: 21-24.

Segal, G., and Ron, E.Z. 1996. Regulation and organization of the *groE* and *dnaK* operons in eubacteria. FEMS Microbiol. Lett. 138:1-10.

Selifonova, O., Burlage, R. and Barkay, T. 1993. Bioluminescent sensor for detection of bioavailable Hg^{2+} in the environment. Appl. Environ. Microbiol. 59: 3083-3090.

Seligman, P. F, Valkirs, A. O. and Lee, R.F. 1986. Degradation of tributyltin in San Diego Bay, California Water. Environ. Sci. Technol. 20.1229-1235.

Showalter, R. E., Martin, M.O. and Silverman, M. R. 1990. Cloning and nucleotide sequence of *lux* R, a regulatory gene controlling bioluminescence in *Vibrio harveyi*. J. Bacteriol. 172: 2946-2954.

Silver, S. and Misra, T.K. 1988. Plasmid-Mediated Heavy Metal Resistances. Annual Review of Microbiology. 42: 717-743.

Silver, S. and Walderhaug, M. 1992. Gene regulation of plasmid- and chromosome-determined inorganic ion transport in bacteria. Microbiol. Rev. 56:195-228.

Silver, S. and Ji, G.G. 1994. Newer systems for bacterial resistances to toxic heavy metals. Environ. Health Perspect. 102: 107-113.

Silver S. and Phung L.T. 1996. Bacterial heavy metal resistance: new surprises. Annu Rev Microbiol 50:753–789

Silver, S. 1998. Genes for all metals—a bacterial view of the periodic table. J. Indust Microbiol. Biotechnol 20:1–12

Singbal, S.Y.S., Sanzgiri, S. and Sengupta, R. 1978. Total mercury concentrations in the Arabian Sea waters o. the Indian coast. Ind. J. Marine Science 7: 124–126.

Soly, R., and Meighen, E. A. 1991. Identification of the acyl transfer site of fatty acyl-protein synthetase from bioluminescent bacteria. J. Mol. Biol. 219: 69-77.

Smedley, P. L. and Kinniburgh, D. G. 2002. The review of source, behavior and distribution of arsenic in natural waters. Appl. Geochemistry 17:517- 568.

Steinberg, S. M. 1995. A review of environmental applications of bioluminescence measurements. Chemosphere: 30: 2155-2197.

Stevens, A. M., Greenberg, E.P. 1997. Quorum sensing in *Vibrio fischeri*: essential elements for activation of the luminescence genes. J. Bacteriol. 179: 557-562.

Stewart G.S., Williams P. 1992. *lux* genes and the applications of bacterial bioluminescence. J. Gen. Microbiol. 138 (7): 1289-300.

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

Sutherland, I. W. 1985. Biosynthesis and composition of Gram negative bacterial extracellular and cell wall polysaccharides. Ann. Rev Microbiol. 39: 243–262.

Sutherland, I. W. 1998. Novel and established applications of microbial polysaccharides. Trends Biotechnol. 16 (1): 41-6.

Suzuki, S. and Fukagawa, T. 1995. Tributyltin resistant bacteria: a summary of recent work. J. Indus. Microbiol. 14.154-158.

Tauriainen, S., Karp, M., Chang, W. and Virta, M. 1998. Luminescent bacterial sensor for cadmium and lead. Biosen. Bioelectronics. 13: 931–938.

Tescione, L. and Belfort, G. 1993. Construction and evaluation of a metal ion biosensor. Biotechnol. Bioeng.42: 945-952.

Thompson, F.L., Iida, T. and Swings, J. 2004. Biodiversity of *Vibrios*. Microbiol. Mol. Biol Reviews: 68: 403-431.

Thomulka, K. W. and Lange, J. H. 1995. Use of the bioluminescent bacterium *Vibrio harveyi* to detect biohazardous chemicals in soil and water extractions with and without acid. Ecotoxicol. Environ. Safety 32: 201-204.

Thomulka, K. W., and Lange, J. H. 1996. A mixture toxicity study employing combinations of tributyltin chloride, dibutyltin dichloride and tin chloride using the marine bacterium *Vibrio harveyi* as the test organism. Ecotoxicol. Environ. Safety 34: 76-84.

Tu, S.C. and Mager, H.I. 1995. Biochemistry of bacterial bioluminescence. Photochem Photobiol. 62(4): 615-24.

Ulitzur, S. and Weiser, I. 1981. Acridine dyes and other DNA-intercalating agents induce the luminescence system of luminous bacteria and their dark variants. Proc. Natl. Acad. Sci. U.S.A. 78: 3338-3342.

Ulitzur, S. 1989. The regulatory control of the bacterial luminescence system – a new view. J. Biolumin. Chemilumin. 4: 317-325.

Vallee, B. L., and Ulmer, D. D. 1972. Biochemnical effects of mercury, cadmium, and lead. Annu. Rev. Biochem. 41:91-128.

Virta, M., Karp, M. and Vuorinen, P. 1994. Nitric oxide donor-mediated killing of bioluminescent *Escherichia coli.* Antimicrob. Agents Chemotherapy 38: 2775– 2779.

Virta, M., Lampinen, J., and Karp, M. 1995. A luminescence-based mercury biosensor. Anal. Chem. 67: 667-669.

Volesky, B. and Holan, Z. R. 1995. Biosorption of heavy metals. Biotechnol Prog 11: 235-250.

Von Bodman, S. B. Majerczak, D. R. and Coplin, D. L. 1998. A negative regulator mediates quorum-sensing control of exopolysaccharide production in *Pantoea stewartii* subsp. *stewartii.* Proc. Natl. Acad. Sci. 95: 7687-7692.

Van Bogelen, R. A., Kelley, P. M., and Neidhardt, F.C. 1987. Differential induction of heat shock, SOS, and oxidation stress regulons and accumulation of nucleotides in *Escherichia coli.* J. Bacteriol. 169: 26-32.

Wada, M., Kogure, K., Ohawada, K., and Simidu, U. 1992. Coupling between the respiratory chain and the luminesnescent System of *Vibrio harveyi,* J. Gen. Microbiol. 138, 1607–1611.

Watras, C. J., Back, R. C., Halvorsen, S., Hudson, R. J. M., Morrison, K. A., and Wente, S. P. 1998. Bioaccumulation of mercury in pelagic freshwater food webs, Sci. Total. Environ. 219: 183.

Weast, R.C. 1984. CRC handbook of chemistry and physics, 64 edn. CRC, Boca Raton, Fla

Wedler, F.C. 1987. Determination of molecular heat stability. In: Henle, K.J. (Eds.), Thermotolerance, Vol. II, Mechanisms of Heat Resistance, CRC Press, Boca Raton, FL, pp: 2-18.

Weiser I., Ulitzur S. and Yannai S. 1981. DNA-damaging agents and DNA-synthesis inhibitors induce luminescence in dark variants of luminous bacteria. Mutation Res. 91: 433-450.

Wilson, T. and Hastings, J. W. 1998. Bioluminescence. Annu. Rev. Cell. Dev. Biol. 14: 197-230.

Winship, K. A. 1984. Toxicity of inorganic arsenic salts. Adv. Drug React. Ac. Pois. Rev. 3:129-160.

Wireman, J., Liebert, C. A., Smith, T and Summers, A.O. 1997. Association of mercury resistance with antibiotic resistance in gram-negative fecal bacteria of primates. Appl. Environ. Microbiol. 63: 4494–4503.

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

Zeigler, M. M. and Baldwin, T.O. 1981. Biochemistry of bacterial bioluminescence. Curr. Top. Bioenerg. 12; 65-112.

Chapter-II

Physico-chemical characterization of environmental water samples, isolation, maintenance, identification, biodiversity studies and environmental optima of the bioluminescent bacterial isolates.

MATERIALS AND METHODS

Sampling sites for collecting environmental samples

Marine and estuarine water samples were collected from different sites of Goa Shipyard Ltd (GSL), Western India Shipping Ltd (WISL) Vasco-Da-Gama, Dona Paula, Miramar, Bambolim, Anjuna, Benaulim, Colva and Majorda from west coast of India. Water samples from surface level and bottom levels were collected from some particular locations like, vicinity of ships and other ship building areas around the Goa Shipyard, estuarine and marine networks. Water samples, from the sampling sites were collected in sterile polycarbonate bottles and transferred immediately to the laboratory and stored at 4°C. All the samples were used within two days of collection for studying physico-chemical, bacteriological and biochemical parameters. The water sample bottles were mechanically shaken on an orbital shaker prior to use and kept for 10 mins to settle down the heavy particles. After 10 min, the upper layer of water was taken for physico-chemical and bacteriological examination.

Physicochemical analysis of water samples

The physicochemical parameters of different water samples such as dissolved oxygen (D.O) nitrites, nitrates, phosphates and salinity were estimated as per the procedure of Grasshoff (1983).

Salinity (NaCl)

This method consists of titrating halide ions in seawater to a standard silver nitrate solution using potassium chromate as an indicator (Grasshoff, 1983). Exactly 10 ml of seawater sample was pipetted into a 250 ml conical flask and about 20 ml of distilled water was added to it, followed by six drops of potassium chromate indicator solution (10 % stock solution) (Appendix- B.1). The content was mixed well and titrated against silver nitrate solution. When silver nitrate solution is run down from a burette a white precipitate begins to form and the solution turns yellow. The first rough colour change appeared when solution becomes dull red by the local excess addition of silver nitrate and then disappears on shaking. From the point onwards silver nitrate solution was added drop

wise every time stirring well and observing the color of the solution. This procedure was repeated till the end point is reached i.e. when the entire solution becomes dull red / dirty orange in color which persists for at least 30 sec by the addition of one drop of silver nitrate solution. The burette reading i.e. the volume of silver nitrate solution is determined and salinity was calculated using Harvey's table.

pH

50 ml of the sample is taken in a beaker and the pH electrode is inserted into the beaker. The values are read within 30 seconds (Grasshoff, 1983).

Alkalinity

Total alkalinity of the sample is the number of milliequivalents of H^+ ions that are neutralized by 1 litre of seawater when excess of acid is added (Grasshoff, 1983). 10 ml of potassium hydroxide is taken in a conical flask; three drops of methyl orange indicator was added. HCl is taken in the burette; the end point is the color change from yellow to pink. 25 ml of standard 0.01 N HCl into a beaker. 10 ml of the seawater is added and contents are mixed thoroughly and total alkalinity is determined in $m.eq.l^{-1}$.

Temperature

50 ml of the water sample is taken into a beaker and a digital thermometer is dipped into the beaker. The values are read within 5 seconds. (Grasshoff, 1983).

Dissolved oxygen (D.O)

The dissolved oxygen is determined by iodometric titration according to the Winkler method. Manganous hydroxide is precipitated in the bottle filled with water sample, by the reaction of manganous chloride with alkaline potassium iodide solution. The dissolved oxygen present in the sample quickly oxidizes the manganous hydroxide to brown manganese hydroxide. (Koroleff, 1983). 100 ml of the water sample is taken in a bottle. The air bubbles should be driven out by knocking at the bottles till it become brimful. Without intermediate stoppering 0.5 ml of Wrinklers A & B reagents are added separately

using pipette. The bottle is shaken vigorously for 2 mins to bring each molecule of dissolved oxygen in contact with manganous hydroxide. After the fixation of oxygen, the precipitate is allowed to settle for 20-30 mins. The titration is carried out immediately against sodium thiosulphate till light yellow color develops, and then 1 ml of starch solution is added and titrated till the blue color disappeared (Appendix B.2).

Nitrite content (NO_2)

Determination of nitrite in seawater is based on the reaction of NO_2 with an aromatic amine leading to the formation of diazonium compound, which couples with a second aromatic amine to form an azo dye (Koroleff, 1983). 10 ml of sample was taken into 50 ml graduated tubes and 10 ml of distilled water was added followed by 1ml of Sulphanilamide and 1ml of N-napthyl ethylene diamine-di- hydrochloride. (Annexure B.3). The volume of the solution was made up to 50 ml with distilled water and the absorbance was measured after 20 mins using Spectrophotometer (SHIMADZU, UV-1601) at the wavelength of 543 nm. The concentration of nitrite is determined using standard curve of sodium nitrite, which was prepared by plotting concentration of NO_2 Vs O.D. at 543 nm.

Nitrate content (NO_3)

The reaction is based on reduction of nitrate to nitrite, which is then determined via the formation of an azodye. The reduction of nitrate is performed in the heterogeneous system using cadmium granules (Koroleff, 1983). 10 ml of sample was taken in 125 ml polypropylene bottle and the volume was made up to 100 ml with distilled water. 2 ml of concentrated ammonium chloride solution (Appendix B.4) was added to the above solution, before passing it through the cadmium column. About 40 ml of the solution was eluted through the cadmium column and concentration of nitrate was determined indirectly from nitrite.

Phosphate content (PO_4)

Phosphate dissolved in water when treated with acidic ammonium molybdate gives phosphomolybdic complex, which can be reduced with ascorbic acid to give phosphomolybdenum blue (Koroleff, 1983). 10 ml of sample was taken in 50 ml graduated

tube and distilled water was added to make the final volume of 50 ml. This solution was treated with 1 ml of mixed reagent (Solutions of Ammonium molbydate solution, H_2SO_4 and Potassium antimonyl tartarate) (Appendix B.5) and mixed thoroughly. 1 ml of ascorbic acid was added to this and shaken well. After 15 mins the absorbance of the solution was measured at 880 nm using spectrophotometer. Standard curve of phosphate was used to estimate the concentration of phosphate in water samples.

Determination of viable counts of luminous bacterial isolates

Optimum levels of As (III), As (V) and Sn (II) were determined previously, which is non-inhibitory for bioluminescence of the bacterial isolates. 0.1 ml of each sample was surface spreaded on to thiosulfate-citrate-bile salts-sucrose (TCBS) agar and Glycerol based marine agar plates separately supplemented with 2 mM As (III), 2 mM As (V) and 5 mM $SnCl_2.2H_2O$. The respective plates were kept for incubation at room temperature for 24 hrs, 48 hrs to check the colony-forming unit (CFU/ml). After respective incubation, bacterial colony count was determined and viable counts/ mL was expressed and tabulated in the (Table 2.2).

Growth behavior of the isolates in minimal and rich media.

The growth behavior of each isolate was determined, using separate Erlenmeyer flasks, containing 200 ml of GBM broth and MSM supplemented with 2 % NaCl and 0.4 % glucose separately and inoculated with 2 % of overnight grown culture. Erlenmeyer flasks were incubated at 28±2 °C with agitation of 160 rpm on an orbital shaker. Cell growth was followed by measuring the absorbance at 600 nm using an Elico Colorimeter, every four hours up to 24 hrs. The bacterial growth behavior was studied in GBM and optimized mineral salts medium (MSM) + 2 % NaCl and 0.4 % glucose separately.

Utilization of carbon sources

Optimum levels of the carbon sources were determined prior to the experiment. The luminous bacterial strain VB23 was grown in optimized mineral salts medium (MSM) broth + 2 % NaCl with different carbon source separately viz. Glucose, cellobiose, galactose, glycerol and sucrose were tested at their optimal concentrations to check the growth

response. Strain VB23 was grown overnight in GBM broth, harvested at 5000 rpm and washed in MSM broth + 2 % NaCl. The washed cells were used to inoculate the test media to a starting Optical density of 0.02 at 600 nm. Samples were taken at every 4 hrs time interval and the absorbance at O.D.600 was measured to check the growth behavior in different carbon sources (Fig 2.16).

Optimization of environmental parameters for growth:

Determination of optimum pH for growth of isolates:

For determination of optimum pH, the growth of five potential isolates VB6, VB9, VB23, BR9 and DN1W was determined by, inoculating 2 % of overnight grown culture in optimized MSM containing 2 % NaCl +0.2 % glucose+ 2 mM As (V). These flasks were separately adjusted to three different pH values i.e. 5.0, 7.4 and 9.0 before inoculation and were incubated on an incubator-shaker for 36 hrs at 28±2 ^{o}C. Growth was determined in terms of absorbance at 600 nm and result was recorded as graphical representation of growth (Absorbance) of Vs pH values.

Determination of optimum temperature for growth of marine isolates

Optimum temperature for the growth of five potential luminous bacterial isolates VB6, VB9, VB23, BR9 and DN1W, was determined by, inoculating 2 % of overnight grown cultures separately in MSM containing 2 % NaCl +0.2 % glucose and incubating on incubator-shaker for 36 hrs at three different temperatures i.e. 28 ^{0}C, 37 ^{0}C and 42 ^{0}C. Growth was determined in terms of absorbance at 600 nm and result was recorded as graphical representation of growth (Absorbance) Vs temperature.

Determination of optimum salt (NaCl) concentration for the growth of marine isolates

Optimum NaCl concentration for the growth of five potential luminous bacterial isolates VB6, VB9, VB23, BR9 and DN1W was determined by 2 % of overnight grown cultures separately in MSM+ 0.2 % glucose with gradually increasing concentration of NaCl (2 %, 3 %, 6 % and 8 %). Flasks were incubated in an incubator-shaker for 36 hrs at 28±2 ^{o}C

Growth of all the five isolates was determined in terms of absorbance at 600 nm and result was recorded through graphical representation of growth (Absorbance) Vs NaCl concentration.

Maintenance of the luminous bacterial strains in heavy metals

The bacterial isolates, which were growing on GBM agar with 2 mM $NaAsO_4$, were sub cultured separately in GBM agar with increasing concentration of $NaAsO_4$. All the bacterial isolates were checked for their growth up to 5 mM concentration of $NaAsO_4$. The predominant isolates, which were growing well on these plates after repeated subculture, were selected for further studies. These heavy metal resistant isolates were then maintained on GBM agar supplemented with 5 mM $NaAsO_4$ at room temperature. The strains were designated as VB6, VB9, VB23 and DN1W for experimental convenience.

Preservation of the luminous bacterial strains

Luminous bacterial cells were suspended in a protective medium of low ionic strength i.e. (1 % NaCl) supplemented with 10 % lactose and 2 % soluble starch and lyophilized (Abrashev et al. 1998). The freeze-dried samples were stored at 4°C and designated as VB01, VB02, VB03, VB04, VB1, VB2, VB3, VB4, VB5, VB6, VB9, VB18, VB22, VB23, VB40, VB50, BR9, DN1W, DN1R, DN2R and DN2W. Periodic viability tests were done to ascertain the viability of the cultures. For long-term storage, cells from GBM agar plates were transferred into GBM broth supplemented with 50 % glycerol as a cryoprotective agent and stored at -20°C for 6 months.

IDENTIFICATION OF LUMINOUS BACTERIAL ISOLATES:

Microscopy

Phase Contrast Microscopy

The cells were examined during the exponential phase in the GBM broth at an O.D.600 nm of approximately 0.4-0.7. The cell preparations were gently pelleted and resuspended in 0.9 % (v/v) filter sterilized saline. The overnight grown bacterial cultures of VB6, VB23, VB9, and BR9 were air dried on clean sterile glass slides. Gram staining was performed

using the standard protocol (Gerhardt et al. 1994) and cell morphology was observed with a standard microscope using phase-contrast illumination and a 100 X oil immersion objective (Olympus BXF40).

Scanning electron microscopy

Sample preparation

The bacterial cultures of VB6, VB23, VB9, BR9, were centrifuged at 5000 rpm for 5 mins and the supernatant is discarded. The pellet was dispersed in 50 mM phosphate buffer pH.7 and smeared on the stubs; smear on the stub was fixed in 2 ml of 2.5 % glutaraldehyde fixative (pH 7.2-7.4) overnight at room temperature. The stubs were placed in phosphate buffer and then in 30% acetone solution and allowed to stand for 30 mins. The acetone dehydration procedure was repeated likewise with 50 %, 70 % and 90 % acetone for 10 mins, each and finally in 100 % acetone for 30 mins. The stubs were then put in critical pressure drying (CPD) device wherein the acetone gets replaced by liquid carbon dioxide at high pressure. This was evaporated by raising the temperature to 45 °C and liquid CO_2 gets converted to gaseous CO_2 and escapes, which takes 1hr. The stubs were placed on the sputter coater (spi-module) specimen holder, after drying. The position of the stage is set such that the specimen is approximately 50 mm from the bottom of sputter head. After sputtering the specimen with 10-15 mm, then film was coated with gold particles. The stubs were placed onto the electron microscope sample chamber of JOEL-5800 LV scanning electron microscope and cells were visualized at 1500 X magnification

Physiological and biochemical characterization of luminous bacteria

Luminous bacterial cultures were grown at 28±2 °C. Cell morphology and Gram staining was determined after 24 hrs incubation and growth on GBM agar. Biochemical, physiological and morphological tests for eight selective metal resistant luminous bacterial strains i.e. VB6, VB9, VB23, BR9, DN1W, VB01 and VB02 were performed according to the *Bergey's Manual of Systemic Bacteriology* (Krieg and Holt, 1984) and Alsina and Blanch's set of keys for biochemical identification of *Vibrios* (Alsina and Blanch 1994; (Gerhardt et al. 1994).

The following physiological and biochemical properties for 9 selective bioluminescent bacterial isolates were examined: oxidation/fermentation of glucose, reduction of nitrate, catalase activity, gelatin liquefaction, arginine dihydrolase, lysine decarboxylase, ornithine decarboxylase, acetoin production (Voges-Proskauer test), sodium requirement (0, 3, 6 and 8 % (w/v) NaCl, indole and H_2S production. Oxidase test was performed using a filter paper moistened with a few drops of Kovac's oxidase reagent and a small amount of bacterial growth was smeared onto the moist filter paper with a spatula or loop. The hydrolysis of starch, lipid, and casein was determined on the plates of basal nutrient agar containing 0.4% starch, 1% Tween 80 and 5 % of skimmed milk powder respectively. Acid production from carbohydrates was determined in tryptone broth supplemented with 0·001 % (w/v) bromocresol purple and one of the following substrates at a concentration of 1 % (w/v); L-arabinose, sucrose, glycerol, D-mannitol, galactose, lactose and glucose. Susceptibility to antibiotics was tested by the conventional diffusion plate technique using Mueller Hinton agar and discs impregnated with antibiotics by using Kirby Bauer's method (Bauer et al. 1966). The commercial miniaturized API20E-system. (Identification of Enterobacteriaceae and other gram negative rods, Biomerieux, France) strips were also used for selective cultures VB6 and VB23 in parallel with conventional biochemical tests.

Chitin agar plate assay for luminous bacteria

Colloidal chitin was prepared by the method of Rodriguez-Kabana (1983) by partial hydrolysis of chitin (Sigma, U.S.A.) with 10 N HCl for 2 hr at room temperature. The colloidal chitin was washed several times with large volumes of distilled water to adjust the pH to 7.0. The culture medium used for the chitinase activity was colloidal chitin The culture medium was composed of 1.0 % swollen chitin, 0.5 % peptone, 0.5 % yeast extract, 0.1 % KH_2PO_4 and 0.01 % $MgSO_4.7H_2O$ (pH 8.0) (Hiraga et al.1997). The medium was sterilized by autoclaving at 121°C for 15 mins and incubated at 30°C for 72 hrs. Overnight grown luminous *Vibrio harveyi* isolates VB6, VB9, VB20, VB23, VB45, VB32, VB50, BR9, DN1W, VB1, VB2, VB01, VB02, and VB03 were streaked on the chitin agar plates. The plates were incubated at 30°C for three consecutive days, congo red (0.01%) was overlaid onto chitin agar plates and incubated fro 30 mins at room temperature. Clearance zones around the colonies were examined to detect chitinase activity of luminous bacterial isolates.

β -lactamase bioassay for luminous bacteria

β –lactamase was assayed by the acidometric method (Cattabiani et al. 1992). Briefly 125 µl of aqueous solution containing 0.2 % of soluble starch (w/v) and 1% benzyl penicillin (w/v) was added to the wells of 96 well microtitre plates. A heavy suspension (50 µl) of luminous bacterial isolates VB6, VB9, VB20, VB23, VB45, VB32, VB50, BR9, DN1W, VB1, VB2, VB01, VB02, and VB03 was prepared and mixed with the solution. The release of penicillinoic acid was determined by adding 125 µl of aqueous solution containing 0.5 % of Iodine (w/v) and 1 % of potassium iodide (KI) (w/v). The reaction was positive when a rapid change (< 3 mins) i.e. decolourization of the starch iodine mixture was observed.

Genomic DNA extraction from luminous bacteria

Bioluminescent bacterial strains were cultured at 28±2 °C, for 16-18 hrs in Glycerol based marine broth. About 1.5 ml of culture suspension was transferred to a micro centrifuge tube and cells were harvested for 2 mins. The supernatant was decanted and drained well onto a Kim wipe. Resuspended the pellet in 467 µl of TE buffer by repeated pipetting. Added 30 µl of 10 % SDS and 3 µl of 20 mg/ml proteinase -K, mixed well and incubated for 1 hr at 37 °C. Equal volume of phenol: chloroform was added and mixed well by inverting the tube until the phases are completely mixed. The sample was harvested at 9000 rpm at 4°C for 10 mins. The upper aqueous phases are transferred to a new microfuge tubes and added an equal volume of phenol: chloroform. The sample was harvested at 9000 rpm at 4°C for 5 mins. The upper aqueous phases were transferred to new tubes. Added $1/10^{th}$ volume of 3 M sodium acetate. 0.6 volumes of isopropanol was also added and mixed gently until the DNA precipitates. The sample was centrifuged at 9000 rpm at 4°C for 10 mins to pellet down the DNA. The pellet DNA was washed with 0.5 ml of 70 % ethanol and resuspended the dried pellet DNA in 100 µl of TE buffer. The concentration of DNA was measured by diluting 10 µl of DNA into 1 ml of TE buffer (1:1000 dilution) and measured absorbance at 260 nm. Concentration of genomic DNA was determined using the following formulae: DNA µg/ml = Abs x 100 x 50 µg/ml (Jones and Bartlet 1990).

Gel electrophoresis of nucleic acids

Agarose gel electrophoresis

Agarose gel electrophoresis of genomic DNA was performed using horizontal slab gels BG-100, 10.5.8 cm apparatus (B. Genei) and 0.8 % agarose dissolved in 1X electrode buffer). Electrophoresis was performed using 1x TAE electrophoresis buffer at 80 V for 90 mins with 0.5 µg/ml ethidium Bromide in the gel. Samples containing approximately 5 µg of genomic DNA or plasmid DNA or restriction enzyme digested genomic DNA or PCR products were mixed with 6X DNA loading buffer. Samples were briefly spun using an Eppendorf centrifuge before being loaded into the gel. Large-scale DNA gels were performed using a BG-200 apparatus (B. Genei), approximately 10x20 cm, capacity 100 ml of agarose gel solution with ethidium bromide) for 4 hours (80 V, 1xTAE electrophoresis buffer). The run was usually stopped when the dye front had run up to $2/3^{rd}$ of the gel. The gel was viewed and photographed using a Gel documentation system (BioRad, U.S.A). Appropriate molecular weight markers were run on gels in parallel with samples were used as DNA size markers for size determination (Sambrook et al. 1989; Ausubel et al. 1992).

Molecular identification of *Vibrio harveyi* isolates

It is accomplished by amplification of 16 s rDNA using species-specific primers VH-1 and VH-2 designed for *Vibrio harveyi* isolates. Oligonucleotides were synthesized by Integrated DNA technologies U.S.A. PCR reactions were performed using a Gene Amp thermal cycler (Applied Biosystems, U.S.A). PCR amplification was performed according to the Expand^{Tm} Long Template PCR system' (Boehringer Mannheim) protocol. The PCR mixtures (25 µl) contained 10 pmol of each appropriate primer, and approximately 200 ng of genomic DNA. The DNA sequence of F' and R' primers are mentioned below (Oakay et al. 2003).

VH-1 F' (5' ACC GAG TTA TCT GAA CCT TC 3')

VH-2 R' (5' GCA GCT ATT AAC TAT ACT ACT 3'),

PCR results in specific amplification of a 413 bps internal fragment of the 16 s rDNA sequence from a number of *Vibrio harveyi* isolates. The reaction mixture (25 µl) contained 5 µl of 10 X DNA polymerase buffer, 3 mM Magnesium chloride, 200 µM each dNTP, 1U per 50 µl *Taq* DNA polymerase, 10 pmol each primer and 50-250 ng template genomic DNA per 50 µl. The thermal cycler was programmed to perform 35 cycles consisting of initial denaturation at 94 °C for 2 mins, 94 °C for 1 min, 65 °C for 1 min, 72 °C for 1 min followed by a final extension step at 72 °C for 2 mins and storage step at 4°C for infinity. The same primers were used to set up a negative control with *Escherichia coli* as template DNA. On gel electrophoresis of 10 µl of PCR product through 0.8% agarose gel and staining with ethidium bromide, amplification of 413 bps amplicons were visualized, in order to confirm the size of the PCR product. 50 bp and 100 bp DNA ladders were used as standard markers, and the agarose gel picture with PCR product was recorded by gel documentation system.

Fatty acid Methyl ester analyses (FAME)

Isolates VB6 and VB23 were grown on tryptic soya agar (TSA) supplemented with 2.5% NaCl at 28 °C for 24 hrs. Whole-cell fatty acids were extracted from cell material according to the MIDI protocol (Sasser, 1990). Fatty acid methyl esters (FAME) were treated with N, O-bis- (trimethylsilyl)-trifluoroacetamide to convert hydroxy acids to their corresponding trimethylsilyl (TMSi) ethers for analysis by gas chromatography (GC) and GC mass spectrometry. Double bond position and geometry of mono-unsaturated fatty acid methyl esters were determined after the formation of dimethyl–disulphide (DMDS) adducts prepared according to methods described previously (Sasser, 1990).

Gas chromatographic analyses were performed on a Sherlock microbial identification system, Newark, U.S.A. fitted with a cross-linked methyl silicone fused capillary column (25 m. 0.2 mm i.d.) and flame ionization detector (FID) and a sampler. Helium was the carrier gas. Samples were injected in split less mode at an oven temperature of 50 °C. After 1 min, the oven temperature was raised to 170 °C at 30 °C min^{-1} then to 270 °C at 2 °C min^{-1} and finally to 300 °C at 5 °C min^{-1}. GC-MS analysis of the FAME was performed using a GCQ Plus GC/MS System (Thermoquest, USA) fitted with on-column injection set at 45 °C. Samples were injected using a sampler into a retention gap attached to an Ultra

2.50 m. 0.32 mm i.d. and 0.17 l m film thickness column using helium as the carrier gas. The chromatograms and mass spectra were applied using internal software. Peaks were identified by comparison with known standards, the library included with the software, and by consideration of the mass spectra. The total number of carbon atoms designate fatty acids: number of double bonds, followed by the position of the double bond from the terminal (x) end of the molecule. The suffixes 'c' and 't' indicate cis and trans geometry.

Biodiversity of luminous bacterial isolates

PCR amplification of Universal 16 s rRNA genes using genomic DNA templates

Genomic DNA was extracted from the luminous bacterial isolates according to the procedure mentioned earlier (Jones and Bartlett 1990). Universal primers (Oligonucleotides) for 16 s rRNA genes were synthesized from Integrated DNA technologies U.S.A. Two PCR primers were used to amplify approximately 1,300 bp of a consensus 16 s rRNA gene primers. PCR reactions were performed using a Gene Amp thermal cycler (Applied Biosystems, U.S.A). PCR amplification of 16 s rDNA was performed according to the Expand^{Tm} Long Template PCR system's (Boehringer Mannheim) protocol. The PCR mixtures (25 µl) contained 10 pmol of each appropriate primer, and approximately 200 ng of genomic DNA. The following universal primers were used for PCR amplification of 16 s rDNA (Marchesi et al. 1998)..

Forward primer 63 f (5'-CAG GCC TAA CAC ATG CAA GTC-3')

Reverse primer 1387 r (5'-GGG CGG TGT GTA CAA GGC-3')

Briefly, 25 µl of PCR master mix was prepared. The reaction mixture contained 5 µl of 10 X DNA polymerase buffer, 3 mM Magnesium chloride, 200 µM each dNTP, 1U per 50 µl *Taq* DNA polymerase, 10 pmols of each primer and 50-250 ng of template genomic DNA per 50 µl. The cycle profile was as follows: initial denaturation at 94 °C for 2 minutes, 94 °C for 1 min, 65 °C for 1 min, and 72 °C for 1 min, followed by a final extension step at 72 °C for 5 mins and storage step at 4°C for infinity. The same primers were used to set up a negative control with *Escherichia coli* as *template*. The PCR products and 100 bp marker (Promega) were visualized by agarose gel electrophoresis (1 % agarose gel using 1X TAE, 80 V for 90 mins).

Restriction Enzyme Digestion of 16 s rDNA amplicon (RFLP analysis)

PCR products 6–8 µl each were taken in separate microfuge tubes followed by adding 2 µl of 10X buffer, 1-2 µl of restriction enzyme *Hha* I (MBI Fermentas), and deionized sterile distilled water to bring the final reaction volume to 20 µl. The digestion contents were gently spinned for a few seconds in a microfuge and incubated at 37 °C for 360 min according to the manufacturer's instructions. The restricted DNA's were analyzed by horizontal electrophoresis in 1 % agarose gel. Electrophoresis was carried out at 100 V for 45 mins with a mini-gel electrophoresis apparatus (BioRad, U.S.A.) in TAE electrophoresis buffer. After electrophoresis, the gel was stained in ethidium bromide solution for 10 mins. 100 bps molecular weight DNA ladder was used as standard marker and the agarose gel picture with PCR digested products were recorded by using gel documentation system.

Results and Discussion

Details of sampling sites

In the present study 10 sampling sites were selected for sampling of environmental water samples, out of which six sites were from Mandovi and Zuari estuarine network systems viz. WISL, GSL, Dona Paula Bay, Miramar beaches 1, 2 and Bambolim beach. Four sampling sites were selected from marine habitats viz. Anjuna, Benaulim, Colva and Majorda beaches. The geographical locations of the sampling sites ranged from (latitudes 15° 35' 061' N to 15° 27' 703' N) and longitudes (73° 44' 213' E to 73° 49' 985' E) (Fig 2.1, Table 2.1). The coastal regions of Goa are affected by extensive mining operations, shipping activities, industrial effluent waste disposals and various other natural biogeochemical and anthropogenic activities. Goa shipyard (GSL) and Western India shipping limited (WISL) are among the biggest shipyards in the west coast of India, which substantially contribute several metal and organo-metal pollutants in the surrounding vicinities of the marine environment. The environment pollutants generated may include both the heavy metals viz. (Hg, Cr, As, Pb, Zn, Sn, Fe, Mn etc) and organometals viz. (TBT and DBT). These contaminants often share critical properties such as toxicity, high-level persistence.

Physico-chemical characteristics of environmental samples

The environmental samples were collected during the months of June-October, 2002. The estuarine network (Mandovi and Zuari) receives a large influx of fresh water from the rainfall, this in turn causes changes in the salinity, alkalinity, temperature, dissolved oxygen and inorganic nutrients. The distribution of nutrient elements in the marine and estuarine waters is controlled by constant circulation, mixing and other physical process, along with biological, sedimentological and chemical processes (Aston, 1980). The biogeocycling of inorganic nutrients in the estuaries plays a dominant role than in the marine waters. Likewise the levels of dissolved oxygen, inorganic nutrients (phosphates, nitrites and nitrates) increases in estuaries. A correlation exists between dissolved oxygen and salinity indicating that an increase in the salinity decreases the dissolved oxygen, as the solubility of dissolved oxygen is relatively more in the fresh waters with compared to the saline waters (Vaz, 2005). Oxygen plays a key role in bacterial bioluminescence and oxidizes both $FMNH_2$ and a long-chain aliphatic aldehyde (Makemson, 1986). The energy liberated by this process is not used for H+ gradient building, osmotic work, or ATP synthesis, but directly dissipated in the form of light (Bourgois et al. 2001).

During the present study, the levels of physico-chemical parameters in estuarine waters (GSL, WISL, Miramar, Dona Paula bay and Bambolim) and marine waters (Anjuna, Benaulim, Colva, and Majorda) showed the variation in temperature (27-29 °C); pH (7.5-8.05); salinity (24-28% in Estuaries and 33-34 % in marine waters); Alkalinity (2.14- 2.28 meq. L^{-1} in estuaries and 2.08-2.17 meq. L^{-1} in marine waters); Dissolved oxygen (2.88-3.58 ml. L^{-1} in estuaries and 2.40- 2.88 ml. L^{-1} in marine waters); Phosphates (0.88 -1.24 mol. dm^{-3} in estuaries and 0.45-0.64 mol.dm^{-3} in marine); nitrates (3.62-4.04 µmol.dm^{-3} in estuaries and 2.64-3.05 µmol.dm^{-3} in marine waters); nitrites (0.40-0.58 µmol.dm^{-3} in estuaries and 0.18-0.28 µmol.dm^{-3} in marine waters) respectively. The highest densities of luminous bacterial isolates were detected in estuarine water samples (Mandovi and Zuari rivers) with compared to the marine waters along the west coast of Goa (Table 2.1).

The ambient inorganic nutrient concentrations in the estuarine network (Mandovi, Zuari) and marine waters during the monsoon was reported Vaz (2005). The values ranged as salinity (17-20 % in Estuaries and 32-34 % in marine waters); Alkalinity (2.1 - 2.18 meq.

L^{-1} in estuaries and 2.17- 2.26 meq. L^{-1} in marine waters); Dissolved oxygen (1.45 - 3.0 ml. L^{-1} in estuaries and 2.62- 3.9 ml. L^{-1} in marine waters); Phosphates (0.4- 0.6 $mol.dm^{-3}$ in estuaries and 0.6-1.24 $mol.dm^{-3}$ in marine); nitrates (2.5-3.0 $\mu mol.dm^{-3}$ in estuaries and 3.5–5.0 $\mu mol.dm^{-3}$ in marine waters); nitrites (0.2-0.4 $\mu mol.dm^{-3}$ in estuaries and 0.4 -1.02 $\mu mol.dm^{-3}$ in marine waters) respectively. With reference to the above physico-chemical parameters of marine samples the nitrites, nitrates and phosphate contents in the Indian Ocean were found to be 0.7 µg/L, 100-280 µg/L and 3.1-31 µg/L respectively (Lobban et al.1985). Salinity of surface waters in Arabian Sea varies between 34-37 % (Quasim and Sengupta 1981; Subramanyam and Sambamurthy, 2000).

Determination of viable counts of luminous bacteria

The total luminous bacterial counts of water samples from estuarine network of Mandovi and Zuari rivers (GSL, WISL, Miramar, Dona Paula bay and Bambolim) ranged between a range of $1.9x10^2$ to $3.3x10^2$ Cfu/ml, when plated on GBM agar plates and $1.6x10^2$ – $2.3x10^2$ Cfu/ml on TCBS agar plates. Whereas in marine waters (Anjuna, Benaulim, Colva, and Majorda), the luminous bacterial counts ranged between a range of $1.6x10^2$ to $1.8x10^2$ Cfu/ml on GBM agar plates and $1.1.6x10^2$ to $2.3x10^2$ Cfu/ml on TCBS agar plates (Table). The total luminous counts were also determined on GBM agar plates supplemented with sub lethal concentrations of selective heavy metals. The luminous counts in estuarine network ranged between a range of $1.26x10^2$ to $2.14x10^2$ Cfu/ml, and in marine waters ranged between $1.6x10^2$ to $1.7x10^2$ Cfu/ml, when plated on GBM agar plates supplemented with 5 mM NaAs (III); Viable luminous counts on GBM agar plates supplemented with 5 mM NaAs (V) ranged between a range $1.26x10^2$ to $2.04x10^2$ Cfu/ml in the estuarine waters and $1.26x10^2$ to $1.61x10^2$ Cfu/ml in the marine waters; The luminous counts on GBM agar plate supplemented with 5 mM $SnCl_2$ ranged between a range of $1.31x10^2$ to $1.61x10^2$ Cfu/ml in the estuarine network and $1.316x10^2$ to $1.61x10^2$ Cfu/ml in the marine waters respectively (Table 2.2).

Environmental factors seem to control the population dynamics of free-living luminous marine bacteria and also account for the geographic distribution of different types of luminescent bacterial strains due to their seasonal fluctuations. Our results indicate that the distribution pattern of luminous bacteria in the west coast of India (Goa) is presumably

affected by at least four major factors viz. water temperature, dissolved oxygen, inorganic nutrient concentrations, and salinity. The adaptability to these environmental parameters governs their distribution. When such parameters are favorable the nutrient loading may stimulate the abundance of luminous bacteria in the transects of estuarine networks as compared to the marine waters (Grimes et al. 1986). The relative constancy of the population density in the estuarine water body of the west coast is mainly due to the stability of the temperature (21 to 28 °C) and dissolved oxygen (1.45-3.0 $ml.1^{-1}$) throughout the water profile. Among all the examined sampling sites, the densities of luminous bacterial isolates were apparently higher at Bambolim and Miramar followed by Dona Paula. The highest luminous population densities were present in the estuarine regions, which are markedly higher in nutrient concentrations, primary production and chlorophyll *'a'* content than the marine waters (Quasim and Sengupta 1981). Since the highest densities of luminescent *Vibrios* were detected in water samples from Mandovi and Zuari estuarine network (Table 2.2), which also gave the highest counts of total viable bacteria, since water samples from these two sites showed the highest levels of dissolved oxygen, NO_3^{2-} and $P0_4^{2-}$. The abundance of luminous *vibrios* in waters from these two sample sites may be attributed been due to the combined effect of these environmental factors (Table 2.1).

Optimization of growth media for luminous bacteria

The luminous *Vibrio harveyi* VB23 isolate when grown in presence of modified MSM broth i.e. (MSM + 2 % NaCl+ 0.2 % glucose, the growth was relatively quiet slow as compared to cells grown in rich media, glycerol based marine broth (GBM). After 24 hrs of growth cycle, the isolate VB23 reached an optical density of 2.1 when grown in GBM, whereas the O.D is 1.46 in the modified MSM broth (Fig 2.15). For selection of the best carbon source, the cells were grown in MSM broth + 2 % NaCl, supplemented with optimal concentrations of different carbon sources such as glucose, cellobiose, galactose, glycerol and sucrose. The medium when supplemented with 3 % glycerol enhanced the growth of the test organism. Likewise the following concentrations of glucose (0.4 %), cellobiose (0.5 %), galactose (0.5 %) and sucrose (0.5 %) showed enhanced growth, when supplemented. Cellobiose showed relatively less stimulation of growth as compared to

rest of carbon sources, which in modified MSM broth indicates low utilization of cellobiose by luminescent bacterium *Vibrio harveyi* VB23 (Fig 2.16).

Optimization of environmental parameters for growth

Optimum temperature

The five selected isolates were grown in MSM + 2 % NaCl+ 0.2 % glucose at three different temperatures viz. viz. 28 °C, 37 °C and 42 °C (Fig. 2.18), in order to check the optimal temperature for growth of the isolates. The apparent seasonal variation in the occurrence of luminous bacterial isolates suggests that relatively warm temperatures favor growth of the organisms. Growth behavior of each isolate was determined separately by checking the absorbance at 600 nm after 24 hrs incubation. In all five isolates growth was observed at all three temperatures, whereas the luminescence was observed only at 28 °C but not at 37 °C and 42 °C (Fig 2.18). The isolates VB6 and VB23 showed relatively higher growth as compared to isolates VB9, BR9 and DN1W after 24 hrs incubation. Much slower growth was observed at 25°C, and no growth occurred below 20°C, over the 12 hr period of observation. However, after 48 hrs of incubation, slight growth was noted at 20°C. The isolates were unable to grow above 42°C. Changes in the colony morphology was observed when the plates were incubated at 42°C temperature.

Optimum pH

The five selected isolates were grown in MSM + 2 % NaCl+ 0.2 % Glucose at four different pH viz. 5.5, 8.0, 8.5 and 9.0 in order to check the optimal pH for growth of the luminous bacterial isolates (Fig. 2.17). Growth behavior of each isolate was determined separately by checking the absorbance at 600 nm after 24 hrs of incubation. In all five isolates growth was observed at all pH ranges, whereas the luminescence was observed only at pH. 8.5, but not at 9.25 and 9.5. The isolates VB6 and VB23 showed considerably higher growth as compared to other isolates VB9, BR9 and DN1W after 24 hr incubation. Maximum growth was obtained at pH 8.5, but the isolates also grew well at pH. 9.25 and 9.5. Relatively much slower growth was observed below pH. 5.5 and above 9.5 and no growth occurred at pH of 5.0 and pH. 10.

Optimum salinity

The five selected isolates were grown in MSM + 0.2 % glucose at four different NaCl concentrations viz. 2%, 3%, 6 % and 8%, in order to check the optimal salinity for growth of the isolates (Fig. 2.19). Growth behavior of each isolate was determined separately by checking the absorbance at 600 nm after 24 hrs of incubation. In all five isolates growth was observed at all four NaCl concentrations, whereas the luminescence was observed at 2 %, 3% and 6 % but not at 8 %. The growth was optimal in media containing 2.0 to 3.0 % NaCl, and no growth occurred in media containing < 0.1 % NaCl or > 6.0 % NaCl. Whereas the isolates VB6 and VB23 showed relatively high growth as compared to other 3 isolates. The present results indicated that moderate salinity (2-3 %) had a favorable influence on the occurrence of luminous bacterial isolates. Sites of low salinity (estuaries) often resulted in abundance of luminescent bacterial isolates, but sites of relatively high salinity (marine) were seldom positive for luminous bacteria. Na^+ ions are known to be indispensable for the growth and luminescence of marine luminous bacteria. The functional role of Na+ in bioluminescence was suggested to be due to creation of the required ionic strength and maintenance of the osmotic pressure (Dunlap, 1985; Waters and Lloyd 1985). Luminescent marine *Vibrios,* such as *V. fischeri* and *V. harveyi*, also possessed a primary Na^+ pumps (Na^+-NQR) and their role in luminescence seemed to link to the sodium motive force bioenergetically (Waters and Lloyd 1985; Wada and Kogure 2000).

Screening, isolation, maintenance and preservation of luminous bacterial isolates

The luminous bacterial isolates which were screened initially on GBM agar and MSM agar supplemented with 2 mM As (III) and 5 mM As (V) were repeatedly sub cultured separately on MSM agar + 2 % NaCl + 0.2 % glucose and also supplemented with increasing concentrations of As (V) up to 7 mM and 0.25 mM TBT and 0.5 mM DBT respectively. The predominant luminous bacterial isolates, which were resistant to both heavy metals and organometals, were selected for impact assessment, exopolysaccharide production (EPS), protein profiles, pigment studies and molecular biological and mutagenic studies. The selected isolates (VB6, VB9, VB23, BR9, DN1W and VB01 were periodically maintained on MSM with 2 mM As (V) and incubated at ambient temperature of 28±2 °C. Fifteen of the selected luminous bacterial isolates were

lyophilized according to standard protocol provided by Abrashev et al. (1998) and preserved in a protective medium of low ionic strength. Most of the luminous bacterial isolates could not grow at high concentrations of heavy metals and organo-metals due to the cellular toxicity and inhibitory effect on metabolic process and viability of bacterial strains (Thomulka and Lange 1995, 1995) Periodic viability tests were performed every six months by adding the fresh GBM broth and revived the luminous cultures.

Morphological, Physiological and Biochemical characterization

Scanning electron microscopy (S.E.M.) and phase contrast microscopic examination of the luminous bacterial strains revealed that these bacteria possess long curved rod-shaped cells (Fig 2.2 and Fig 2.3). The luminous bacterial strains were selectively grown in TCBS agar, VH agar and enriched in GBM agar plates (Appendix A.1, A.2, A.3). All strains of luminous bacteria isolated in the present study were gram-negative bacilli, facultative anaerobes, grew well at 28 °C on a GBM and minerals salts media containing 2 % NaCl and 3 % glycerol. However, the growth of these strains on this medium was inhibited at temperatures above 35 °C and below 4 °C, pH above 9.2 and below 6.0 respectively. Luminous bacterial isolates were capable of growth up to 6% sodium chloride (NaCl). It failed to grow on thiosulphate citrate bile salt sucrose agar and MSM agar and medium without 1.5 % NaCl. These isolates were positive for luminescence, amylase, gelatinase, catalase, indole production and nitrate reduction, and negative for urease activity and citrate utilization (Fig 2.5). Carbohydrates such as arabinose, rhamnose, inositol, gluconate, glucouronate, actetate, pyruvate, xylose, sorbitol, and sucrose were not utilized. Among non-sugar carbohydrate substrates, glycerate and ethanol produced significant growth and succinate and hexadecane produced slight growth, whereas malate, mannitol, sorbitol and gluconate were not utilized. Gram-negative, halophilic rods that ferment glucose were identified as *Vibrios* as described by Simidu and Tsukamoto (1980). ONPG, Lysine decarboxylase, and Ornithine decarboxylase positive and Voges-Proskauer, lactose, inositol, arabinose, and tryptophan deaminase negative strains were classified as luminous *Vibrio harveyi* isolates (Alsina and Blanch 1994).

A total of 43 luminous bacterial isolates were screened on GBM and TCBS agar plates supplemented with selective heavy metals such as As (III), As (V) and Sn (II) heavy metals. Out of these only seven isolates were selected for the morphological, physiological and biochemical identification studies, 43 isolates for molecular identification and biodiversity studies and 2 isolates (VB6 and VB23) for identification by API 20E strips (Biomeriux, France) employing 1.7 % sterile saline as an inoculum diluent and Fatty acid methyl ester (FAME) identification analysis. The similarity index of the luminous bacterial isolates VB6 and VB23 for FAME identification were compared with the internal database library TSBA404.10 (FAME) of 0.519 similarities matching to *Vibrio fumisii* of 50 % similarity (Sasser, 1990) (Fig 2.13 and Fig 2.14). Fatty acids methyl ester (FAME) profiling was evaluated by the earlier researchers for the differentiation of *Vibrionaceae* species (Lambert et al. 1983; Bertone et al.1996). FAME profiling is generally very useful as a chemotaxonomic marker. The biochemical characteristics of luminous strains VB6, VB9, VB23, BR9, DN1W, VB01 and VB02 were identified and compared to literature of luminous bacterial reference strains according to Bergey's Manual of Systematic Bacteriology (Baumann and Schubert 1984; Krieg and Holt.1984) and Alsina and Blanch's set of keys for biochemical identification of *Vibrio* sp (Alsina and Blanch 1994) (Table 1). There have been many studies on physiological and biochemical features of the growth and luminescence of luminous bacteria *P. leiognathi* and *P. phosphoreum* (Baumann and Baumann 1981; Baumann and Schubert 1984; Ramaiah and Chandramohan 1993), whereas very few reports are available on free-living *Vibrio harveyi* (Table 2.3)

Chitinase and β- lactamase producing luminous bacteria

Luminous bacterial isolates from various sampling sites of marine and estuarine sources were grown on the selective medium containing colloidal chitin. Forty-three luminous bacteria were tested for the detection of chitinase and β- lactamase activities, out of which only 18 isolates were able to show a clearance zone around the colony after 72 hrs of incubation. 70 % of the chitinase positive luminous bacterial isolates were screened from Mandovi and Zuari estuaries. The previous report states that chitinase is also produced by many non-luminous *Vibrio harveyi* and most frequently found in all the vibrionaceae members along with *Alteromonas* spp. and *Pseudomonas* spp. (Coleman et al. 1996). All these three species of marine bacteria are the major chitin degraders in the estuarine and

marine ecosystem (Cottrell et al. 2000). Most of the marine *Vibrios* spp. are able to break down chitin, a homopolymer of *N*-acetyl-D- glucosamine, which is one of the largest pools of amino sugars in the oceans (Cottrell et al. 2000; Riemann and Azam 2002). *Vibrio harveyi*, for instance, excretes at least ten different chitin-degrading enzymes (Svitil et al.1997). Chitinase genes have been detected in *Vibrio harveyi* isolates by using *chiA* gene probes (Wood et al. 2000; Cottrell et al. 2000; Ramaiah et al. 2000).

β-lactamase production was determined by acidometric method (Cattabiani et al. 1992). Almost all the luminous bacterial isolates were able to cleave the β-lactam ring by producing penicillinoic acid. Out of the 43 strains tested, more that 95 % of luminous *Vibrio harveyi* isolates with high MIC values for β-lactam antibiotics antibiotics (e.g. ampicillins and cephalosporins), showed the evidence for production of β -lactamase. Resistance to β-lactams is often the result of β-lactamases that inactivate the antibiotics (Devar and Dermody 1991). This indicates that the high MIC values against the two β-lactam antibiotics may directly relate to the production of β-lactamases. β-lactamase production was also detected in most of the Vibrionaceae family and *Pseudomonas* (Corkill et al.1991; Silva et al.2000). Several workers have reported the production of β-lactamase by bacteria with high resistance to β-lactam antibiotics (Nandivada and Amyes, 1990; McArthur and Tuckfield 2000; Rahman Khan and Malik, 2001). Recently two novel ß-lactamase genes (*bla*VHW-1) and (*bla*VHH-1), from two *Vibrio harveyi* isolates W3B and HB3 have been cloned and their nucleotide sequences were determined by Jeanette et al. (2000).

Species specific identification of *Vibrio harveyi* isolates by PCR amplification

Genomic DNA from the luminous bacterial isolates was prepared according to standard protocol of Jones and Bartlet (1990) (Fig 2.6). The species-specific PCR primers VH-1 and VH-2 used in the current study corresponded to variable regions of *Escherichia coli* 16 s ribosomal gene sequence (Gene bank accession J01859) at bases 59-87 and 453-473. It was consequently found that these primers corresponded to *Vibrios* as described by Dorsch et al. (1992). The PCR amplified amplicon of VH-1 and VH-2 primers is 413 bp from all the isolates of luminous *Vibrio harveyi* (Fig 2.7). This PCR method has been found to reduce the time and resource required for confirmation of identity of *Vibrio*

harveyi up to species level. The identification of *Vibrio* spp. isolated from the aquatic environment has been imprecise and labour-intensive, requiring many biochemical and physiological tests (Devar and Dermody 1991) or dichotomous key that takes weeks to perform (Alsina and Blanch 1994). Several highly powerful molecular tools, e.g. amplified fragment length polymorphism (AFLP) (Rademaker et al. 2000; Gurtler and Mayall 2001) and repetitive extragenic palindromic elements polymerase chain reaction (REPPCR) (Versalovic et al. 1991), have become readily available for the identification of bacteria, including vibrios (Thompson et al. 2001). With our PCR based technique, an isolate suspected to be luminous *Vibrio harveyi*, could be confirmed in less than 24 hrs, and positive PCR cultures would be further confirmed by growth characteristics, Voges Proskeur test, and use of mannose, L-leucine, acetate or propionate as a sole carbon source. But this PCR based taxonomic identification of *Vibrio harveyi* could easily initiate antibacterial therapy and thus prevent the rapid spread of specific bacterium *Vibrio harveyi* in the estuarine and fresh water aquaculture.

Biodiversity of luminous bacterial isolates

16S rRNA gene amplification RFLP analysis

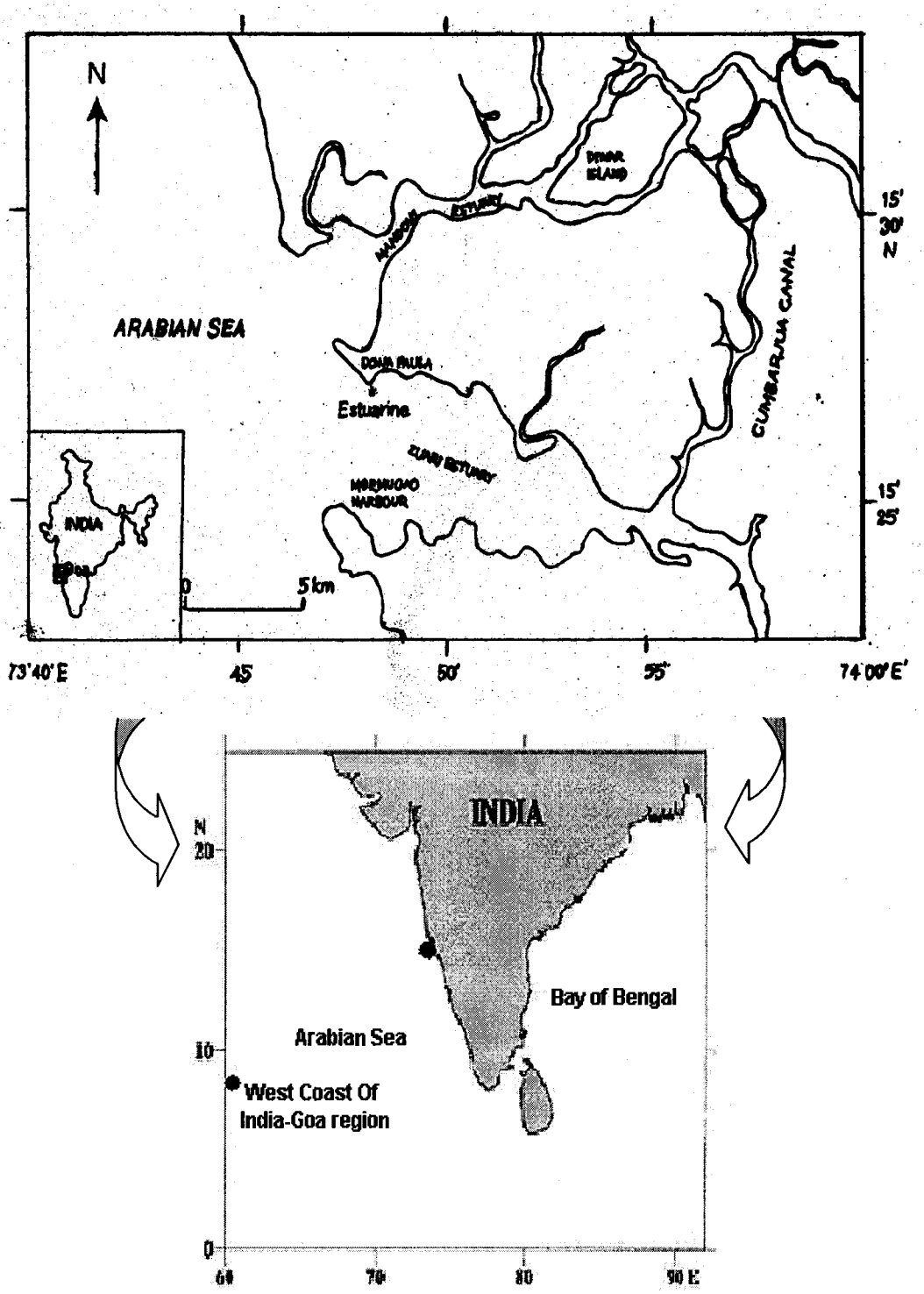
The luminous bacteria are highly abundant in aquatic environments, including estuaries, marine coastal waters, sediments, and aquaculture settings worldwide (Ortigosa et al. 1994; Urakawa et al. 2000). Several cultivation-dependent and independent studies have showed that luminous *Vibrios* appear at particularly high densities in and/or on marine organisms, e.g., corals, fish, molluscs, seagrass, sponges, shrimp and zooplankton (Gomez-Gil et al. 1998; Gurtler and Mayall 2001; Vandenberghe et al. 2003). The luminous *Vibrios* (Vibrionaceae strains) belong to the Gammaproteobacteria, are gram negative, usually motile rods, are mesophilic and chemoorganotrophic, have a facultative fermentative metabolism, and are found mostly in aquatic habitats. They are generally able to grow on marine agar and on the selective medium thiosulfate-citratebile salt-sucrose agar (TCBS) and are mostly oxidase positive. Whereas few species of *Photobacterium* do not grow on the TCBS agar.

Genomic DNA was isolated from all 46 strains of luminous bacterial strains by using the method of Jones and Bartlet (1990) (Fig 2.8). The analysis of 16 s rRNA genes was aided

by using PCR to amplify target sequences in the strains of *Vibrio harveyi.* Two PCR primers viz. forward primer 63f (5'-CAG GCC TAA CAC ATG CAA GTC-3') and reverse primer 1387r (5'-GGG CGG TGT GTA CAA GGC-3') (Marchesi et al. 1998) were used to amplify approximately 1,300 bp of a consensus 16 s rRNA gene (Fig 2.9). All the strains produced 16 s ribosomal DNA amplicon of 1300 bps. This size corresponded to the predicted size of the 16 s rRNA genes from the primer pair used in this study.

RFLP analysis of 16 s ribosomal DNA amplicons was carried out for the 36 natural isolates using restriction endonuclease *Hha* I. Four 16 s rDNA genotypes were obtained by digestion with the restriction endonuclease (*Hha* I) viz. *Vibrio harveyi, Vibrio fischeri, Vibrio splendidus* and *Photobacterium leiognathi* respectively. The RFLP analysis showed different banding patterns with the environmental isolates, corresponding to four genotypes grouping all of them into the genus *Vibrio* and *Photobacterium* (Garcia, 1997) For the preliminary identification of the 15 environmental luminous bacterial strains, RFLP of 16 s rDNA genes was used. All *Photobacterium* spp. produced identical fragment pattern by digestion with *Hha* I and were clearly distinguished from other *Vibrio* spp. *Vibrio harveyi* strains produced five fragments, whereas three clear fragments were observed for the strains of the genus *Photobacterium* (Fig's. 2.10, 2.11 and 2.12). Fragment sizes obtained in this study were in accordance with previously predicted restriction fragment sizes by Urakawa et al. (1997). It is difficult to distinguish *Vibrio* from other facultatively anaerobic fermentative marine rods by phenotypic characteristics. However, PCR–RFLP analysis shows as a rapid tool to distinguish the genus *Vibrio* from other genera such as *Photobacterium*, and some species of *Shewanella* (Urakawa et al. 1998). The use of 16 s rRNA in the classification of bacterial species has been well established, and its impact on biology has been profound. Certain regions of rRNA have been extremely highly conserved during evolution, and sequence homology studies in rRNAs are currently widely used to indicate evolutionary relationships between organisms (Woese et al. 1990). The general approach of determinative and environmental studies has been targeted at discrete regions of the rRNA's genes. Since the rRNA gene sequences are conserved, the targeting of regions where greater or lesser conservation occuring offers exquisite control for bacterial classification purposes.

The west coast of India (Goa) is presumably affected by at least four major environmental factors viz. water temperature, nutrient concentration, pH and salinity. The adaptability of the different luminescent bacteria to these environmental parameters govern their distribution and diversity in different econiches of Goa. In the Mediterranean Sea, seasonal temperature fluctuations seem to select for thermal and taxonomic types of bacteria (Grimes et al.1986). However, the luminescence of these strains on GBM medium was inhibited at temperatures above 30 °C. Scanning electron microscopy (SEM) examination of the two strains viz. VB9 and DN1W revealed that these bacteria were long curved rods. These strains differed from one another in the efficiency of utilization of D-mannitol, sucrose, gas production from glucose, swarming on solid media, growth at pH above 9.0, and growth at 8 % NaCl concentration and different temperature maximas. The differentiating biochemical characteristics of luminous bacterial strains are given in (Table 2.4, Fig 2.4) One of these strains isolated from Bambolim was attributed to typical representatives of *Vibrio splendidus*, and the other isolate from WISL, Goa was identified as *Photobacterium leiognathi,* whereas *Vibrio harveyi* were determined as predominant species among the luminous bacterial strains isolated from all sampling sites of west coast of India (Goa).



(Fig 2.1) Sampling Map for collection of bioluminescent bacterial strains from marine and estuarine habitats of Goa.

(Table 2.1) Physico-chemical characteristics of environmental samples from various (marine and estuarine) habitats of Goa region.

Sl.N	Sampling sites	Depth	pH	Salinity ‰	Alkalinity ($meq.l^{-1}$)	Temperature (°C)	Dissolved O_2 ($ml.l^{-1}$)	Nitrites $\mu mol.dm^{-3}$	Nitrates $\mu mol.dm^{-3}$	Phosphates $\mu mol.dm^{-3}$
1	Goa Shipyard Limited (GSL)	B	7.83	28.03	2.28	28.0	2.88	0.58	3.84	1.24
2	Western India shipping (WISL)	B	7.92	28.07	2.24	27.5	2.94	0.62	4.04	1.20
3	Dona Paula Bay	S	8.22	25.0	2.18	28.5	3.46	0.50	3.62	0.94
4	Miramar –1	S	7.97	26.02	2.20	28.0	3.52	0.58	3.98	0.98
5	Miramar-2	S	8.04	24.06	2.14	28.0	3.58	0.64	4.16	1.14
6	Bambolim	S	7.86	25.10	2.28	27.6	3.12	0.40	3.76	0.88
7	Anjuna	S	7.88	33.08	2.10	28.6	2.40	0.28	2.64	0.45
8	Benaulim	S	7.86	34.03	2.01	28.8	2.88	0.32	2.92	0.52
9	Colva	S	7.98	34.06	2.14	28.4	2.56	0.18	3.05	0.64
10	Majorda	S	7.92	34.08	2.17	28.0	2.67	0.26	3.02	0.56

(Table 2.2). Total viable counts of selected bacterial isolates from marine and estuarine water samples collected from various habitats of Goa

Sampling site	Sampling Date	Geographical positions		Luminescence counts (cfuX10^2 cells/ml)± SE				
		Latitude	Longitude	GBMA counts	TCBS counts	GBMA + 5 mM AsO_2	GBMA + 5 mM AsO_4	GBMA + 5 mM $SnCl_2$
GSL	15/9/2002	15°27' 703'N	73°49' 985'E	1.9±0.08	1.76±.14	1.26±0.04	1.26±0.04	1.32±0.04
WISL	15/9/2002	15°27 628'N	73°49' 842'E	1.9±0.13	1.8±0.19	1.31±0.05	1.32±0.04	1.31±0.05
Dona Paula Bay	24/9/2002	15°27' 15.7'N	73°48' 15.2'E	2.7±0.45	1.6±0.13	1.51±0.06	2.04±0.28	1.6±0.17
Miramar-1	27/9/2002	15°27' 64.8'N	73°48' 12.5'E	3.06±0.43	1.9±0.24	1.74±0.23	1.9±0.37	1.8±0.28
Miramar-2	28/9/2002	15°27' 68.12'N	73°48' 18.6'E	3.2±0.43	2.3±0.46	2.14±0.28	1.7±0.04	1.7±0.04
Bambolim	29/9/2002	15°27' 42.8'N	73°54' 37.2'E	3.3±0.46	2.1±0.34	1.7±0.37	1.8±0.28	1.61±0.18
Anjuna	4/10/2002	15°35' 061'N	73°442' 13'E	1.8±0.28	1.7±0.31	1.6±0.17	1.26±0.04	1.31±0.05
Benaulim	10/10/2002	15°14' 663'N	73°55' 351'E	1.61±0.18	1.7±0.37	1.26±0.04	1.31±0.05	1.36±0.04
Colva	14/10/2002	15°16' 499'N	73°54' 815'E	1.71±0.08	1.7±0.43	1.32±0.04	1.6±0.17	1.26±0.04
Majorda	14/10/2002	15°15' 524'N	73°55" 650'E	1.6±0.17	1.6±0.49	1.32±0.04	1.61±0.18	1.61±0.18

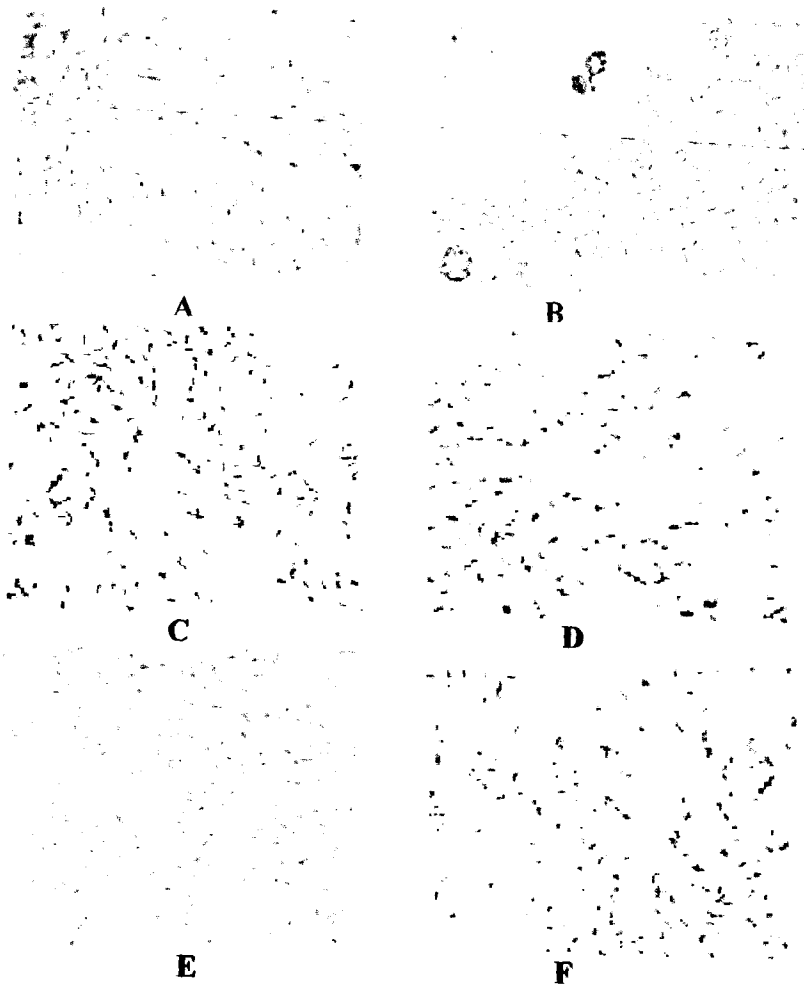
(Table 2.3). Biochemical Characteristics of selected Luminous bacterial isolates

Biochemical tests	VB6	**VB23**	**VB9**	**VB1**	**BL9**	**DNIW**	**VB01**
Gram Character	gram–ve	gram–ve	gram–ve	gram–ve	gram–ve	gram–ve	gram–ve
Production of H_2S	-	-	-	-	-	-	-
Indole	+	+	+	+	+	+	+
Acetoin (VP test)	-	-	-	-	-	-	-
Methyl Red	-	-	-	-	-	-	-
Presence of							
ß-Galactosidase	+	+	+	+	+	+	+
Arginine dihydrolase	-	-	-	-	-	-	-
Lysine decarboxylase	+	+	+	+	+	+	+
Ornithine decarboxylase	+	+	+	+	+	+	-
Tryptophan deaminase	-	-	-	-	-	-	-
Gelatinase	+	+	-	-	+	+	+
Cytochrome oxidase	+	+	+	+	+	+	+
Catalase	+	+	+	+	-	+	+
Urease	+	+	-	-	+	+	+
Amylase/Starch	+	+	-	-	+	+	+
Lipase/Tween 80	+	+	+	+	+	+	+
Chitinase	+	+	+	+	+	+	+
Nitrate reductase	+	+	+	+	+	+	+
L-Asparginase	+	+	+	+	+	+	+
Utilization of sugars							
Glucose	+	+	+	+	+	+	+
Inositol	-	-	-	-	-	-	-
Arabinose	-	-	-	-	-	-	-
Mannitol	+	+	-	+	+	+	+
Galactose	+	+	+	-	+	+	+
Rhamnose	-	-	-	-	-	-	-
Maltose	+	+	-	-	+	+	+
Cellobiose	-	-	-	+	-	-	-

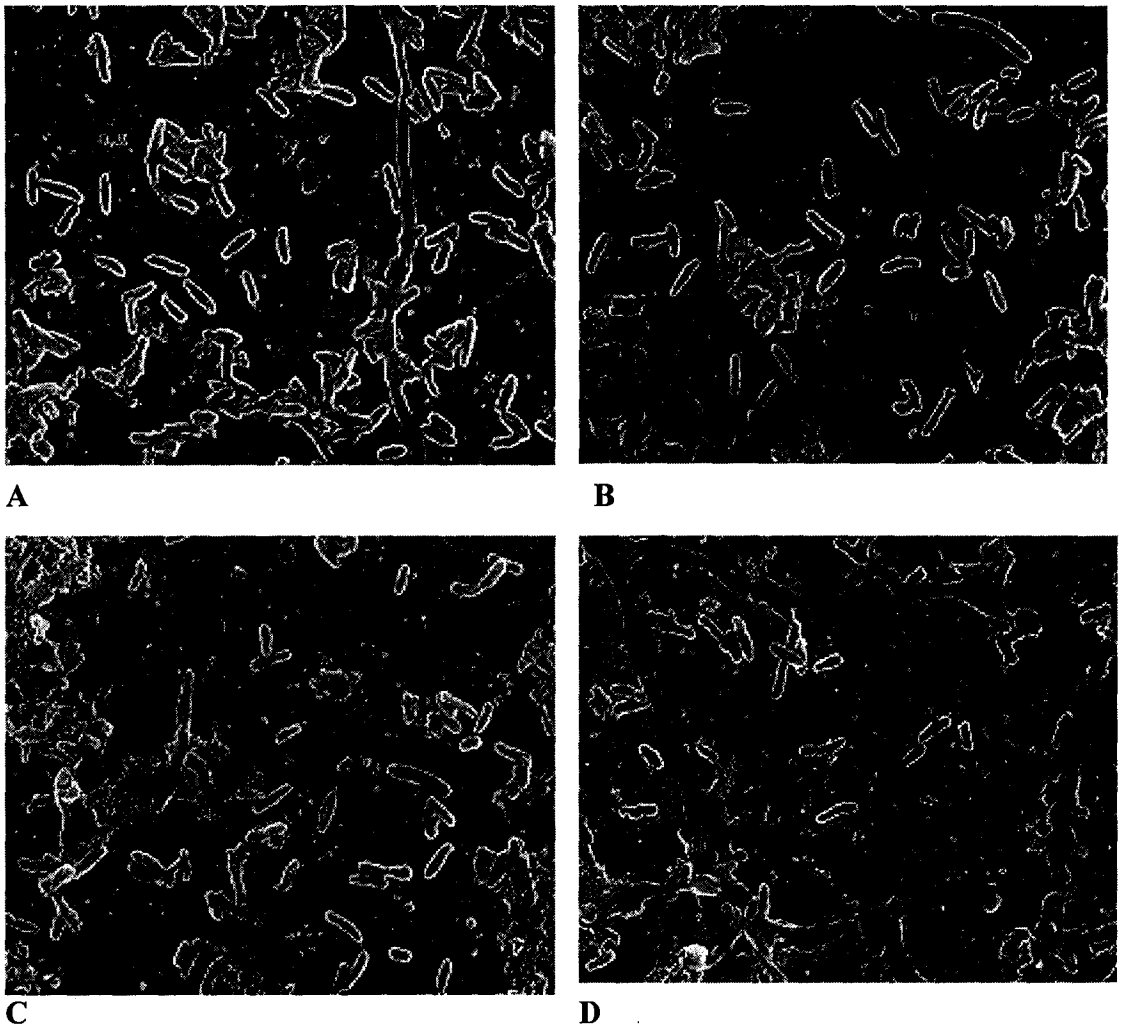
D-Gluconate	-	-	+	-	-	-	-
D-Glucouronate	-	-	-	-	-	-	-
L-Proline	+	+	+	-	+	+	+
D-Lactate	-	-	+	+	-	-	-
Acetate	-	-	+	-	-	-	-
L-Pyruvate	-	-	+	+	-	-	+
D-Xylose	-	-	-	+	-	-	-
Citrate	+	+	-	+	+	+	+
Sucrose	+	+	-	+	+	+	-
Diverse functions							
Luminescence	+	+	+	-	+	+	+
Growth on TCBS	+	+	-	+	+	+	+
Growth on GBM	+	+	+	-	+	+	+
Gas from Glucose	-	-	-	+	-	-	-
Swarming on solid media	-	-	-	-	-	-	-
Growth at 0°c	-	-	-	-	-	-	-
Growth at 4°c	-	-	-	-	-	-	-
Growth at 28°c	+	+	+	+	+	+	+
Growth at 35°c	+	+	+	+	+	+	+
pH 5.5	-	-	-	-	-	-	-
pH 8.5	+	+	+	+	+	+	+
pH 9.5	+	+	-	+	+	+	-
Growth in 0% NaCl	-	-	-	-	-	-	-
3% NaCl	+	+	+	+	+	+	+
6% NaCl	+	+	-	+	+	+	+
8 % NaCl							
Utilization of sole carbon energy sources (0.2% v/v)							
Glycerol	+	+	+	+	+	+	+
Succinate	+	+	+	+	+	+	+
Ethanol	+	+	-	-	+	+	+
L-Aspartate	+	+	+	+	+	+	+

(Table 2.4). Morphological and colony characteristics of potential heavy metal resistant marine and estuarine luminous bacterial isolates on GBMA

S.No	Sampling sites	Bacterial isolates	Colony characteristics on GBM agar	Identified bacterial isolates
1	Goa Shipyard Limited (GSL)	VB6	Circular, cream, entire, opaque, raised, motile, gram-negative curved rods.	***Vibrio harveyi***
2	Western India Shipping (WISL)	VB9	Circular, cream, entire, opaque, raised, motile, gram-negative straight rods.	***Photobacterium leiognathi***
3	Dona Paula Bay	VB23	Circular, cream, entire, opaque, raised, motile, gram-negative curved rods.	***Vibrio harveyi***
4	Miramar –1	VB32	Circular, cream, entire, opaque, raised, motile, gram-negative curved rods.	***Vibrio harveyi***
5	Miramar-2	VB51	Circular, cream, entire, opaque, raised, motile, gram-negative curved rods.	***Photobacterium leiognathi***
6	Bambolim	DN1W	Circular, cream, entire, opaque, raised, motile, gram-negative curved rods.	***Vibrio harveyi***
7	Anjuna	VB1	Circular, cream, entire, opaque, raised, motile, gram-negative curved rods.	***Vibrio fischeri***
8	Benaulim	VB01	Circular, cream, entire, opaque, raised, motile, gram-negative curved rods.	***Vibrio splendidus***
9	Colva	VB02	Circular, swarming, cream, entire, opaque, raised, motile, gram-negative rods.	***Vibrio harveyi***
10	Majorda	BR 10		



Figures (2.2) Phase contrast microscopy of Luminous *Vibrio harveyi* isolates A. VB6, B. VB9, C. VB23, D. BR9, E. DN1W.



(Figure 2.3) Scanning electron microscopy images of luminous bacterial strains A. VB9, B. VB6, C. VB23, D. BR9

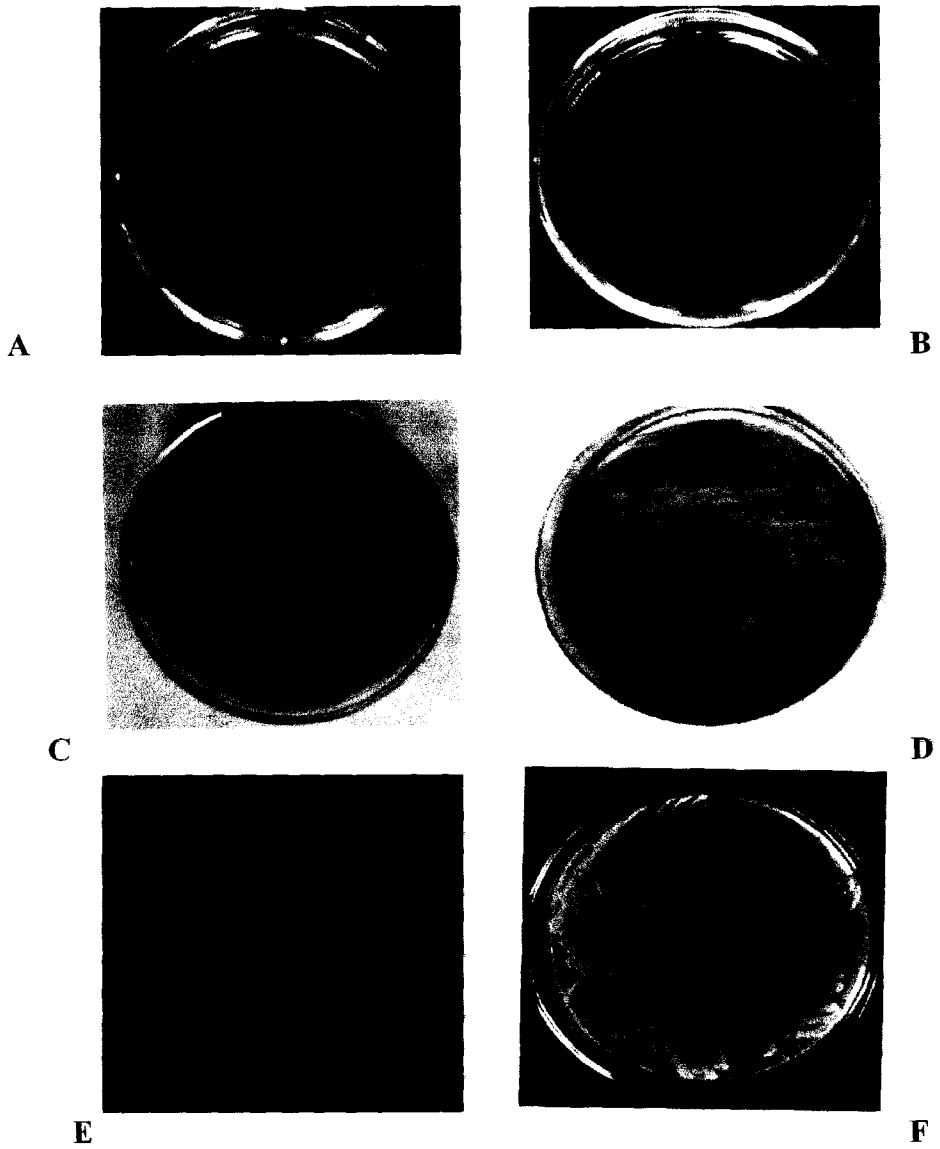
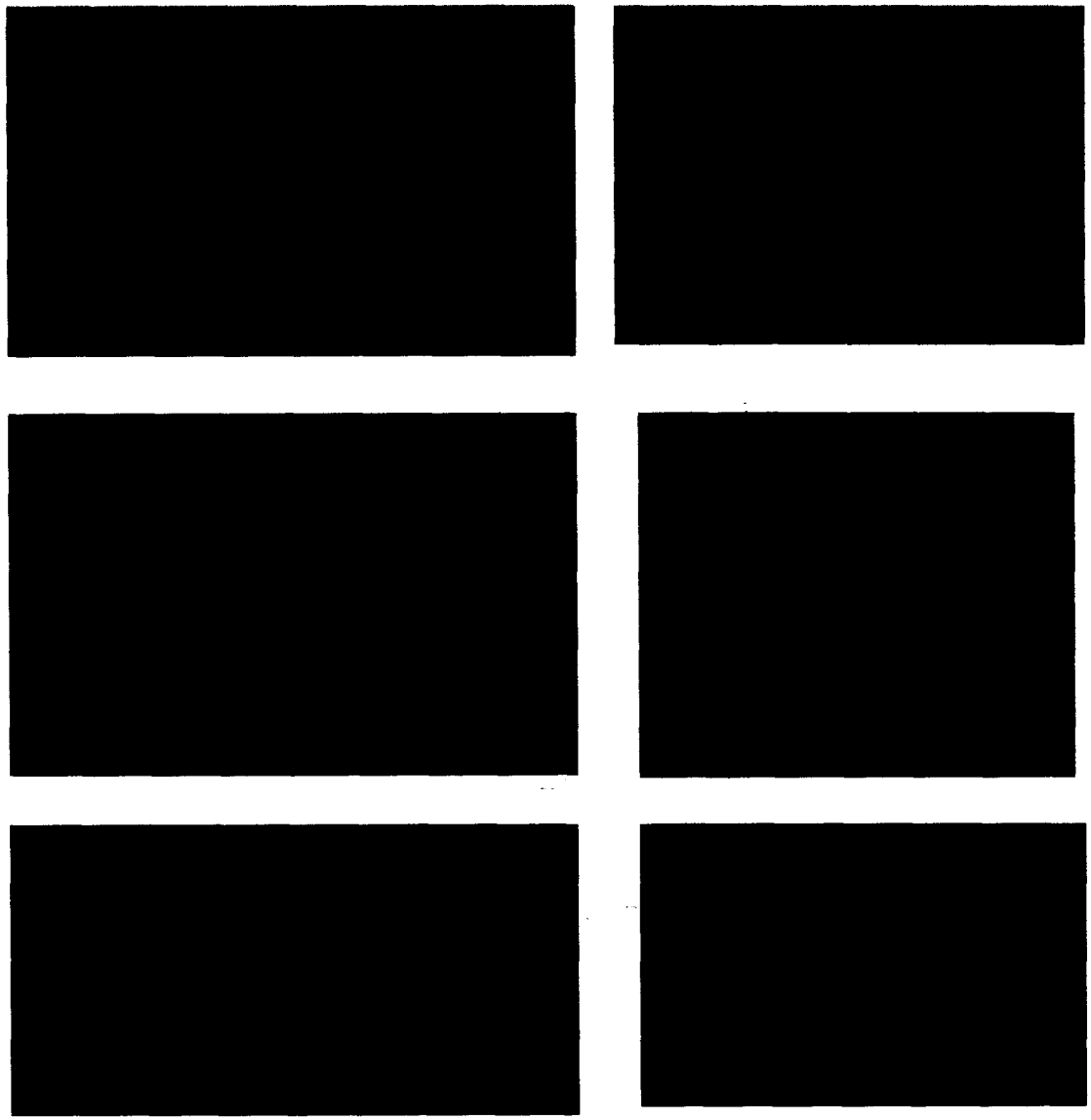


Fig. 2.4. Luminous Bacterial isolates *Vibrio harveyi* (VB23) grown in presence of **A.** Vibrio agar, **B.** *Vibrio harveyi* agar, **C.** *Vibrio harveyi* (VB23) grown in TCBS agar, **D.** *Vibrio splendidus* grown in TCBS agar, **E** and **F** , *Vibrio harveyi* (VB23) grown in GBM agar



(Fig 2.5). Images of Luminous bacteria in flasks, tubes and plates

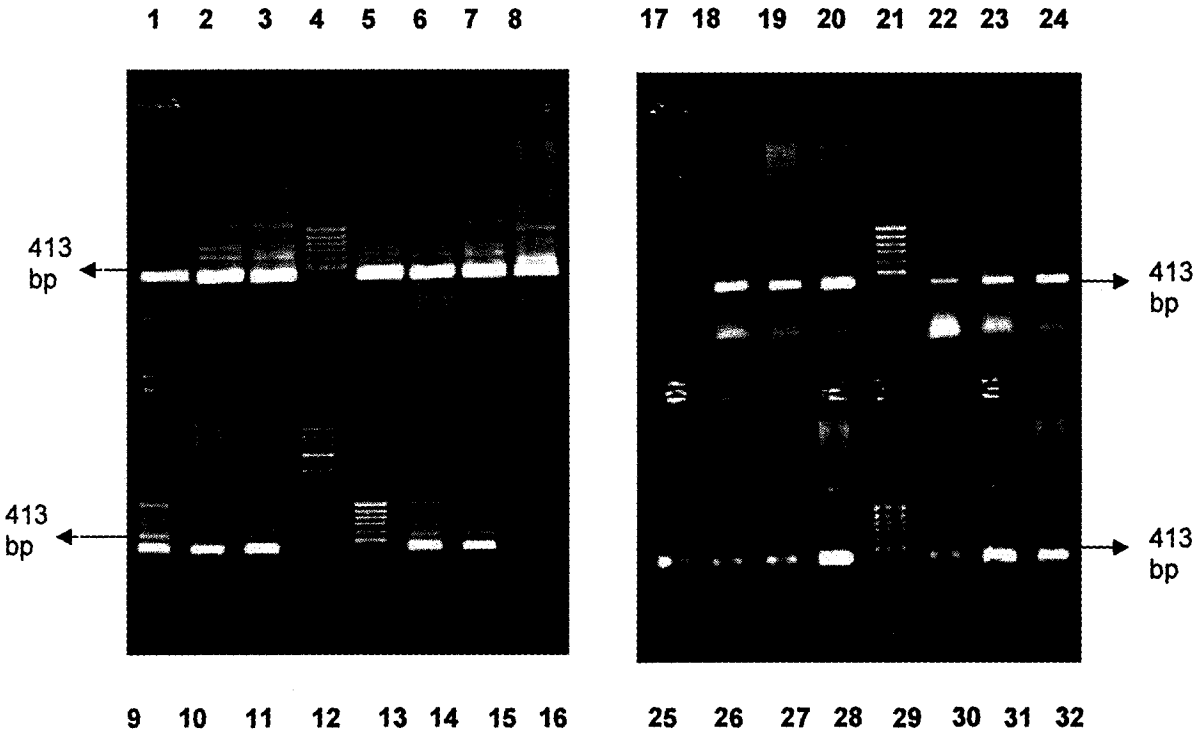
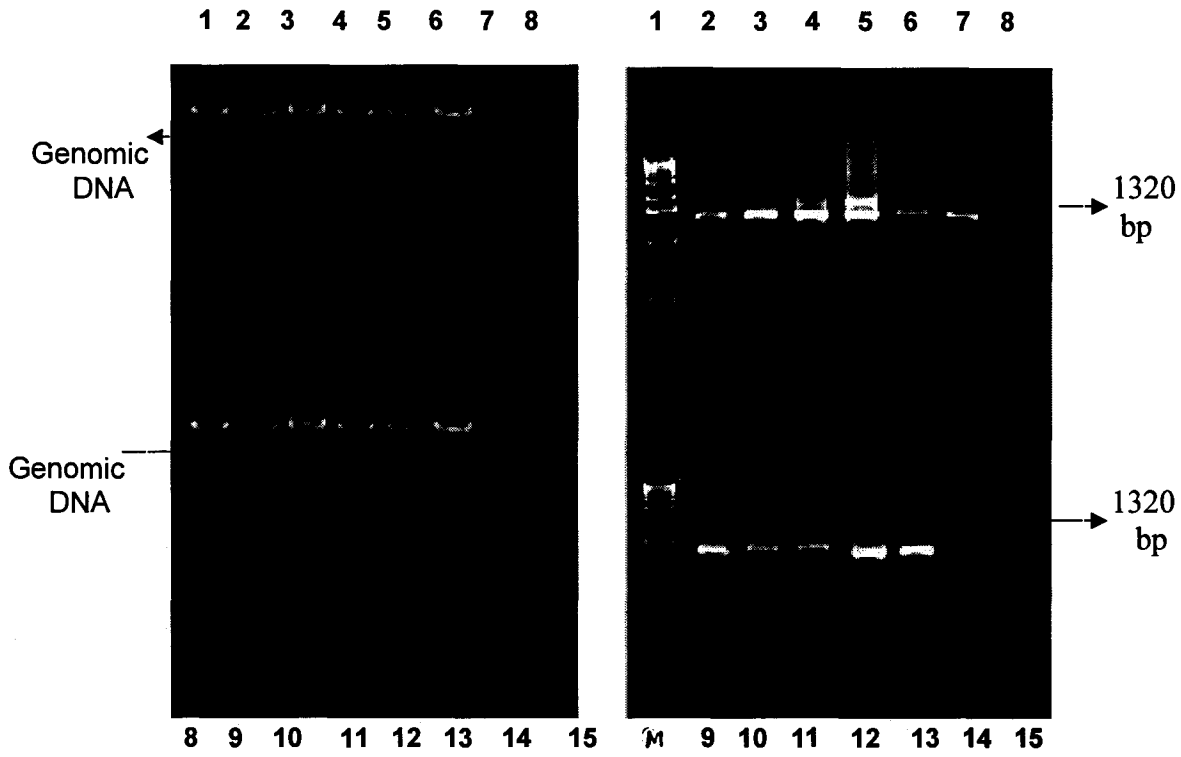


Fig 2. 6 and 2.7. Agarose gel electrophoresis of 16 s ribosomal DNA (PCR products). Lanes 4, 12, 21 and [illegible], (100 bp DNA ladder), Lane 13, (50 bp ladder) Lanes 1-3, 5-11, 13-15, 17-20, 22-28, 30-32- PCR products amplified from genomic DNA of various luminous bacterial isolates. Lanes 16 and 17 negative control – *E. coli*

Primers- **VH1 F' 5' ACC GAG TTA TCT GAA CCT TC 3'**
VH2 R' 5' GCA GCT ATT AAC TAC ACT ACC 3'

***Vibrio harveyi* species specific 16 s rDNA gene segment amplification using PCR**

Fig 2. 6 and 2.7. Rapid PCR method to identify *Vibrio harveyi* bacterial isolates from west coast of Goa using species specific primers



A. Isolation of Genomic DNA from luminous bacterial isolates
B. Universal 16 s rDNA gene amplification using PCR

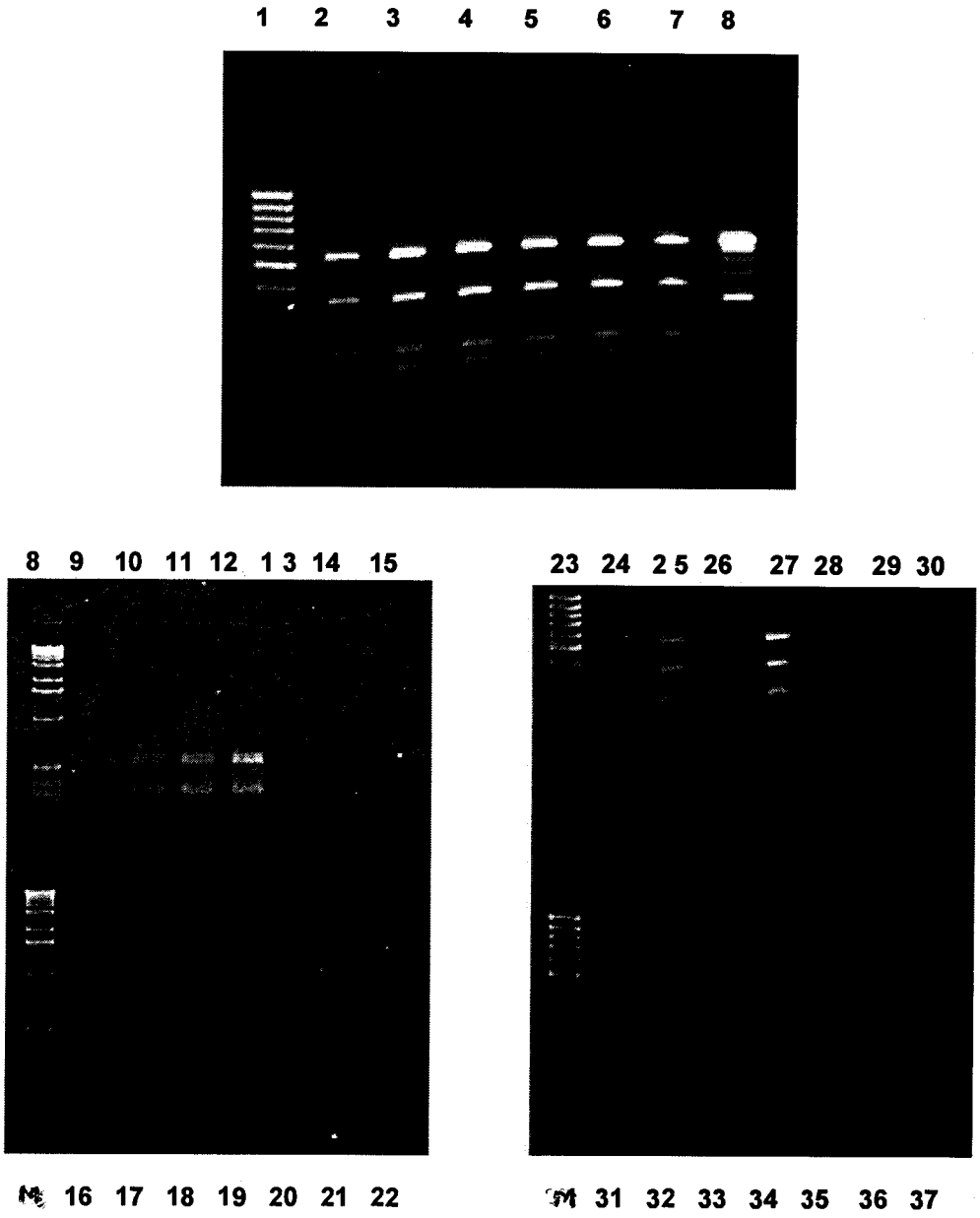
Fig 2. 8 Agarose gel electrophoresis of genomic DNA.
Lanes 1-6-10, 8-13 genomic DNA from various luminous bacterial isolates, Lanes 7 and 14 DNA marker, lanes 8 and 15 no DNA

Fig 2. 9. Universal 16 s *rDNA* gene amplification using PCR

Agarose gel electrophoresis of 16 s ribosomal DNA (PCR products). Lanes 1 and M (500 bp DNA ladder), Lanes 1-7, 9-13 PCR products amplified from genomic DNA of various luminous bacterial isolates, Lane 8, negative control-milli Q water, lanes 14-15 , no sample

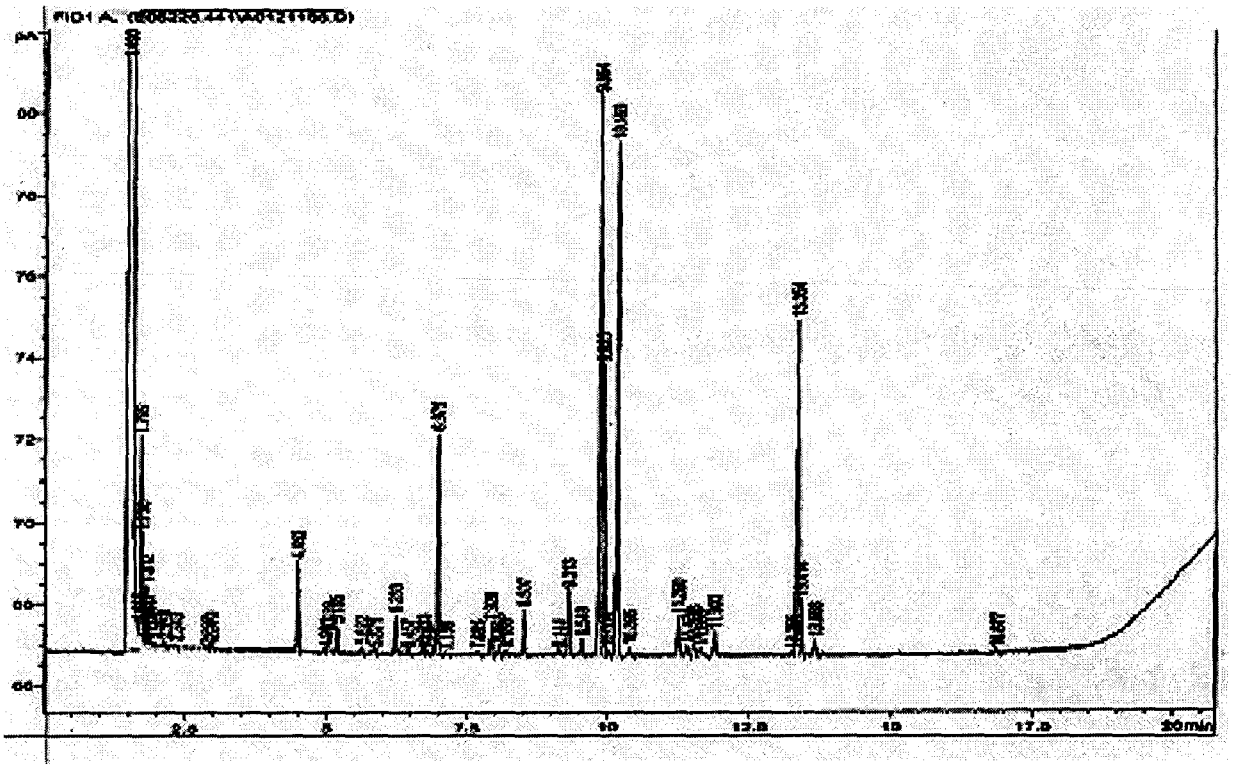
Universal 16 s rDNA gene primers

63f	**(5'-CAG GCC TAA CAC ATG CAA GTC-3')**
1387r	**(5'-GGG CGG WGT GTA CAA GGC-3')**

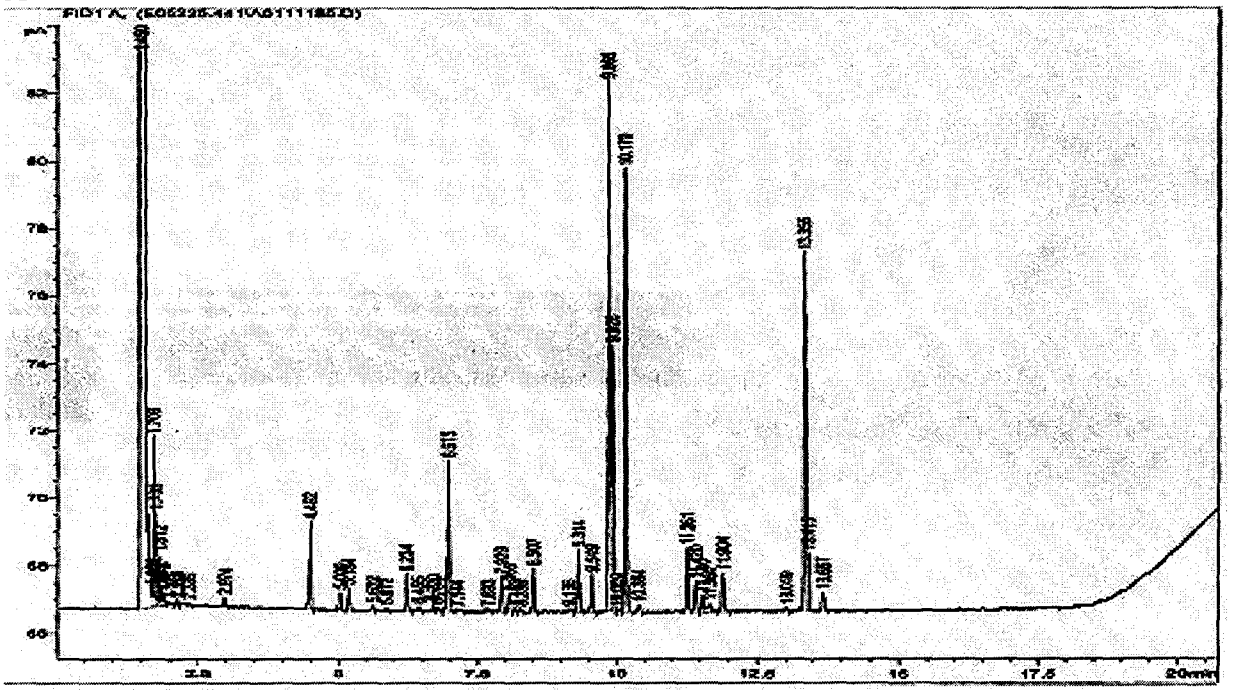


Fig's 2.10, 2.11 and 2.12. Agarose gel electrophoresis of Restriction fragment pattern of PCR amplified Universal 16 s *rDNA* gene of the genus *Vibrio* and *Photobacterium* digested with *Hha* I enzyme to distinguish the various luminous bacterial strains. Lanes 1, 8, M, 23 and M, 100 bps DNA ladder, Lanes 2-7, *Vibrio harveyi* isolates, Lane 8, *Photobacterium* isolate, Lanes 9-14, 16-21, *Photobacterium* isolates, Lanes 24-30, 31-33 *Vibrio fischeri* and *Vibrio splendidus,* Lanes 30, 34-37, undigested products

Fig's 2.10, 2.11 and 2.12. Restriction fragment pattern of PCR amplified universal 16 s *rDNA* gene of the genus *Vibrio* and *Photobacterium* digested with *Hha* I

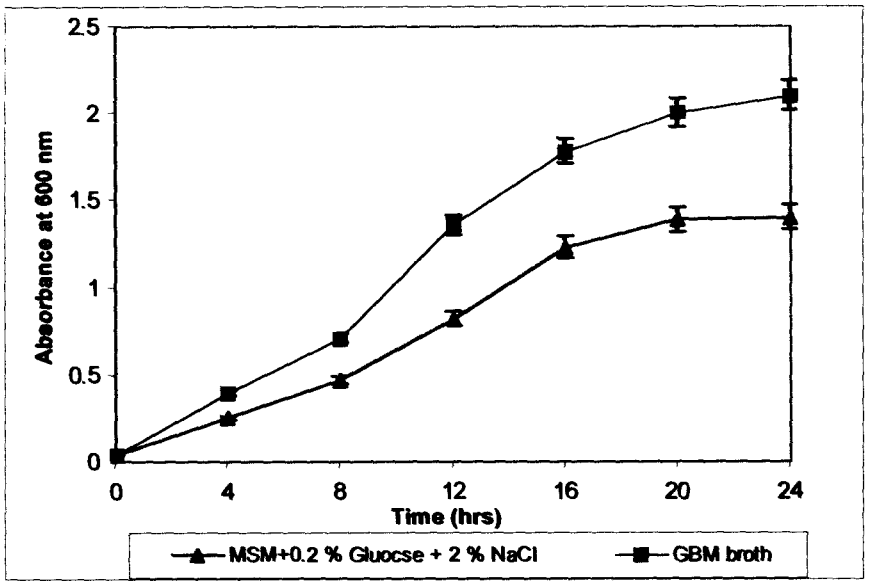


A

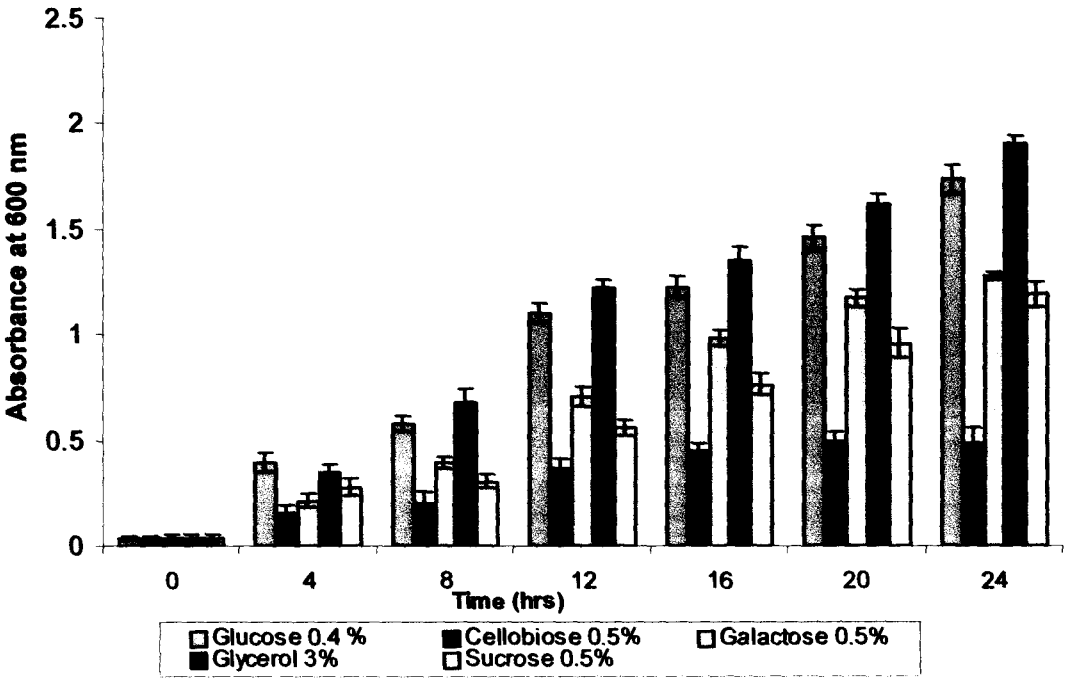


B

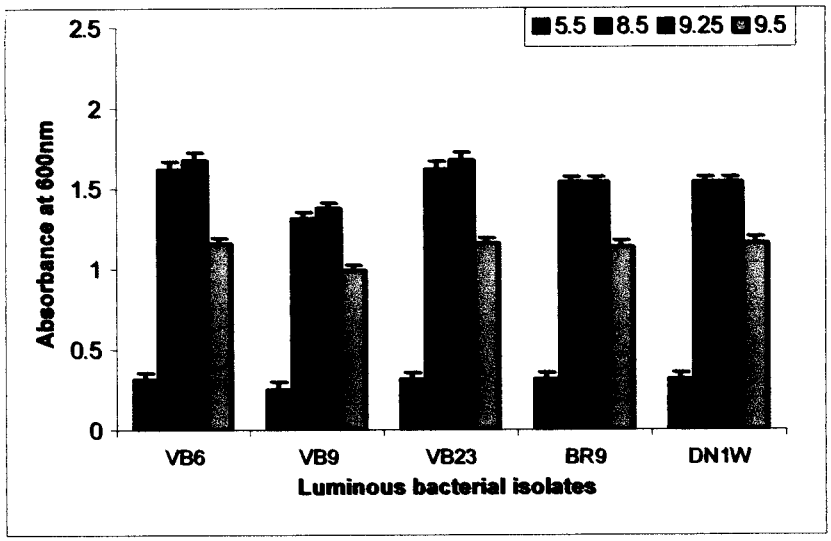
(Fig 2.13 and 2.14) Fatty acid methyl ester profile of Luminous bacterial isolates VB6 and VB23



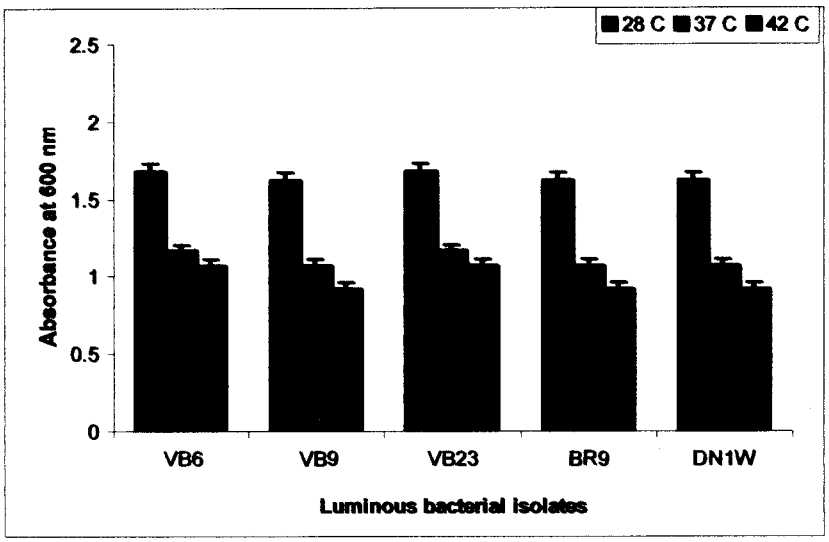
(Fig.2.15). Growth behavior of VB23 in MSM and Glycerol based marine broth



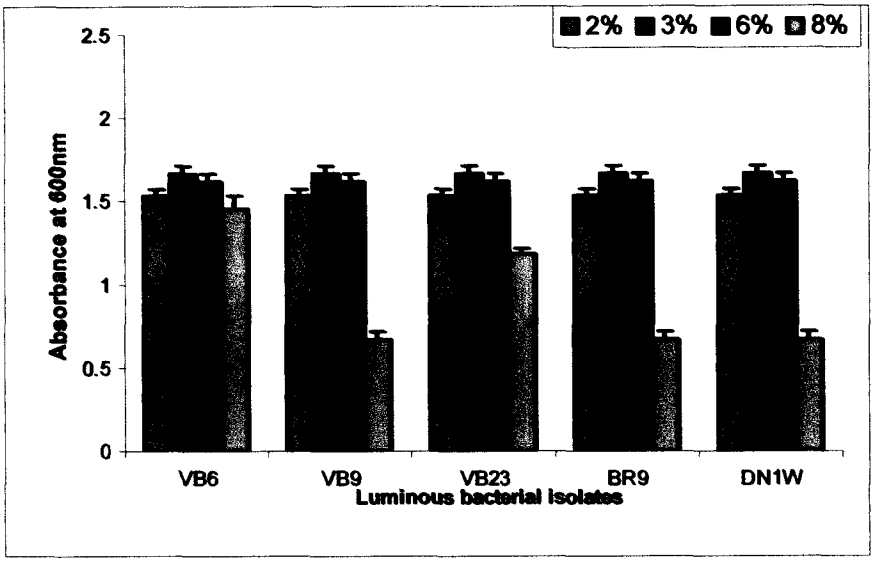
(Fig.2.16). Utilization of various carbon sources by *Vibrio harveyi* VB23



(Fig.2.17). Optimum pH for the growth of luminous bacterial isolates grown in MSM + 2% NaCl +0.2% Glucose .



(Fig. 2.18). Optimum Temperature (°C) for growth of luminous bacterial isolates grown in MSM + 2% NaCl + 0.2% Glucose .



(Fig.2. 19). Optimum NaCl (%) concentration for luminous bacterial isolates grown in MSM + 2% NaCl +0.2%Glucose.

References

Abrashev, I., Sotirova, A., Ilieva, K. and Donev, T. 1998. Long-term preservation of vitality and light emission of luminous bacterial strains. J. Culture Collections. 2: 26-29.

Alsina, M. and Blanch, A. R. 1994. A set of keys for the biochemical identification of environmental *Vibrio* species. J. Appl. Bacteriol 76: 79–85.

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

Ausubel, F.M., Brent, R., Kingston, R.E., Moore, D.D., Seidman, J.G., Smith, J. A. and Struhl, K. 1992. Short protocols in molecular biology, second edition. Green Publishing Associates and John Wiley & Sons, New York.

Bauer, A.W., Kirby, W.M.M, Sherris, J.C. and Turck, M. 1966. Antibiotic Susceptibility testing by a standard single disc diffusion method. Am. J. Clin. Pathol. 45: 493-496.

Baumann, P. and Baumann, L. 1981. The marine Gram-negative eubacteria: Genera *Photobacterium, Beneckea, Alteromonas, Pseudomonas, and Alcaligenes,* p. 1302–1331. In: M. P. Starr, H. Stolp, H. G. Truper, A. Balows, and H. G. Schlegel (ed.), The prokaryotes. Springer-Verlag, Berlin.

Baumann, P. and Schubert. R.H.W. 1984. Facultatively anaerobic Gram-negative rods, Family II.

Bertone, S., Giacomini, M. Ruggiero, C., Piccarolo, C. and Calegari, L. 1996. Automated systems for identification of heterotrophic marine bacteria on the basis of their fatty acid composition. Appl. Environ. Microbiol. 62:2122–2132.

Bourgois, J.J., Sluse, F. E., Baguet, F. and Mallefet, J. 2001. Kinetics of light emission and oxygen consumption by bioluminescent bacteria. J. Bioenergetics and Biomembranes, 33: No. 4,

Cattabiani, F, Brindani, E, Freschi, M, Ossiprandi,C. and Attivita, C. 1992. β-lattamasica in Vibrionaceae. Archivio Veter Ital. 43:1–12.

Coleman, R.H, Shaffer, J. and True, H. 1996. Properties of β-Lactamase from *Pseudomonas syringae.* Current Microbiology 32: 147–150.

Corkill, J. E, Hart, C. A, Mc Lennan., A. G. and Aspinall. S. 1991. Characterization of a β-lactamase produced by *Pseudomonas paucimobilis.* J Gen Microbiol 137:1425–1429

Cottrell, M.T., Wood, D.N., Yu, L. and Kirchman, D.L. 2000. Selected chitinase genes in cultured and uncultured marine bacteria in the alpha- and gamma-subclasses of the Proteobacteria. Appl. Environ. Microbiol. 66: 1195-1201.

Dorsch, M., Lane, D. and Stackebrandt, E. 1992. Towards a phylogeny of the genus *Vibrio* based on 16S rRNA sequences. Int. J. Syst. Bacteriol. 42: 58–63.

Dever, L. A., and Dermody, T.S. 1991. Mechanisms of bacterial resistance to antibiotics. Arch. Intern. Med. 151:886–895. 215–220.

Dunlap, P. 1985. Osmotic control of luminescence and growth in *Photobacterium leiognathi* from pony fish light organs. Arch. Microbiol. 141: (1), 44–50.

García, O. M 1997. Caracterización molecular de especies marinas del género Vibrio. BSch Tesis. Universidad Autónoma de Baja California. Tijuana, Baja California, México

Grasshoff, K. 1983. Determination of salinity. *In:* Methods of seawater analysis. Ed. 2nd. By Grasshoff, K., Ehrhardt. M., Kremling, K. Verlag chemie publication. p-31-59.

Grimes, D.J., Atwell, R.W., Brayton, P.R., Palmer, L.M., Rollis, D.M., Roszak, D.B., Singleton, T.L., Tampling, M. L. and Colwell, R.R. 1986. The fate of enteric pathogenic bacteria in estuarine and marine environments. Microb. Sci. 3: 324-329.

Gomez-Gil, B., Roque, A., Turnbull, J. F. and Tron-Mayen, L. 1998. Species of *Vibrio* isolated from hepatopancreas, haemolymph and digestive tract of a population of healthy juvenile *Penaeus vannamei*. Aquaculture. 163:1–9.

Gurtler, V. and Mayall, B. C. 2001. Genomic approaches to typing, taxonomy and evolution of bacterial isolates. Int. J. Syst. Evol. Microbiol. 51: 3–16.

Hiraga, K., Shou, L., Kitazawa, M., Takahashi, S., Shimada, M., Sato, R. and Oda, K. 1997. Isolation and characterization of chitinase from a flake-chitin degrading marine bacterium, *Aeromonas hydrophila* H-2330. Biosci. Biotech. Biochem. 61: 174- 176.

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

Jones and Bartlet. 1990. "Preparation of Genomic DNA from Bacteria" pp-20-23. Modified from Experimental Techniques in Bacterial Genetics.

Koroleff, F. 1983. Determination of nutrients. *In:* Methods of seawater analysis. Ed. 2nd. By Grasshoff, K., Ehrhardt. M., Kremling, K. Verlag chemie publication. p- 125-187.

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

Lambert, M. A., Hickman-Brenner, F. W., Farmer III, J. J. and Moss, W. 1983. Differentiation of *Vibrionaceae* species by their cellular fatty acid composition. Int. J. Syst. Bacteriol. 33:777–792.

Lobban, C.S., Harrison, P.J. and Duncan M.J. 1985. Nutrients, Chapter 6, In: The Physiological ecology of seaweeds. pp: 75-110. Cambridge University Press, London.

McArthur, J.V. and Tuckfield, R.C. 2000. Spatial patterns in antibiotic resistance among stream bacteria: effects of industrial pollution. Appl. Environ. Microbiol. 66(9): 3722-3726.

Maheswari, N. 1994. Study of arsenic and related parameters along the central west coast of India. pp 209. Ph.D. thesis, Goa University, India.

Makemson, J. C. 1986. Luciferase-dependent oxygen consumption by bioluminescent vibrios. J. Bacteriol. 165(2):461–466

Marchesi, J. R., Sato, T., Weightman, A.J., Martin, T.A., Fry, J.C., Hiom, S.J. and Wade, W.G. 199. Design and evaluation of useful bacterium-specific PCR primers that amplify genes coding for bacterial 16s rRNA. Appl. Environ. Microbiol. 64: 795-799.

Nandivada, L.S. and Amyes, S.G.B. 1990. Plasmid mediated ß-lactam resistance in pathogenic Gram negative bacteria isolated in South India. J. Antimicrob. Chemother. 26: 279-290.

Oakey, H.J., Levy, N., Bourne, D.G., Cullen, B. and Thomas, A. 2003. The use of PCR to aid in the rapid identification of *Vibrio harveyi* isolates. J. Appl. Microbiol. 95: 1293–1303.

Ortigosa, M., E. Garay, and M. J. Pujalte. 1994. Numerical taxonomy of Vibrionaceae isolated from oysters and seawater along an annual cycle. Syst. Appl. Microbiol. 17:216–225.

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

Rademaker, J. L., Hoste, B., Louws, F. J., Kersters, K., Swings, J., Vauterin, L., Vauterin, P. and de Bruijn, F. J. 2000. Comparison of AFLP and rep-PCR genomic fingerprinting with DNA–DNA homology studies: *Xanthomonas* as a model system. Int. J. Syst. Evol. Microbiol 50: 2, 665–677.

Rahman Khan, M. K. and Malik, A. 2001. Antibiotic resistance and detection of b-lactamase in bacterial strains of *Staphylococci* and *Escherichia coli* isolated from foodstuffs. World J. Microbiol. Biotechnol. 17: 863- 868.

Ramaiah, N. and Chandramohan, D. 1993. Ecological and laboratory studies on the role of luminous bacteria and their luminescence in coastal pollution surveillance. Mar Poll Bull 26:190–201.

Ramaiah, N., Hill, T.R., Chun, J., Ravel, J., Matte, M.H., Straube, W.L. and. Colwell, R. R. 2000. Use of a *chiA* probe for detection of chitinase genes in bacteria from the Chesapeake Bay. FEMS Microbiology Ecology 34: 63-71.

Riemann, L., and Azam, F. 2002. Widespread *N*-acetyl-D-glucosamine uptake among pelagic marine bacteria and its ecological implications. Appl. Environ. Microbiol. 68: 5554–5562.

Rodriguez-Kabana, R., Godoy, G., Morgan-Jones, G. and Shelby, R.A. 1983. The determination of soil chitinase activity: conditions for assay and ecological studies. Plant Soil. 75: 95-106.

Sambrook, J., Fritsch, E. F. and Maniatis, T. 1989. Molecular cloning: a laboratory manual (second edition). Cold Spring Harbour, New York.

Sasser, M. 1990. Identification of bacteria by gas chromatography of cellular fatty acids. Newark, DE, USA: Microbial ID.

Silva, J., Anguilar, C., Ayala, G., Estrada, M.A., Garza-Ramos, U., Lara-Lemus, R., Ledezma, L. 2000. TLA-1: a new plasmid mediated extended spectrum ß-lactamase from *E. coli.* Antimicrob. Agents Chemother. 44: 997-1003.

Simidu, U., Tsukamoto, K. 1980. A method of the selective isolation and enumeration of marine *Vibrionaceae*. Microbiol. Ecol. 6: 181–184.

Svitil, A. L., Chadhain, S. M. N., Moore, J. A. and Kirchman, D. L. 1997. Chitin degradation proteins produced by the marine bacterium *Vibrio harveyi* growing on different forms of chitin. Appl. Environ. Microbiol. 63:408– 413.

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

Thompson, F. L., Hoste, B., Vandemeulebroecke, K and Swings, J. 2001. Genomic diversity amongst *Vibrio* isolates from different sources determined by fluorescent amplified fragment length polymorphism. Syst Appl Microbiol 24, 520–538.

Urakawa, H., Kita-Tsukamoto, K., and Ohwada, K. 1997. 16S rDNA genotyping using PCR/RFLP (restriction fragment length polymorphism) analysis among the family *Vibrionaceae*. FEMS Microbiol. Lett. 152: 125–132.

Urakawa, H., Kita-Tsukamoto, K., and Ohwada, K. 1998. A new approach to separate the genus *Photobacterium* from *Vibrio* with RFLP patterns by *Hha*I digestion of PCR-amplified 16S rDNA. Current Microbiol. 36: 171–174.

Urakawa, H., Yoshida, T., Nishimura, M. and Ohwada, K. 2000. Characterization of depth-related population variation in microbial communities of a coastal marine sediment using 16S rDNA-based approaches and quinone profiling. Environ. Microbiol. 2: 542–554.

Vandenberghe, J., Thompson, F.L., Gomez-Gil, B. and Swings, J. 2003. Phenotypic diversity amongst *Vibrio* isolates from marine aquaculture systems. Aquaculture. 219:9–20.

Vaz, B. 2005. Studies on nutrients in the Mandovi and Zuari estuarine systems. M.Sc. Dissertation, Goa University.

Versalovic, J., Koeuth, T. and Lupski, J. R. 1991. Distribution of repetitive DNA sequences in eubacteria and application to fingerprinting of bacterial genomes. Nucleic Acids Res 19, 6823–6831.

Wada, M. and Kogure, K. 2000. Membrane bioenergetics in reference to marine bacterial culturability. pp. 47-55. In: Nonculturable Microorganisms in the Environment, American Society for Microbiology, Washington, DC.

Waters, P. and Lloyd, D. 1985. Salt, pH and temperature dependencies of growth and bioluminescence of three species of luminous bacteria analyzed on gradient plates. J. Gen. Microbiol. 11: 2865–2869.

Winship, K. A. 1984. Toxicity of inorganic arsenic salts. Adv. Drug React. Ac. Pois. Rev. 3:129-160.

Wood, M. T., Yu, L. and Kirchman, D.L. 2000. Selected chitinase genes in cultured and uncultured marine bacteria in the alpha and gamma-subclasses of the proteobacteria. Appl. Environ. Microbiol. 66: 1195–1201.

Woese, C.R., Kandler, O., and Wheelis, M. L. 1990. Towards a natural system of organisms: proposal for the domains *Archaea*, Bacteria, and Eucarya. Proc. Natl. Acad. Aci. USA. 87: 4576-4579.

Chapter III

Heavy metal and Organo-metal Impact assessment study using selective marine and estuarine bioluminescent bacteria with reference to Growth behavior, bioluminescence (light emission), induced protein profiles, exopolysaccharide EPS characterization and induced pigment profiles.

Material and Methods

Metal and organometals used for the impact studies

All chemicals were analytical grade reagents obtained from Merck and other reputed chemical companies. For toxicity assessment, chemicals used are as follows: organo metals Tributyltin chloride (1M) and Dibutyltin chloride (1M);- E. Merck, AG (Darmstadt, Germany) dissolved in absolute ethanol (Merck). Heavy metal and salts, $HgCl_2$ (10 mM), $CdCl_2$ (10 mM) (Merck), $NaAsO_2$ (50 mM) (E.Merck), $NaAsO_4$ (100 mM) and $SnCl_2.2H_2O$ (10 mM) (Qualigens). All stock metal solutions, with the exception of $SnCl_2$, were made in double-distilled water, membrane-filtered (0.22 µm, Millipore) into sterile glass vials. $SnCl_2$ was dissolved in 50 % ethanol and stored in sterile polypropylene tubes. These solutions are prepared fresh and stored at 4 °C (Appendix D.1-D.3).

Screening and isolation of metal and organo-metal resistant luminous bacteria

Metal and organo-metal resistant luminous bacteria were screened and isolated by plating diluted samples on GBM agar plates supplemented with the following heavy metals; 2 mM $NaAsO_2$, 2 mM $NaHAsO_4$, 5 mM $SnCl_2.2H_20$, 2 µM $HgCl_2$, 50 µM $CdCl_2$ and 0.5 mM TBT and DBT respectively. These metal solutions were added from filter sterilized stock solutions to the media after autoclaving and cooling to 45–50°C. The plates were incubated at 28±2 °C, and resistant colonies were screened after 24 hrs. Ten randomly selected metal resistant colonies were isolated from the selective plates containing heavy metals separately and further sub cultured on respective metal containing plates.

Impact of heavy metals on Growth behavior of *Vibrio harveyi* VB23

Effects of heavy metals and organo-metals on the growth behavior of bioluminescent *Vibrio harveyi* VB23 was analyzed in optimized mineral salts medium (MSM) supplemented with 2% NaCl and 0.2% glucose at pH 7.5. The medium (100 ml) dispensed in sterilized 250 ml Erlenmeyer flasks and inoculated with 2 % (v/v) of overnight grown culture and incubated at 28±2 °C, for 24 hrs in environmental shaker at 180 rpm. The sub samples (5 ml) were drawn at regular intervals of 4 hours for determination of impact of heavy metals on growth response. Controls were carried out under same conditions, without the addition of metals and organometals. Bacterial growth was determined by measuring the absorbance at 600 nm using an ELICO colorimeter. Final

concentrations of the heavy metals were $HgCl_2$ (2, 5, 7 and 10 µM) $CdCl_2$ (25, 50, 75, 100, 150 µM), Chromate (25, 50, 75, 100 and 150 µM), $SnCl_2$ (0.5, 1, 2.5 and 5 mM), $NaAsO_2$ (2.5, 5, 7.5 and 10 mM), $NaHAsO_4$ (1, 2.5, 5, 10 mM); Organo-metals were Tributyltin Chloride (0.25, 0.5, 0.75 and 1 mM) and Dibutyltin Chloride (0.1, 0.25, 0.5 mM and 1 mM). Each metal treatments and controls were done in triplicate. Bacterial growth response as (Absorbance at 600nm Vs time (hrs) was determined for different metal concentrations and plotted accordingly.

Percent survival curves of *Vibrio harveyi* VB23 in metals and organo-metals

An inoculum of (1.2 $X10^6$ cells/ml) grown in GBM broth was inoculated in MSM + 2.0 % NaCl + 0.2 % glucose supplemented with gradually increasing levels of heavy metals and organo-metals. After inoculation the cultures were incubated with shaking at 28±2 °C and optical density at A_{600} nm was measured at 24hrs of incubation. Absorbance of control culture is considered as 100% survival. The percent survival of luminous *Vibrio harveyi* under the stress of metals viz. Hg (II), Cr (VI), Cd (II), As (III), As (V), Sn (II) and organo-metals viz. TBTC and DBTC were determined as compared to control and sub lethal concentrations (LD 50) were obtained by plotting metal / organometals concentrations vs survival (%).

Disc inhibition assay for determination of metal resistance

The heavy metal resistance patterns of luminous bacterial strains (VB6, VB9, VB23, and DN1W) were tested by the filter paper-disk diffusion method using Mueller-Hinton agar No:2 medium. Plates were swabbed with a faintly opalescent culture suspension of (1 × 10^6 CFU/ml), prior to placement of filter discs of the following concentrations of heavy metals and organo-metals; $HgCl_2$ (2, 5, and 7.5 µM), $CdCl_2$ (5, 10, 25, and 50 µM), $NaAsO_2$ (5, 7.5 and 10 mM), $NaHAsO_4$ (5, 7.5 and 10 mM), $SnCl_2$ (5 and 10 mM), and TBTC (50, 70 and 100 µM), DBTC (50, 70 and 100 µM). 10 mm size of the presterilized filter paper discs are placed and 5,10,15 µl of the above metal stock solutions are subsequently dispensed on to the filter paper disc and observed for the zone of inhibition. The plates were incubated at 28±2 °C for 24 hrs. A control was carried out under the same conditions to the tests, without the addition of metals and organometals.

Regulation of metal toxicity by thiols and chelating agents

In order to find out the non-inhibitory level of thiol and chelating agents (Glutathione, Cysteine, Dithiotreitol, β-mercaptoethanol and Na_2EDTA), the growth behavior of bioluminescent *Vibrio harveyi* VB23 was analyzed in optimized mineral salts medium (MSM) +2 % NaCl + 0.2 % glucose at pH 7.5. The growth medium was supplemented with different concentrations of thiols and chelating agents. The medium (100 ml) dispensed in sterilized 250 ml Erlenmeyer flasks and inoculated with 2 % (v/v) of overnight grown culture and incubated at 28±2 °C for 24 hrs on an environmental shaker at 180 rpm. The sub samples (5 ml) were drawn at regular intervals for determination of growth response. Controls were carried out under same conditions but without the addition of treatments. Bacterial growth was determined by measuring the absorbance at 600 nm using an ELICO colorimeter. Non – inhibitory concentrations of thiol and chelating agents was determined and the same concentration was used for further experiments to study regulation of metal toxicity.

2 % (v/v) overnight grown culture VB23 was inoculated in modified MSM with non-inhibitory concentration of thiol and chelating agents along with the MIC of toxic heavy metals, $HgCl_2$ (5 µM), $CdCl_2$ (100 µM) and TBT 0.75 mM. The tubes were kept at 28 ±2 °C on an environmental shaker at 180 rpm for 24 hrs. The growth was determined using colorimeter at 600 nm and the graphs were plotted as Absorbance (600 nm) Vs time (hrs) with reference to various treatments of the culture. Control cultures were incubated under identical growth conditions to the tests but without the addition of metals, thiols and chelating agents.

Antimicrobial susceptibility testing (Kirby Bauer method)

Antibiotic susceptibility tests for the metal, organo-metal resistant isolates of *Vibrio harveyi* and *Photobacterium leiognathi* (VB6, VB1, VB23, VB30, VB50, BR9 VB9, and DN1W were performed by the disk diffusion method. The strains were grown in Mueller-Hinton broth at 28 ±2 °C for 16 hrs. Plates were swabbed with a faintly opalescent culture suspension of (1×10^6 CFU/ml) and then the octadiscs with antibiotics dispends by a disc dispenser on Mueller–Hinton agar plate. Following antibiotics were tested: Kanamycin (Km, 30 µg), Ampicillin (Ap, l00 µg), Tetracycline (25 µg) Chloramphenicol (Cm 30 µg);

Gentamycin (G 30 µg); Co-Trimoxazole (25 µg), Amikacin (30 µg), Nalidixic acid (Na, 30 µg), Norfloxacin (10 µg), Cephalothin, (30 µg); Mecillinam (33 µg), Mecillinam (Mc 33 µg), Ciprofloxacin (Cp, 5 µg), Erythromycin (E 15 µg), Cephalothin (Cf, 30 µg), Amoxycillin (Am 30 µg), Novobiocin (Nv 30 µg), Nitrofurantoin (Nf-300 µg), Trimethoprim (Tr 5 µg), Polymixin-B (Pb-300 µg), Vancomycin (Va 30 µg), Streptomycin (S 100 µg) Rifamycin (Rf 100 µg) and Norflaxacin 10 µg) (Appendix D.5). Plates were incubated at 28±2 °C and the zones of inhibition were measured after 24 hrs. Isolates were considered as resistant, intermediate, and susceptible following the standard antibiotic disc sensitivity testing method. (Table 3.1.4). *E. coli* type strain ATCC 11775 was used as control and the zones of inhibition (mm) were compared with the results obtained with selected luminous bacterial isolates.

Percent survival curves of VB23 in broad range antibiotics

An inoculum of (1.2 $X10^6$ cells/ml) grown in GBM broth was inoculated in MSM supplemented with 2.0 % NaCl + 0.2 % glucose at different concentrations of broad range antibiotics. After inoculation the cultures were incubated with shaking at 28 ±2 °C and optical density at 600 nm was measured at 24 hrs. The percent survival of luminous *Vibrio harveyi* VB23 in presence of novobiocin, kanamycin, nystatin, chloramphenicol, tetracycline, valinomycin, cyclohexamide, rifamycin, ampicillin and streptomycin (Appendix D.5) were determined and the sub lethal concentrations were obtained by plotting broad range antibiotics concentrations Vs survival (%).

MIC determination of antibiotics (Tube dilution method)

Minimal inhibitory concentrations (MIC's) of different antibiotics for selected bacterial isolates VB6 VB9, VB23, BR9, DN1W and VB02 isolates were determined in Mueller Hinton broth 5 ml supplemented with 1.5 % NaCl. One milliliter of the bacterial culture suspension (1 × 10^6 CFU/ml) was added to each tube. The tubes were incubated for 18 h at 28 ±2 °C and visual turbidity was noted. The antibiotics used were as follows; Protein and DNA inhibitors: Chloramphenicol (0, 10, 20, 50, 75, 100 µg/ml), Erythromycin (0, 10, 20, 50, 75, 100 µg/ml); Nalidixic acid (0, 2, 5, 10, 15, 20 µg/ml), Neomycin (0, 10, 20, 50, 75, 100); and Streptomycin- 0, 10, 20, 50, 75, 100,150. β-lactam antibiotics: Ampicillin (0, 25, 50, 75, 100, 150, 200, 250), Penicillin (0, 25, 50, 75, 100, 150, 200, 250), Carbenicilin (0, 25, 50, 75, 100, 150, 200), Amoxycillin (0, 25, 50, 75, 100, 150, 200), and Cephalothin

(0, 25, 50, 75, 100, 150, 200). The lowest concentration that inhibited growth compared with the control tube was defined as the MIC of the antibiotic. Controls were maintained under same conditions but without the addition of antibiotics (Table 3.1.3)

Bioluminescence

Chemicals used for bioluminescence (Relative light intensity) experiment:

The heavy metals and organometals used were Mercuric chloride, Cadmium chloride, stannous chloride, Sodium arsenite, Sodium arsenate, Tributyltin chloride and Dibutyltin chloride. Metal chelator used is Na_2EDTA and Protein Inhibitors selected were Chloramphenicol, Rifamycin, Kanamycin and Cyclohexamide. The Chemical mutagens used were 2-methoxy-6-chloro-9- (3-(2-chloroethyl) aminopropylamino-acridine × 2HCl (AcOr), Ethidium bromide (Eth-Br), Nitrosoguanidine (MNNG), sodium Azide (SA) and 5-Bromouracil (5-BU). Luciferin substrate, n-decanaldehyde was also used. Concentrations of all the chemicals are mentioned in (Tables 3.2.1-3.2.10).

Measurement of Bacterial bioluminescence

Cultures were grown until the absorbance (600 nm) reached 0.8-1.2 (exponential phase), at which point 5 ml samples were promptly removed into tubes and light emission was recorded as relative light intensity (RLI) immediately after the addition of heavy metals, organo-metals, antibiotics, chemical mutagens, and amino acids separately. The concentrations of the above chemicals selected in the following experiments were minimal growth inhibitory concentrations. The treated samples along with the controls were incubated on orbital shaker at 160 rpm, at 28±2 °C. Bioluminescence was measured using Shimadzu spectroflourimeter RF-5301, calibrated with slit width of excitation: 1.5 nm, slit width of emission: 10.0 nm, spectral emission range (350-850 nm) and excitation wavelength as zero. Light emission was expressed as RLI (relative light intensity). Light emission was measured every 30 mins for heavy metals, organometals, antibiotics, chemical mutagens and amino acids whereas for UV treated samples, light intensity was measured for every 5 min time interval. All experiments were performed in duplicates and the relative light intensity was determined as % RLI Vs Time (mins).

Effect of Heavy metals and Organometals on Bacterial bioluminescence

Effects of heavy metals on bacterial luminescence were investigated as described above by adding to various concentrations of metals and organometals $HgCl_2$ (5, 7, µM) $CdCl_2$ (25 and 50 µM) $SnCl_2$ (5 mM); $NaAsO_2$ (5 and 10 mM); $NaHAsO_4$ (5 and 10 mM); Tributyltin chloride (0.5 mM and 1mM); Dibutyltin chloride (0.5 mM and 1mM), directly to the bacterial culture. Bioluminescence was measured as relative light intensity (RLI) using a spectroflourimeter every 30 minutes up to 120 min after addition of heavy metal and organo-metals in culture. Culture untreated with the metal and organo-metals was considered as control (Appendix E).

Effect of Hg^{2+} on bioluminescence: Regulation of toxicity by chelating agent

Minimal inhibitory concentration (MIC) of $HgCl_2$ (5 µM) were added to the cells, along with chelating agent, Na_2EDTA (10 µM) was maintained in 5 ml cultures of bacteria in the tubes. Bioluminescence of bacterial cells was measured as relative light intensity (RLI) in a spectroflourimeter every 30 minutes up to 150 min after addition of heavy metal and chelating agent separately and also combined in cultures, whereas the culture untreated with heavy metals or chelating agent was considered as control (Appendix E)..

Effect of antibiotics (Protein –inhibitors) on Bacterial bioluminescence

Minimal inhibitory concentration (MIC) of antibiotics (protein –inhibitors) Chloromphenicol (25 µg/ml) Rifamycin (5 µg/ml) Kanamycin (10 µg/ml); and Cyclohexamide (10 µg/ml) directly to 5 ml cultures of bacteria in the tubes. Bioluminescence of bacterial cells was measured as relative light intensity (RLI) in a spectroflourimeter every 30 minutes up to 150 min after addition of chemicals, whereas the culture untreated with antibiotics (protein inhibitors) was determined as control (Appendix E).

Effect of Protein inhibitors and $HgCl_2$ on Bacterial bioluminescence

Minimal inhibitory concentration (MIC) of antibiotics (protein –inhibitors) of Kanamycin (10 µg/ml) and Chloromphenicol (25 µg/ml), Kanamycin (10 µg/ml), was maintained in 5 ml cultures of bacteria in the tubes. Bioluminescence of bacterial cells was measured as relative light intensity (RLI) in a spectroflourimeter every 30 minutes up to 150 min after addition of antibiotics and heavy metal (Hg^{2+}) separately and also combined. Whereas the

cultures untreated with antibiotics (protein inhibitors) and $HgCl_2$ were determined as control (Appendix E).

Effect of chemical mutagens on Bacterial bioluminescence

Minimal inhibitory concentration (MIC) of chemical mutagens on bacterial bioluminescence was investigated by adding various concentrations of Acridine Orange (10 µg/ml), Ethidium bromide (10 µg/ml), Nitrosoguanidine (MNNG) (10 µg/ml, 25 µg/ml), sodium azide (SA) (10, 25 and 50 µg/ml) and 5-Bromouracil (5-BU) (10, 25 and 50 µg/ml) to 5 ml cultures of bacteria into the tubes. Luminescence of bacterial cells was measured as relative light intensity (RLI) in a spectroflourimeter every 30 minutes up to 150 mins after addition of chemicals. The culture untreated with chemical mutagens was considered as control (Appendix E).

Effect of UV light on Bacterial bioluminescence

Effect of UV light (254 nm, 15 watts germicidal lamp) on bacterial luminescence was investigated as described above. Following UV irradiation for (0- 40 mins) the samples of 5 ml of each treatment were withdrawn every 5 minutes after irradiation and the luminescence of bacterial cells was measured as relative light intensity (RLI) in a spectroflourimeter until 40 mins after UV irradiation, whereas the culture unexposed to UV irradiation was considered as control.

Effect of antibiotics (Protein-inhibitors) and UV irradiation on bacterial bioluminescence

Minimal inhibitory concentrations of Kanamycin (10 µg/ml) and Chloromphenicol (25 µg/ml), along with the control were incubated for 0-10 mins, followed by UV irradiation (15 and 30 mins) subsequently. The samples of 5 ml of each treatment were withdrawn every 5 minutes after irradiation and the luminescence of bacterial cells was measured as relative light intensity (RLI) in a spectroflourimeter up to 40 min after UV irradiation, whereas the culture untreated with UV irradiation was considered as control.

Effect of n-Decanaldehyde on bacterial bioluminescence

Non-inhibitory concentrations of n-Decanaldehyde on bacterial luminescence was investigated by adding various concentrations of n-decanal (0.05, 0.1, 0.2, 0.5 mM) to 5

ml cultures of bacteria in the tubes. Bioluminescence of bacterial cells was measured as relative light intensity (RLI) in a spectroflourimeter for every 30 minutes until 120 min after addition of chemicals, whereas the culture untreated with substrate was determined as control (Appendix E).

Growth and Bioluminescence by serial transfer in (heavy metals and organo-metals)

Metal and organo-metal resistant luminous bacterial isolates obtained after screening were sub cultured at 9-day interval for a total of eight serial transfers. GBM agar plates supplemented with the following concentrations of heavy metals $HgCl_2$ (5 and 7 µM), $CdCl_2$ (25 and 50 µM), $SnCl_2.2H_2O$ (5 mM), K_2CrO_4 (100 µM), $NaAsO_2$ (5 mM), $NaHAsO_4$ (10 mM) and Organo-metals TBT (0.5 and 1 mM), DBT (0.5 and 1 mM) were prepared in duplicates, whereas control plates were without addition of metal and organo-metals. Growth and bioluminescence of test organism was monitored visually after every subsequent serial sub culturing. Plates were incubated at 28± 2 °C for 24 hrs per each transfer, and tested for their bioluminescence and resistance growth pattern against metals and organo-metals.

Protein profiles of *Vibrio harveyi* VB23 isolate

Induction of metal and organo-metal stress proteins

In order to evaluate the induction of stress proteins over the growth cycle, luminous bacterial strain VB23 was grown in glycerol based marine (GBM) broth with a starting absorbance of 0.1 at 28±2 °C. When absorbance at 600 nm reaches 0.4-0.5, the culture was promptly transferred to MSM broth supplemented with 1.5 % NaCl and 0.2 % glucose, pH 7.5, and growth was monitored at 4hrs time intervals. Induction of stress was achieved by adding heavy metals to the above medium viz. $HgCl_2$ (2, 5, 7 µM), $CdCl_2$ (20, 50 µM), $NaAsO_2$, (1, 2, 5 mM), $NaHAsO_4$ (1, 2, 5 mM) and organometals viz. 0.5 and 1 mM TBTC, (0.5 and 1 mM) DBTC and (0.5 to 2 %) ethanol. Absorbance at 600nm was monitored throughout the growth cycle and 1.5ml culture samples were withdrawn periodically every 4 hrs up to 20 hours to detect induction of stress proteins by SDS-PAGE. After harvesting, the cells were washed several times with 0.05 M Tris buffer (pH 8.0) and concentrated in the same buffer prior to protein extraction. For all experiments,

control cultures were incubated under identical growth condition to the tests but without the addition of metals and organometals.

Protein analysis

Preparation of cellular proteins (Cell lysis and protein extraction)

Samples (5 ml) were harvested during the growth cycle in 100 ml cultures by centrifugation (5 minutes, 10,000 rpm. at 4 °C, Remi cooling centrifuge, C-24 rotor) and cells were resuspended in 50 mM Tris-HCl buffer, pH 8.0 to concentrate these 10-fold. To extract the cellular proteins, the cells were disrupted by sonication (B. Braun. Biotech 450, micro tip, 50 % cycle duty, output 45 Watts) for 3 minutes. The cells were kept on ice containing ethanol during sonication and the tip allowed for cooling between 30 seconds pulse. Cell debris was removed by centrifugation for 15 minutes at 12,000 rpm, using cooling centrifuge. The supernatant was collected and kept at -20°C until use. Similar procedure was followed for the extraction of proteins from the metal treated cultures as well as control. The soluble protein concentration was determined by the Lowry method (Lowry et al.1951) before performing SDS-PAGE. The cells were washed in 50 mM Tris-HCl, pH 8.0, and resuspended in the same buffer. One volume of two-fold protein gel loading buffer was added to the sample then the mixture placed in boiling water for 5 minutes then placed on ice. The samples were centrifuged for 2 minutes (Eppendorf) prior to loading onto the SDS-PAGE gel to remove unlysed cells (Appendix G.1).

Estimation of protein concentration

The method modified from that described by Lowry et al. (1951) was used. Sample (100 µl in 50 mM Tris buffer, pH 8.0) was added to 0.5 ml of solution A (0.1 ml of 5 % $CuSO_4$, 0.9 ml of Na_2CO_3 in 10 ml of 0.5 M NaOH). After incubation at 37 °C for 10 minutes in a water-bath, 1.5 ml of solution B (1 ml Folin-Ciocalteu's, reagent plus 10 ml Milli-Q water) was added and then vortexed immediately. The sample was incubated for 20 minutes, and A_{660} readings were recorded using a Shimadzu spectrophotometer. Standards, ranging from 0 to 100 µg, were treated the same as samples and prepared from 1 mg/ml BSA solution (50 mM Tris buffer, pH 7.2). $CuSO_4$ solution (5 %) was stored at 4 °C and sodium carbonate solution was kept at room temperature. Reagents A and B were prepared immediately before use from stock solutions (Appendix F.6).

Polyacrylamide gel electrophoresis

One-dimensional (1-D) gel electrophoresis (SDS-PAGE)

Proteins were resolved by Polyacrylamide gel electrophoresis (SDS-PAGE) (Laemeli, 1970), which was performed using 10 % resolving gels (Protean II unit, Bio-Rad) overlaid with a 5 % stacking gel. Wells contained either 50 µg of proteins per sample or a constant amount of cellular material (O.D 0.6-0.7) as determined from A_{600} of the original sample and later concentrating. Electrophoresis was carried out in 1X Tris-Glycine electrophoresis buffer with a temperature control achieved by a cooling unit (Remi, Ltd). Samples were electrophoresed at 35 mA until the tracking dye entered the resolving gel, at which time the current was increased to 70 mA for 4 hours. Medium range molecular weight (MWM) (B. Genei) as standard marker was used. The SDS- Polyacrylamide gel was visualized by either Coomassie blue or silver staining (Appendix G.2) and photographed using gel documentation system (Bio Rad, U.S.A).

Polyacrylamide gel staining

Coomassie brilliant blue staining

A 0.5-mg/ml solution of Coomassie brilliant blue R-250 (Sigma) was prepared by dissolving the dye in five parts methanol before addition of one part acetic acid and four parts Milli-Q water. Gels were stained for 30 minutes at room temperature with gentle shaking. The gels were rinsed in Milli-Q water and transferred into destaining solution (one part acetic acid, four parts ethanol and five parts Milli-Q water) then gently shaken at room temperature until blue bands and a clear background were obtained. Fresh destaining solution was added if required. The gels were kept in Milli-Q water overnight after destaining and then dried using a gel air dryer (Appendix G.2).

Silver staining

Silver staining protocol was followed according to the instruction manual (Pharmacia) as follows. The gel was immersed in the fixing solution (one part acetic acid, four parts ethanol, and five parts Milli-Q water) for at least 30 minutes. The gel was placed in the incubation solution for more than 30 minutes. The gel was washed three times, each time for 15 minutes, in Milli-Q water. The gel was put into the silver solution (0.2 gm of silver nitrate, 40 µl of formaldehyde and made up to 200 ml with Milli-Q water) for 40 minutes. The gel was placed into developing solution for 5-10 minutes. Extra formaldehyde was

added if required. The gel was washed in Milli-Q water with one change for 5-10 minutes then preserved in 20 % glycerol as recommended by the manufacturer's instruction (Appendix G.2).

Gel photography

Gel photography was performed using a Fuji Camera; black and white film was used for documentation at a shutter speed of 1/60 to 1/30 seconds with an aperture of F16. Type 665-positive/negative films used at a shutter speed ¼ to 1/2 seconds and an aperture of F16 and alternatively the gel images were captured by Gel documentation system.

EPS Characterization

Screening and culturing of exopolymer-producing luminous bacteria

Exopolymer producing halophilic luminous bacterial strains were isolated from water samples collected from different sampling sites of river Mandovi and Zuari estuarine network connecting the coastal regions of Goa. The luminous bacterial isolates were obtained by serial dilution plating on TCBS Agar and Glycerol based Marine Agar media. A total of about 48 colonies were isolated, and the exopolymer-producing bacteria were screened for their ability to produce exopolymer based on colony morphology (mucoid phenotypes). Among the screened isolates, one halophilic luminous *Vibrio harveyi* strain VB23 having the ability to form a viscous exopolymer after the ice cold ethanol precipitation was identified according to *Bergey's Manual of Systematic Bacteriology* (Baumann and Schubert, 1984; Alsina and Blanch 1994) based on the morphological and physiological characteristics. The isolate VB23 was selected for the isolation, purification and characterization of the exopolymer. The culture VB23 was grown and maintained in optimized mineral salts medium (MSM) containing 0.2 % of glucose and 1.5 % of NaCl in 1 liter of distilled water.

Correlation of Growth with exopolymer production of strain VB23

Luminous bacterial cultures for exopolysaccharide production was grown and maintained as batch cultures in 200 ml of MSM medium supplemented with NaCl to a final concentration of 1.5 % (w/v), 0.2 % glucose in 500 ml Erlenmeyer flasks on a rotary shaker at 28±2 °C for 2 days. The pH of the medium was adjusted to 7.0 with 1 N NaOH. The medium 250 ml was dispensed in 500 ml Erlenmeyer flasks and inoculated with 2 %

(v/v) of an overnight grown culture in the same medium at room temperature (28±2 °C) on a rotary shaker at 160 rpm. The sub samples of 5 ml aliquots were drawn at regular intervals for turbidity measurements of bacterial growth (A_{600} nm) and exopolysaccharide production.

Exopolymer production in different Carbon and Nitrogen sources

Optimum level of carbon and nitrogen sources for EPS production and growth was determined prior to selection of best carbon and nitrogen sources. The effect of carbon source on the production of exopolymer was studied using mineral salts media with 2 % NaCl, 0.1 % of glucose, fructose, galactose, glycerol and mannose and 0.5 % of tryptone, malt extract, peptone, yeast extract and NH_4Cl were added separately. After 28 hrs, when the culture reached the stationary phase, cells were removed by centrifugation at 6000 rpm for 15 mins at 4 °C. The supernatant was passed through 0.22 µm pore size millipore filters. The supernatant is taken and 3 volumes of cold ethanol was added and incubated overnight at 4 °C and wet weight (µg/ml) was determined.

Exopolymer production of Luminous *Vibrio harveyi* in different heavy metal concentrations

The effect of different metals viz. 2 µM $HgCl_2$, 50 µM $CdCl_2$, 2 mM $NaAsO_2$, 2 mM $NaHAsO_4$ and organometals viz. 1 mM TBTC, 0.75 mM DBTC on the production of exopolymer was studied using mineral salts media with 1.5 % NaCl and 0.5 % of glucose. After 28 hrs, when the culture reached the stationary phase, cells were removed by centrifugation at 6000 rpm for 15 mins at 4 °C. The supernatant was passed through 0.22 µm pore size Millipore filters. The supernatant is taken and 3 volumes of cold ethanol (90 %) is added and incubated overnight at 4 °C and wet weight of the exopolymer (µg/ml) was determined.

Extraction and purification of Exopolysaccharide

The culture broth was centrifuged at 15,000 X g for 30 mins at 4 °C. The cell pellets were freeze-dried and weighed. The supernatants were pressure-filtered successively through cellulose nitrate filters with the following pore sizes: 0.8, 0.45 and 0.25 µm (Millipore filters). EPS was precipitated from the final filtrate after the addition of three volumes of cold ethanol (90 %) and the solution was chilled to 4 °C over night. The resulting

precipitate was recovered by vacuum filtration through a sintered glass. An additional 100 ml cold ethanol was added to the filtrate and the solution was placed at –20 °C overnight. The precipitate was recovered as above. The precipitates were washed with 70–100 % ethanol–water mixtures. After washing with ethanol, the recovered EPS was combined and dried in a desiccator and stored at room temperature. To remove excess salts, the EPS were redissolved in distilled water and dialyzed (molecular weight cut-off of 13 kDa, (Sigma–Aldrich Chemie Gmbh, Germany) against distilled water for 24 hrs on a magnetic stirrer at 4°C. Excess water was removed under vacuum dryer before lyophilization. The dialysate was lyophilized in (Labonco Lyophilizer, USA at 3000 psi). The lyophilized sample was stored at room temperature until chemical and physical characterization of the exopolymer.

Alcian blue staining

Anionic carbohydrates were stained for microscopic observation using Alcian Blue (Crayton, 1982). Cell suspensions of a culture in the late logarithmic phase were dried on sterile microscope glass slides. Uronic acids were stained using 0.1 % (w/v) Alcian Blue in 0.5 M acetic acid (pH 2.5) while sulphated sugars were stained using 0.1% (w/v) Alcian Blue in 0.5 M HCl (pH 0.5). Samples were incubated for 30 min and carefully rinsed with distilled water and observed under the phase contrast microscopy (X 100 magnification) and photographed using Olympus digital Camera.

Scanning electron microscopy (SEM)

The exopolymer was fixed for SEM analysis by the following series of treatments: 2% gluteraldehyde (1 h); 0.175 M phosphate-buffer (3 x 15 min); 50% acetone (1 x 15 min); 70 % acetone (1 x 15 min); 90 % acetone (1 x 15 min) and 100% acetone (3 x 15 min). The slides were thereafter dried in a critical point dryer, mounted on studs and coated with gold plasma and examined using the JOEL 5800 LV scanning electron microscope (Kumar et al. 2004) (Appendix C).

Quantitative analysis of exopolysaccharide

Exopolysaccharide extracted from supernatant fluids was lyophilized using Labconco lyophilizer (Kansas City, MO, USA) at 300 psi. Lyophilized exopolysaccharide was hydrolyzed with 2N HCl for 2 hr at 100°C in ampoules flushed with N_2 before sealing

(Read and Costerton 1987). Wet weight and dry weight of the exopolysaccharide are determined (unpublished data). The lyophilized sample is used for further chemical and physical characterization.

Emulsifying activity of exopolysaccharide

The emulsifying activity of EPS was assayed by the modified method of Rosenberg et al. (1979). Lyophilized EPS (0·5 mg) was dissolved in 0·5 ml deionized water by heating at 100 °C for about 15–20 min and allowed to cool to room temperature (28 °C). The volume was then made up to 2 ml using phosphate-buffered saline (PBS). The sample was vortexed for 1 min after the addition of 0·5 ml hexadecane. The absorbance at 540 nm was read immediately before and after vortexing (*A*0). The fall in absorbance was recorded after incubation at room temperature for 30 and 60 min (*A*t). A control was run simultaneously with 2 ml PBS and 0·5 ml hexadecane. The emulsification activity was expressed as the percentage retention of emulsion during incubation for time *t: At/A*0 x100.

Chemical analysis of the exopolysaccharide

The total carbohydrate content of extracted Exopolysaccharide was estimated using the phenol sulphuric acid assay with glucose as standard (Dubois et al. 1956). Uronic acids were assayed using the method of (Dische, 1962) with glucuronic acid as standard. Methyl pentoses (Dische and Shettles 1948). Sulphated sugars were determined by measuring sulphates according to the method of Terho and Hartiala (1971) after hydrolysis of the polymer. K_2SO_4 was used as standard. The protein content of the EPS was determined according to Lowry et al. (1951). Bovine serum albumin (BSA) 1mg/ml was taken as standard (Appendix F.1-F.6).

Analytical gas chromatography of the exopolymer

Alditol acetates derivatives of the monosaccharide sugars released by hydrolysis (Bhosle et al. 1995) from lyophilized EPS were analyzed by a capillary gas chromatography (Perkin -Elmer model 8310) equipped with a fused silica capillary column coated with CP Sil-88, (25 m, i.d. 0·32 mm, ds 0·12) Chrompack, Middleburg, The Netherlands). Flame ionization detector (FID), was used to separate the alditol acetate mixture, and oxygen-free dry helium was used as carrier gas at a flow rate of 25-30 ml. min^{-1}. The resulting

methyl glycosides were converted to their trimethylsilyl derivatives. The gas chromatograph oven temperature was programmed as follows: 70 °C, the oven temperature was then rapidly raised to 150°C and further programmed at 3 °C/min to 230 °C and maintained at this temperature for 40 mins. Inositol (1 mg/ml) was used as an internal standard. The monosaccharides present in the exopolymer are subsequently analyzed.

Fourier transformed infrared (FTIR) spectroscopy of the exopolymer

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

ESI -Mass spectra of the exopolymer

An ESI-Mass spectrum was recorded in the positive mode, on a Q-TOFXL MS/MS Applied Biosystems equipped with Sciex Analyst software. The declustering potential and the collision energy are optimized for MS/MS experiments so as to cause fragmentation of the selected molecular ion species as evident by the appearance of fragment ions and decrease in the intensity of the molecular ions and decrease in the intensity of the molecular ion. ESI –MS was carried out by dissolving the exopolymer (1 mg) in Methanol (MEOH) as a solvent.

Pigment profile

Pigment profile of *Vibrio harveyi* VB23 under metals and organo-metals stress

In order to evaluate the stress of metal and organometals on pigments over the growth cycle, Luminous bacterial cells were grown in 250 mL flask containing 100 ml of optimized mineral salts broth (supplemented with 1.5% NaCl, 0.2 % glucose), inoculated with 2 % overnight grown culture, incubated at 28± 2 °C for 24 hrs with shaking at 160 rpm. The stress conditions were achieved by adding heavy metals viz. 5, 7 µM of $HgCl_2$, 50, 100 µM of $CdCl_2$, 2.5, 5 mM of $NaAsO_2$, 2.5, 5, 7 mM of $NaHAsO_4$ and organometals viz. 0.25, 0.5 mM of TBTC, 0.5, 0.75 mM of DBTC. Growth was monitored throughout the growth

cycle and samples were withdrawn periodically at 4 hour interval up to 24 hours to detect the effect of stress on pigment pigments by UV-Vis spectrophotometry. The cells were washed several times with 0.05 M Tris buffer (pH 8.0) and concentrated in the same buffer prior to pigment extraction. For all experiments, control cultures were incubated under identical growth condition to the tests but without the addition of metals and organometals.

Pigment extraction

Luminous bacterial strain VB23 was harvested from late logarithmic phase the cells were removed by centrifugation at 6000 rpm for 15 mins at 4 °C and washed twice in ice cold 0.05 M Tris-HCl buffer. To extract the intracellular pigments, the cells were disrupted by sonication (B. Braun. Biotech 450, microtip, 50 % cycle duty, output 45 Watts) for 3 minutes. The cells were kept on ice-water containing ethanol during sonication and the tip allowed for cooling between 30 seconds pulse. Cell debris was removed by centrifugation (10 minutes, 12,000 rpm. on cooling centrifuge), the supernatant was collected in various organic solvents viz. methanol, acetone, ethyl acetate, ethanol, chloroform and tetrahydrofuran. The extracted pigment samples were filtered through 0.45 mm membrane filters prior to spectral scans and stored at -20°C until use.

Fourier transformed infrared spectroscopy of the pigment

The major structural groups of the purified pigment were detected using Fourier transformed infrared (FTIR) (Abu et al 1991). Pellets for infrared analysis were obtained by grinding a mixture of 2 mg pigment powder with 200 mg dry KBr, followed by pressing the mixture into a 16-mm diameter mold. The Fourier transform-infrared (FT-IR) spectra were recorded on a SHIMADZU- FTIR 8201PC instrument (Shimadzu, Japan) in the 4000–500 cm^{-1} region and spectra traced with a Hewlett Packard plotter.

HPLC analysis of the *Vibrio harveyi* VB23 pigment

Pigments were separated using HPLC with reverse phase column (Waters Spherisorb ODS 25 mm x 4.6mm x 250 mm) and a detection programme (Waters 2996 phase diode array detector). The samples were filtered through 0.45 mm filter prior to loading. 10 µl of the pigment sample was injected into the HPLC column. The gradient for separation was 0-100 % ethyl acetate in acetonitrile/ water (9:1) over 25 min with flow rate of 1.2 ml/min

and the peaks were detected at 445 nm. The quantity of pigments was calculated from peak area value using ß-carotene as external standard. Identification of pigments was carried out using retention time against standards and using spectral profile of individual peaks using PDA detector in the range of 400-700 nm.

RESULTS AND DISCUSSION

Growth:

Screening and isolation of heavy metal and organometal resistant luminous bacterial isolates

A total of 40 luminous bacterial isolates were isolated from environmental samples of west coast of India (Goa) and only 7 selective isolates were characterized on the basis of morphological, physiological and biochemical characteristics. The status of resistance against different heavy metals i.e. Hg (II), Cd (II), As (III), As (V) and organometals i.e. TBT and DBT was investigated. The present study clearly indicated that 80.0% of the luminous bacterial strains isolated showed resistance to As (V) and Sn (II), whereas 73.3% of the isolates exhibited resistance to cadmium, 71.1% to chromium and zinc and 48.8% to mercury. Approximately 40 % population showed TBT resistance and 35 % were resistant to DBT (Table 3.1.1). The predominant bacterial strains, screened from metal containing plates, were selected for their further characterization. The frequencies (%) of metal resistant isolates were comparable to those reported by earlier workers (Ramaiah and Chandramohan 1993; Malik and Ahmad 1994; Chaudhury and Kumar 1996). Most of the luminous bacterial isolates could not grow at higher metal and organo-metal concentrations due to cellular toxicity and inhibitory effect on metabolic process and viability of bacterial strains (Ulitzur, 1986) (Table 3.1.1).

Impact of heavy metals on growth response of *Vibrio harveyi* VB23

Toxic influence of heavy metals to aquatic organisms depends not only on metal concentration but also on the chemical form of their occurrence (speciation) (Sunda, 1990). However, the speciation depends on a number of factors viz. pH, temperature, salinity, concentration of inorganic ions, dissolved organic matter (Heng et al. 2004). Many bacterial species have been shown to develop resistance to Hg and other heavy metals (Nies, 1992; Silver and Phung, 1996; Nies, 1999). In addition, microorganisms in

the marine environment are thought to undergo selection pressures in the presence of toxic metal pollutants and develop resistance (Hideomi et al. 1977). Microorganisms exhibit several types of metal resistance mechanism (Nies, 1992; Ji and Silver 1995; Nies and Silver 1995; Rosen 1996; Silver 1996; Silver and Phung 1996; Nies, 1999). For example, many microorganisms can mitigate the toxicity of some metals (e.g., divalent mercury and arsenate) through reduction by using the metals as electron acceptors. Toxic metal cations may substitute for physiologically essential cations within an enzyme (e.g., Cd^{2+} may substitute for Zn^{2+}), rendering the enzyme nonfunctional (Silver 1996). Similarly, metal oxyanions, such as arsenate, may be used in place of structurally similar, essential nonmetal oxyanions, such as phosphate. In addition, metals impose oxidative stress on microorganisms (Kachur et al. 1998). Metal toxicity is most commonly ascribed to the tight binding of metal ions to sulfhydryl (–SH) groups of enzymes essential for microbial metabolism.

Sigmodial growth response of luminous *Vibrio harveyi* VB23 was noticed *at* different concentrations of heavy metals and organo-metals (Hg, Cd, Cr, As (III), As (V), Sn (II), TBT and DBT) in MSM with 1.5 % NaCl + 0.2 % glucose. Slight inhibition of growth was observed at 2 µM $HgCl_2$, 50 µM $CdCl_2$, 50 µM $K_2Cr_2O_4$, 2.5 mM AsO_3, 5 mM AsO_4 and $SnCl_2$. But when cells were exposed to higher concentrations of these metals and organometals, significant growth retardation along with extension of lag phase was observed (Figs 3.1.1 – 3.1.8). When cells grown in media supplemented with 5 µM Hg, 7 µM Hg, 100 µM Cr, 100 µM Cd, 0.5, 0.75 mM DBT, 0.75 and 1 mM DBT separately, growth response showed a much longer lag phase than in case of lower concentrations. This could be explained by the toxicity of heavy metals, which cause retardation in the growth of the strains. Growth retardation of *Vibrio harveyi* VB23 may be attributed due to the toxic effect of metals and organometals on the membranes and enzymes involved in various metabolic processes (Silver and Walderhaug 1992). The hierarchal order of toxicity of the heavy metals and organometals to the luminous bacterial strain VB23 is as follows; Hg > Cd > DBTC > Cr > TBTC > Sn > As (III) > As (V). Thus mercury proved to be most toxic metal cation and As (V) is least toxic oxyanion for *Vibrio harveyi* VB23.

Percent survival curves of VB23 in metals and organo-metals

Percent survival curves clearly indicated that 90.96 % cells were killed in presence of Hg (II) 10 µM, whereas survival at 50 % cells survived at 5 µM Hg (II). Likewise, 91.78 % killing was observed in presence of 150 µM Cr (VI), 83.1 % killing was observed at 150 µM Cd (II), 76.5 % killing was observed at 10 mM As (V) and 90 % killing was observed at 10 mM As (III) respectively. Whereas in the presence of organo-metals the killing of 67.32 % cell population was observed in presence of 1 mM TBTC and 82.11 % killing was observed in presence of 1 mM DBTC (Figs 3.1.9 –3.1.10).

In case of bioluminescent bacteria, there are no reports on toxic metals and organo-metals interactions and resistance mechanism, whereas plethora of reports is available in this regard in case of non-luminescent bacteria such as *Vibrio, Pseudomonas, Alteromonas, Flavobacterium, Aeromonas* and *Alkaligenes* spp. (Barkay, 1987; Osborn, et al.1997; Ravel et al. 1998; Muller et al. 2001; Ramaiah et al. 2002). Since several bioluminescent bacteria were isolated from various metal and organometals contaminated marine sites, it was imperative to study the impact of these environmental toxic pollutants on bioluminescent bacterial isolate *Vibrio harveyi* VB23. Similar to Cd^{2+}, Hg^{2+} can also compete for metal binding sites in metalloenzymes thereby inactivating catabolic and anabolic processes (Collins et al. 1989). Microbial populations generally resist Cd^{2+} toxicity by three main processes viz. intracellular sequestration, extracellular sequestration and efflux mediated transport proteins (Ford et al. 1995). Cd^{2+} is a potent scavenger of intracellular sulfhydryl groups and also binds nucleic acid bases with little specificity and typically induces DNA single strand breaks (Hughes et al. 1989). In addition to thiol reactivity, Cd^{2+} has been associated with enzyme inactivation as a result of histidyl and cysteinyl ligand binding (Collins, et al 1989). For these reasons, Cd^{2+} toxicity is commonly associated with both enzyme inactivation and DNA mutagenesis. Although not as well defined, Cd^{2+} can also complex phosphate groups, thereby affecting nucleic acids, phospholipid membrane structure, and phosphorylated metabolic intermediates (Collins et al.1989).

Hg^{2+} and Cd^{2+} were highly toxic metal cations tested for Vibrio harveyi VB23 with reference to survival and growth behavior, since these toxic metal cations are known to inactivate enzymes of metabolism, as a result of binding with –SH groups (Collins et

al.1989; Hughes et al. 1989). Other less toxic heavy metals are Cr^{6+}, As (III), As (V) and Sn (II) for *Vibrio harveyi* VB23 are possibly due to the presence of resistance mechanism such as enzymatic reduction and efflux mediated membrane transport proteins (Nies and Silver 1995; Silver and Phung 1996). Studies on TBT resistance from aquatic environments are extremely limited with few reports demonstrating the presence of plasmids (Cooney and Wuertz 1989; Fukagawa et al. 1994). In most cases it has been demonstrated that the resistance conferring genes are located on chromosomal genome (Suzuki and Fukagawa 1995). Fukagawa et al. (1992) have isolated and characterized tributyltin chloride resistant non luminous marine *Vibrio* spp. Jude et al. (2004) have demonstrated the involvement of MDR efflux pumps in *Pseudomonas stutzeri* for TBT resistance. Gene for TBT resistance in *Alteromonas* spp. M1 has been successfully cloned which is found to be encoding membrane transport protein (Fukagawa and Suzuki 1993).

Disc inhibition assays for the determination of heavy metal resistance

Assays were performed as described as Barkay et al. 1990. Using disc inhibition assays, *Vibrio harveyi* and *Photobacterium leiognathi* isolates showed a wide range of inhibitory responses for Hg, Cd, As, Sn TBT and DBT respectively (Table 3.1.2). Incase of VB9 Hg at a concentration of 7.5 µM showed a zone of inhibition of 8 mm diameter, Cadmium 150 µm showed an inhibition zones in the ranges of 13 mm diameter, whereas the lowest concentrations of Hg and Cd did not show significant zones of inhibition. As (III), As (V) and Sn (II) showed inhibition zones in the ranges of 0-9 mm, however Sn (II) and As (V) tends to be more resistant with compared to As (III). For all the tested strains Cd 25 µM, As (III), As (V), Sn (II) 5 mM TBT 50 µM showed no zones of inhibition at all. Organometals at the concentrations of 50, 70 and 100 µm showed the zones of inhibitions in the ranges of 0- 6 mm. None of the strains tested had zones of inhibition larger than 13 mm around discs containing either 7.5 µM Hg, 150 µM Cd, 10 mM As (III), As (V), 10 mM Sn (II) or 100 µM TBT, DBT respectively. The variable response of bacterial strains to metals and organometals was not surprising because metal resistance is often specified by plasmids and transposons (Belliveau et al. 1987; Mergeay, 1994; Silver and Phung 1996).

Regulation of metal toxicity by thiols and chelating agents

In order to check the regulation of metal and organo-metal toxicity by (Thiols) Glutathione, cysteine, Dithiotriol, β-mercaptoethanol and chelating agent (EDTA-Na_2), non-inhibitory level of these compound was combined with Hg (II) 7 µM, Cd (II) 100 µM and TBT 0.75 mM separately in the growth medium. It was observed that in presence of chelating agent Na_2- EDTA the culture could grow in presence of $HgCl_2$, $CdCl_2$ and TBT up to 9 µM, 150 µM and 1 mM respectively (Fig 's 3.1.1, 3.1.2 and 3.1.13). In case of EDTA, it was observed that the culture could resist up to 1.2 mM of TBTC and decreased its resistance with subsequent increase of TBTC concentration (Fig 3.1.13). In presence of thiol compounds (mercaptoethanol, DTT and sulphur containing amino acid cysteine) (Appendix D.4), the toxicity of all these metals is less ameliorated as compared to chelating agent. the present study the best metal regulation was observed with EDTA with compared to thiol compounds.

It was interesting to note that the culture VB23 which was growing in presence of 5 µM $HgCl_2$, and 100 µM $CdCl_2$ could grow up to 7 µM and 125 µM respectively, when glutathione was supplemented in the medium. This observation indicated that these modifying factors have a similar effect of reducing the heavy metal toxicity. Generally it was observed that heavy metal resistant microorganisms such as yeast, cyanobacteria (*Synechococcus* spp.) and bacteria (*Pseudomonas putida, Vibrio alginolyticus*) induce metallothionein like proteins, which are capable of binding heavy metal ions via thiolate co-ordination. This plays a very important role in sequestering metal ions intracellularly by rapidly binding to them as they enter the cell, thus effectively reducing the toxicity (Higham et al. 1984; Robinson and Tuovinen, 1984; Gadd, 1986; Khare et al. 1997). The present investigation showed that thiols and chelating compounds reduce the toxicity of heavy metals to the cells. A similar kind of observation was also made by Dubey and Rai (1989), in *Anabeana doliolum*, which revealed that mercaptoethanol and dithiotritol reduced the toxicity of chromium and tin, dithiol being more protective than mono-thiols. The better protective efficiency of dithiothreitol and mercaptoethanol could be due to restoration of proton transfer across the biomembrane. The toxicity of the heavy metal or organic compounds gets reduced by formation of complexes with S^- and SO_4 ions, which are unable to cross the interior of biomembranes because they are too large (Dubey and Rai 1989).

In case of *E. coli,* the inhibitory effect of TBTC on the growth was reduced by 10-25% in presence of dithiothreitol (Singh, 1989). It has been reported that some of the organotin compounds, including TBTC, act like organomercurials and inhibit the activity of many enzymes by interacting with thiol groups of protein (Hallas and Cooney 1981). This inhibitory effect of TBTC on above process was completely abolished in presence of various mono- and dithiols (Singh, 1989). In an earlier report the EDTA could strongly compete with the bacterial surface for heavy metals like cadmium and the presence of EDTA significantly diminishes Cd adsorption onto *B. subtilis.* The most possible reason was the formation of Cd-EDTA complex (Fein and Delea 1999). In *Pseudomonas aeruginosa,* β- mercaptoethanol and EDTA act as best desorption agents of heavy metal ions like Cu^{+2}, Hg^{+2}, Pb^{+2} (Chang et al. 1997). Overall EDTA-Na_2 proved to be best ameliorating agent against metal toxicity in *Vibrio harveyi* VB23 (Figs 3.1.11 & 3.1.12).

Antibiotic susceptibility testing

The antimicrobial susceptibility tests were determined in the Mueller Hinton agar with 1% NaCl. The control strain *E. coli* (ATCC 11775) tested on media containing 1% NaCl gave good bacterial growth and produced inhibition zones equal to the standards for each antimicrobial tested. All luminous bacterial isolates (VB6, VB23, VB9, VB30, VB50, BR9, DNIW and VB1) studied were highly susceptible to tetracycline, streptomycin, kanamycin, rifamycin, novobiocin and co-trimaxazole and nitrofurantoin respectively (Table 3.1.4). More than 95% of isolates showed a resistance to β-lactam antibiotics such as ampicillin, cephalothin, mecillinam, penicillin and amoxycillin. Moderate resistance pattern was observed for glycopeptide inhibitor vancomycin and cytoplasmic membrane inhibitor such as polymyxin–B, protein inhibitors such as chloramphenicol, gentamycin, co-trimaxazole, amikacin, erythromycin, streptomycin and rifamycin. Whereas all the luminous bacterial isolates were highly susceptible to the protein inhibitors such as tetracycline, streptomycin and kanamycin. More than 85 % of the isolates are resistant to bacterial nucleic acid and synthesis inhibitors such as trimethoprim, ciproflaxacin, nalidixic acid and norfloxacin, whereas only few isolates viz VB6, VB23, BR9 and DN1W are resistant to nitrofurantoin without significant variation between different species of luminous bacterial isolates. There were hardly few intermediate zone sizes occurred between 5 and 10 % of the strains (Table 3.1.4).

Percent survival of VB23 in broad range antibiotics

65.76 % killing was observed in presence of Novobiocin 100 µg/ml, Likewise, 77.48 % killing was observed in presence of Kanamycin 75 µg/ml, 47.54 % killing was observed at 125 µg/ml Nystatin, 89.76 % killing was observed at 125 µg/ml of chloramphenicol, 97.14 % killing rate was observed at 100 µg/ml tetracycline, 93.25 % killing at 100 µg/ml valinomycin, 93.35 % killing at 100 µg/ml of cyclohexamide, 92.15 % killing at 75 µg/ml rifamycin, 75.38 killing at 100 µg/ml of ampicillin, 93.55 % killing at 100 µg/ml of streptomycin respectively. Highest killing were observed for streptomycin, tetracycline, cyclohexamide and rifamycin and these antibiotics attributes to be more sensitive to VB23 when compared to rest of antibiotics used. The test organism VB23 was resistant to following antibiotics such as novobiocin, ampicillin, nystatin and chloramphenicol, which may be attributed to the antibiotic resistance markers located on plasmids.

MIC determination of antibiotics

The tolerance limits of the heavy metal resistant luminous bacterial isolates to various antibiotics (β -lactam antibiotics and protein and DNA inhibitors) expressed as MIC values are shown in the Table. On the basis of MIC values the strains VB6 and VB23 are highly resistant, the strains BR9, DN1W and VB02 are moderately resistant and the strain VB9 is least resistant to the antibiotics. The MIC (µg/ml) of antibiotics (β-lactam, protein and DNA inhibitors) varied depending upon the resistance pattern in each luminous strain. Often, antibiotic resistance genes encoding resistance to a variety of antibiotics, such as β -lactams, chloramphenicol, and aminoglycosides, are found integrated in a site-specific manner in a mobile gene cassette or integron (Recchia and Hall 1997). Extra-chromosomal genetic elements of the bacterial cells may be the reason for the resistance to different antibiotics.

Earlier reports have exhibited the multiple resistances in *Vibrio* spp. to ampicillin and trimethoprim (Li et al.1999), and to chloramphenicol, tetracycline and ampicillin. Multiple resistances to antimicrobials (nitrofurantoin, novobiocin and sulphonamide) against *Vibrio harveyi* isolates described in this study have been previously reported in luminous bacteria by (Abraham et al. 1997; Li et al. 1998). High-level resistance to broad-spectrum β -lactam antibiotics in gram-negative bacteria is usually due to β-lactamase gene specified on plasmids (Nandivada and Amyes, 1990; Rahman Khan and Malik 2001).

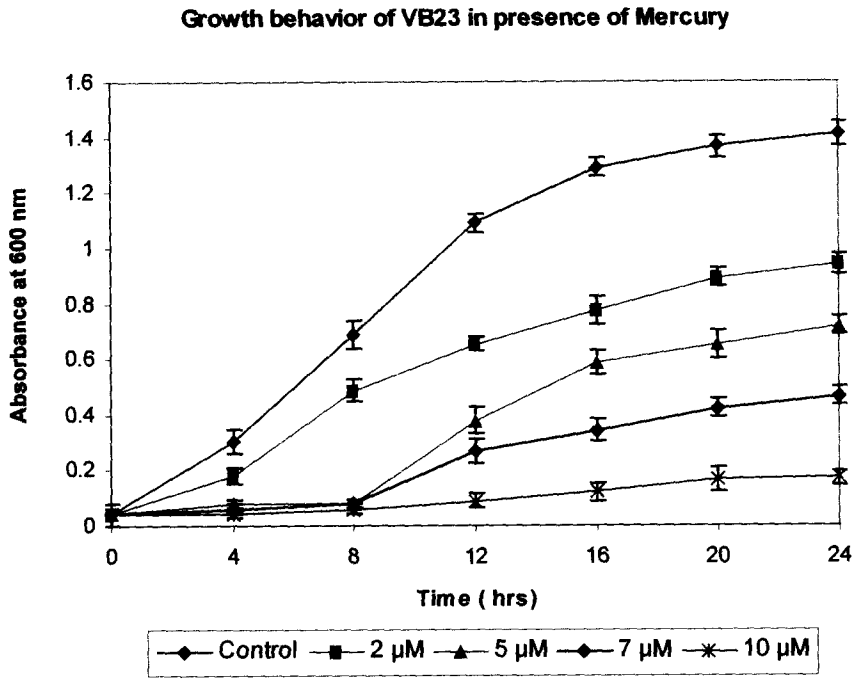
Multiple resistances of luminous bacterial isolates to antimicrobials described in this study have never been previously reported to our knowledge.

Correlation of heavy metal resistance and antibiotic resistance

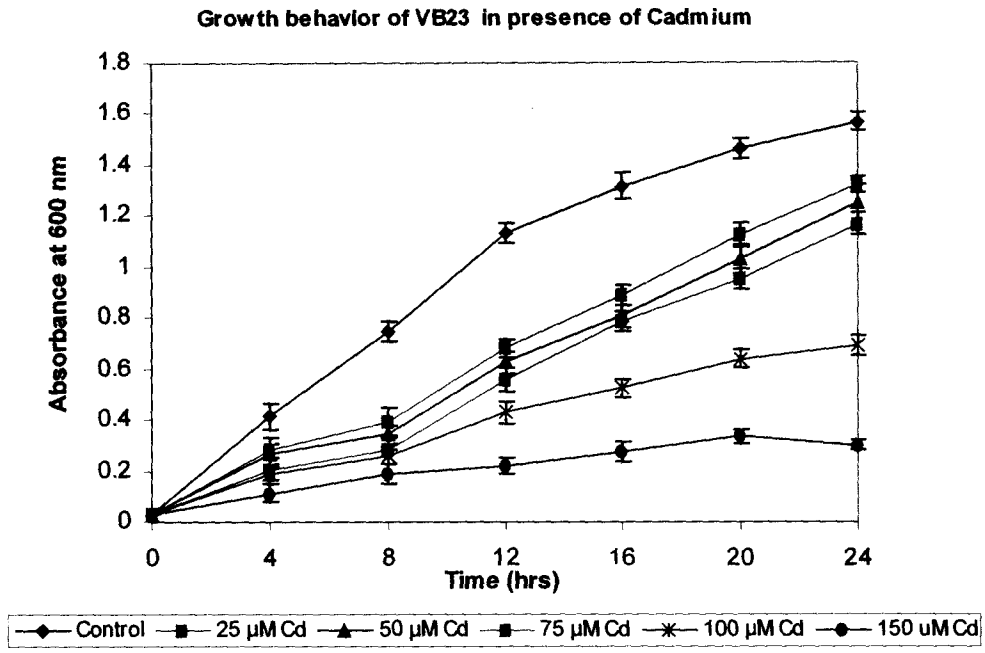
An implication of heavy metal tolerance in the environment is that it may contribute to the maintenance of antibiotic resistance genes by increasing the selective pressure of the environment. Many earlier reports have shown that a correlation exists between metal tolerance and antibiotic resistance in bacteria because of the likelihood that resistance genes to both (antibiotics and heavy metals) may be located closely together on the same plasmid in bacteria and are thus more likely to be transferred together in the environment. Previous studies have demonstrated the role of plasmids in conferring resistance to both antibiotics and metals in number of marine bacterial isolates (Belliveau et al. 1987; De vicente et al.1990; Choudhury and Kumar (1998). It is clear that multiple resistances to metals and antibiotics are widespread among TBT-resistant organisms isolated from both estuarine and freshwater sites. Both antibiotic resistances and heavy-metal resistances can be plasmid mediated (McArthur and Tuckfield 2000).

Heavy metal / Organometals	No: of isolates tested	No: of resistant isolates	% Resistant isolates
Inorganic Heavy metals			
$HgCl_2$ - (Hg^{2+})	40	14	35%
$CdCl_2$ - (Cd^{2+})	40	20	50%
$NaAsO_2$ -As (III)	40	28	70%
$NaAsO_4$ -As (V)	40	35	87.5%
$SnCl_2$ - Sn (II)	40	35	87.5%
CrO_4 - Cr (VI)	40	20	50%
Organo- metals			
TBTC	40	16	40%
DBTC	40	14	35%

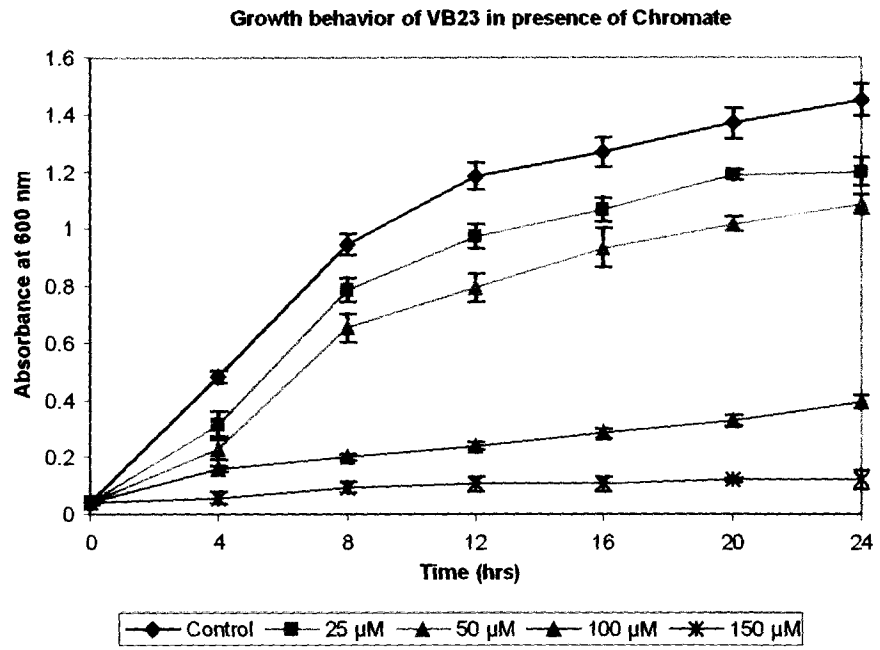
(Table 3.1.1). Screening of metal and organo- metal resistant luminous bacteria



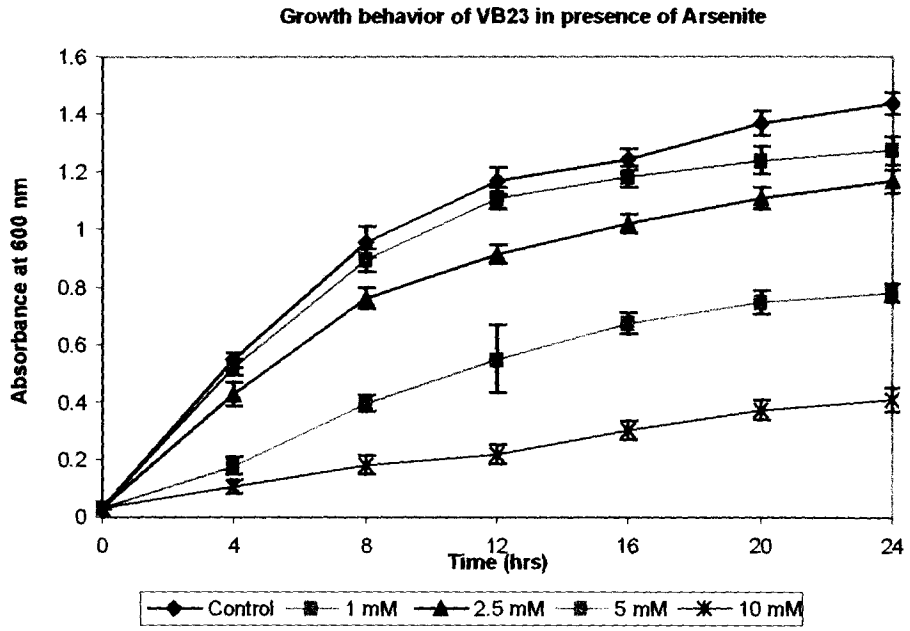
(Fig.3.1.1). Growth behavior of Luminous bacterial strain VB23 in Hg (II)



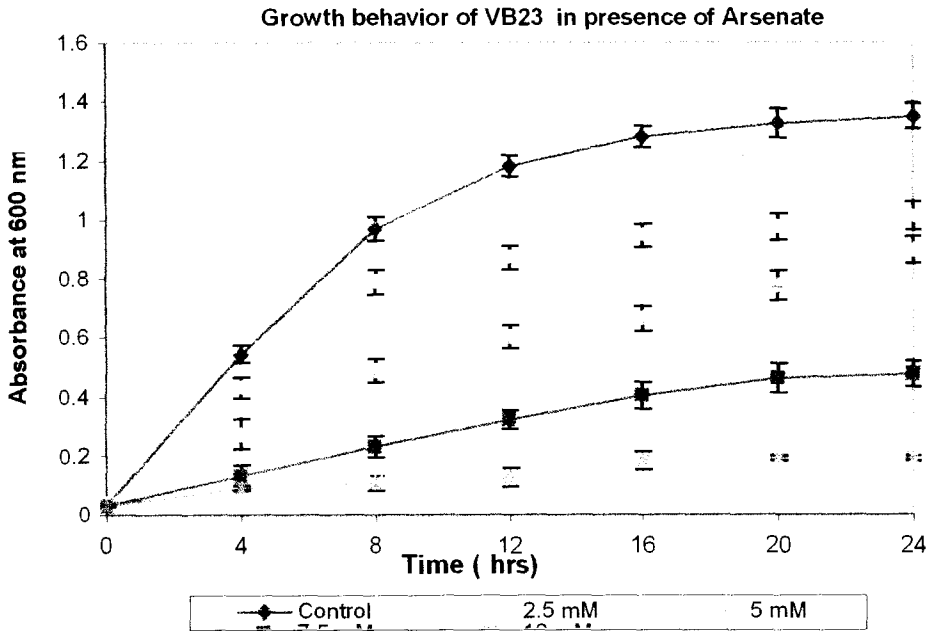
(Fig.3.1.2). Growth behavior of Luminous bacterial strain VB23 in Cd (II)



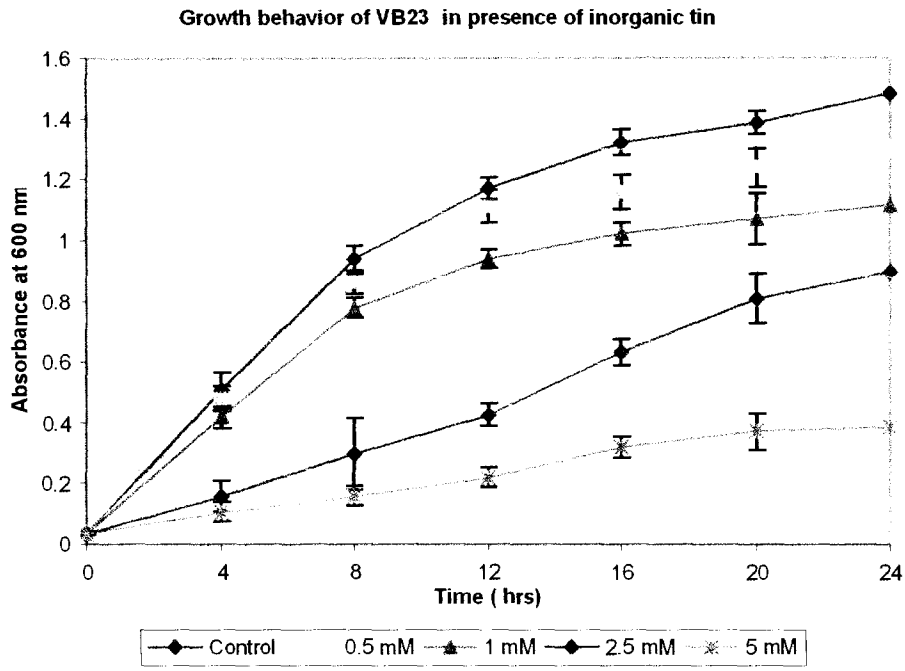
(Fig.3.1.3). Growth behavior of Luminous bacterial strain VB23 in Cr (VI)



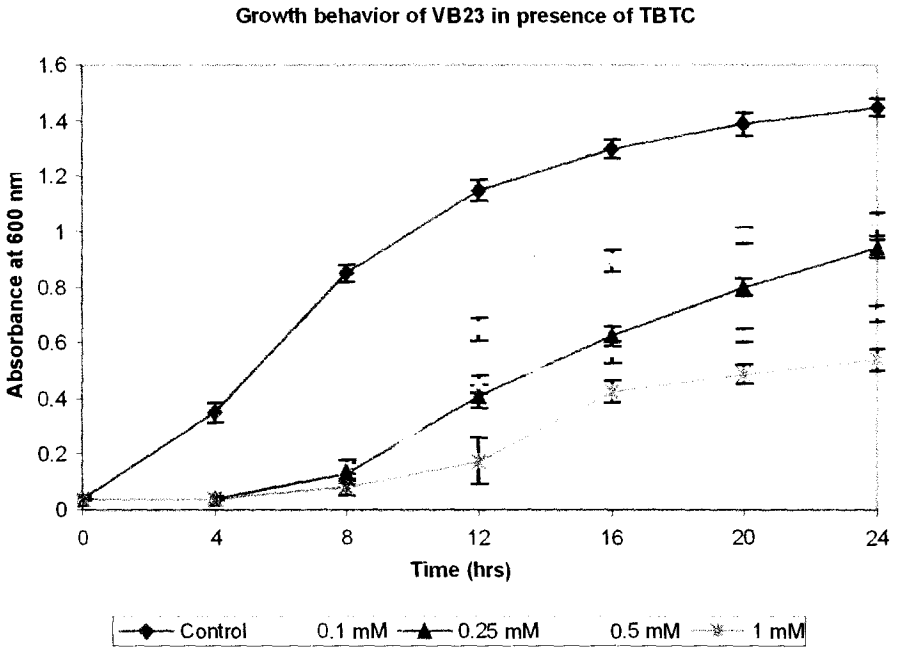
(Fig.3.1.4). Growth behavior of Luminous bacterial strain VB23 in As (III)



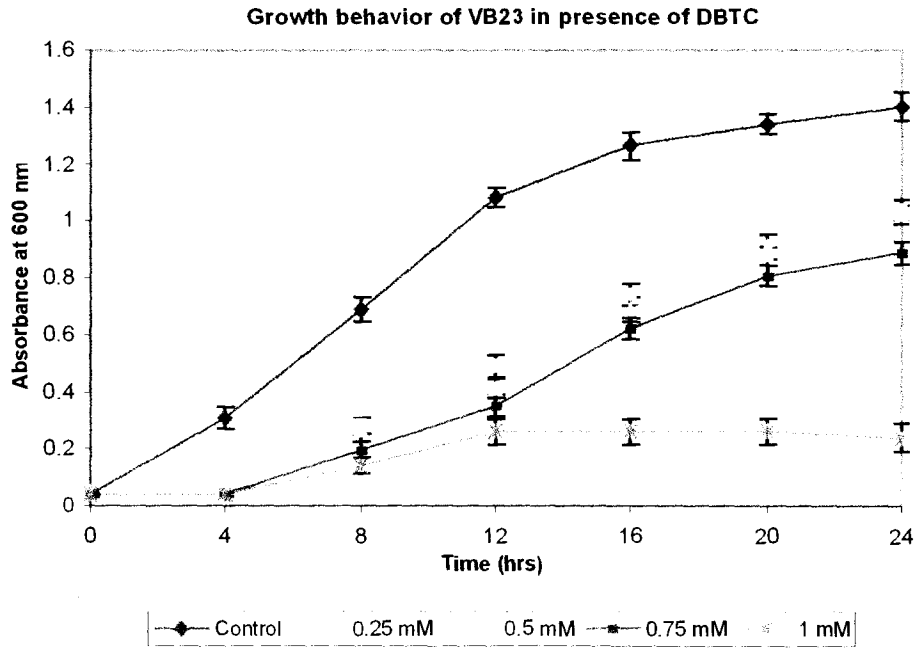
(Fig.3.1.5). Growth behavior of Luminous bacterial strain VB23 in As (V)



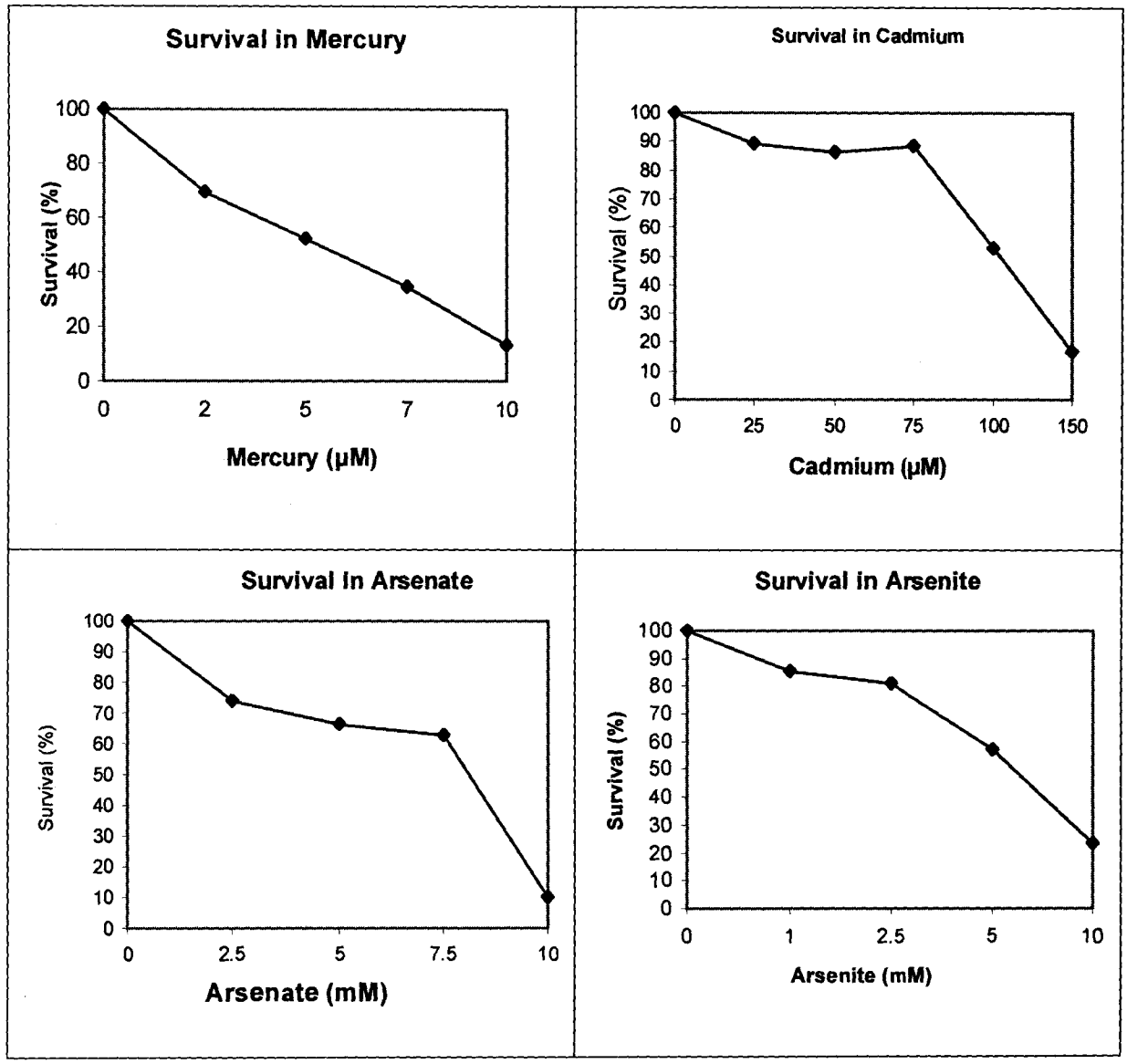
(Fig.3.1.6). Growth behavior of Luminous bacterial strain VB23 in Sn (II)



(Fig.3.1.7) Growth behavior of Luminous bacterial strain VB23 in TBTC



(Fig.3.1.8). Growth behavior of luminous bacterial strain VB23 in DBTC



(Fig 3.1.9). Percent survival of *Vibrio harveyi* strain VB23 in various heavy metals

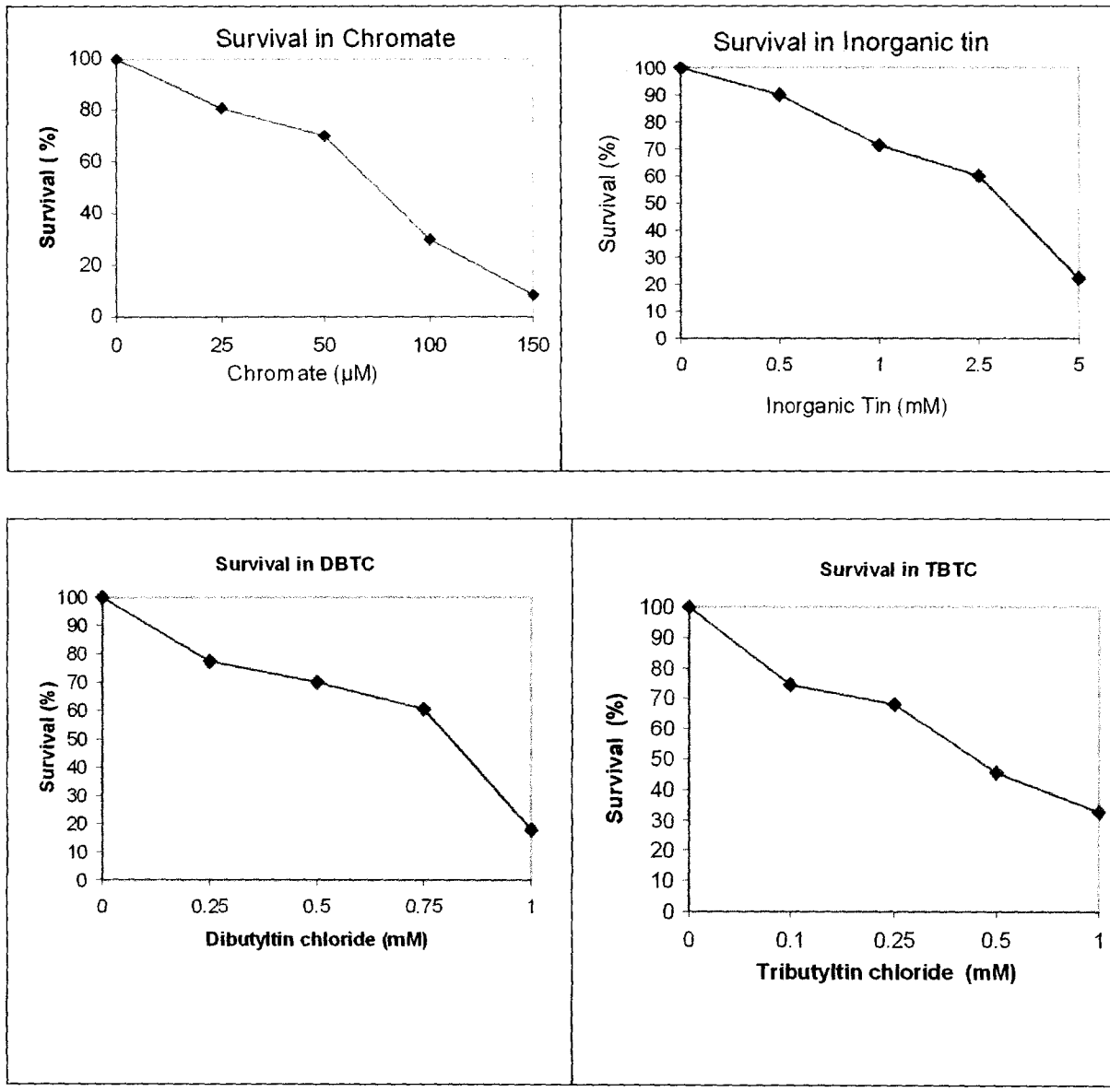
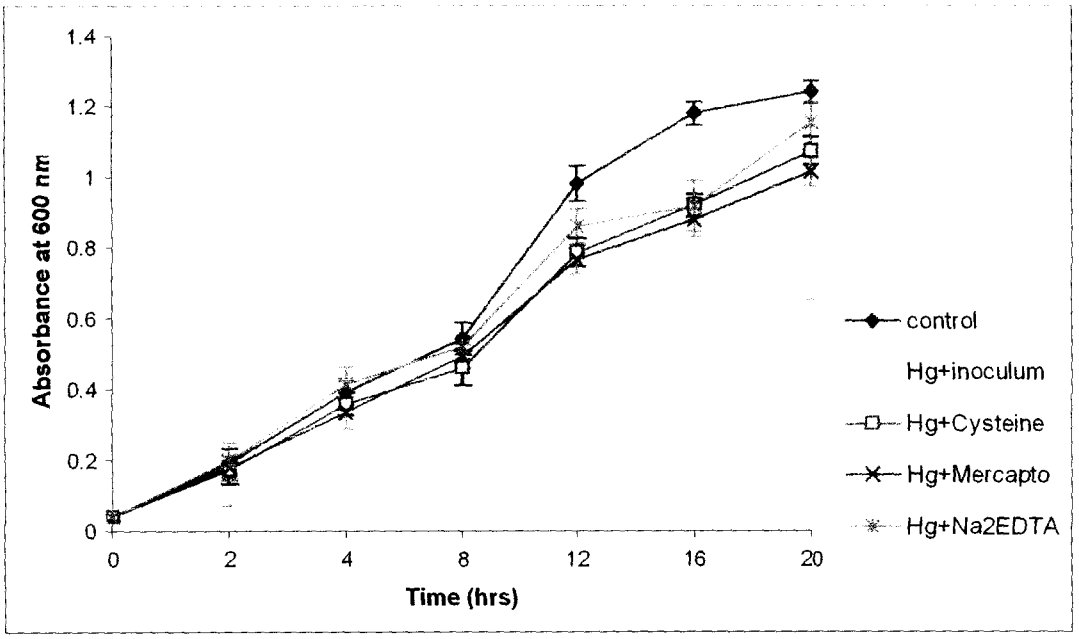


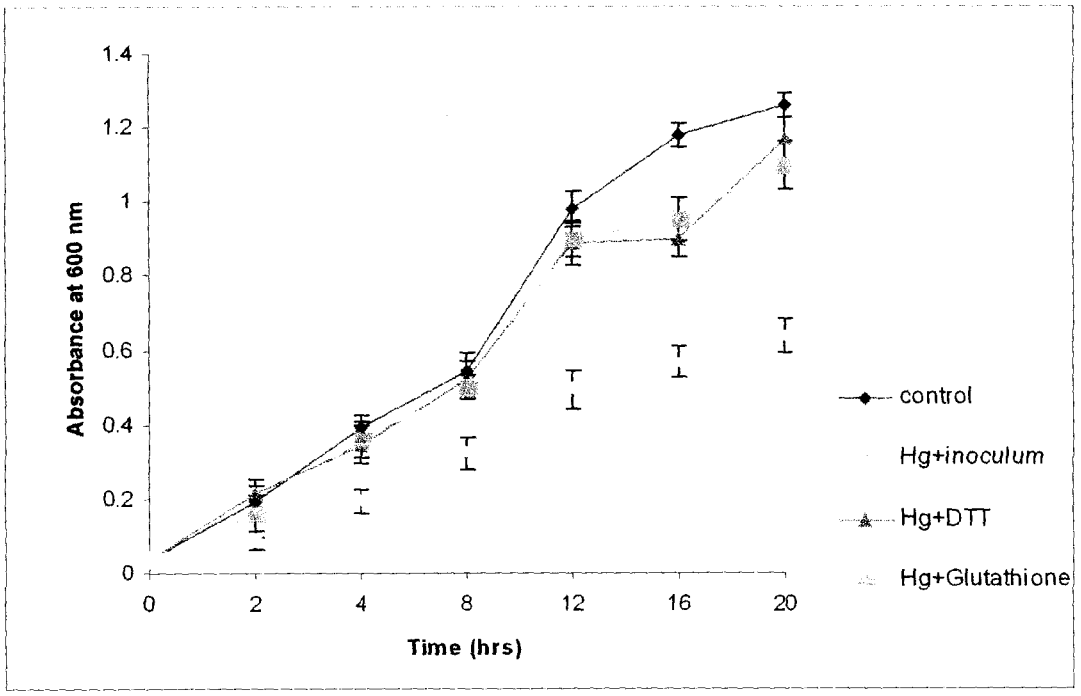
Fig (3.1.10). Percent survival of *Vibrio harveyi* strain VB23 in various metals and organo- metals

S.No	Heavy metal and Organometals	Zones of Inhibition in diameter (mm)			
		VB6	VB9	VB23	DN1W
1	**$HgCl_2 - Hg^{2+}$**				
	5µl-2 µM	2	2	2	2
	10µl- 5 µM	4	6	4	4
	15 µl- 7.5µM	4	8	4	5
2.	**$CdCl_2 - Cd^{2+}$**				
	5µl- 5 µ M	6	6	6	6
	10µl- 10 µM	6	6	6	6
	15 µl- 25 µM	8	11	8	10
	20 µl- 50 µM	8	13	8	10
3.	**$NaAsO_2$- As $^{3+}$**				
	10 µl –5 mM	0	3	0	0
	15µl – 7.5mM	3	6	4	4
	25µ l -10 mM	4	9	4	4
4.	**$NaHAsO_4$ –As $^{5+}$**				
	10 µl- 5 mM	0	3	0	0
	15 µl- 7.5 mM	3	8	3	3
	25 µl—10 mM	6	8	6	6
5.	**$SnCl_2$-Sn $^{2+}$**				
	10 µl- 5 mM	0	2	0	0
	15 µl- 10 mM	2	2	2	2
6.	**CrO_4- Cr^{6+}**				
	10 µl- 50 µM	4	8	4	8
	15 µl- 75 µM	6	10	6	12
	Organometals				
	TBTC-Sn	0	3	0	0
7.	5 µl –50µ M	2	4	2	2
	7 µl – 70 µM	4	6	4	4
	10µl—100 µM				
8.	**DBTC- Sn**	2	4	2	2
	5 µl –50 µ M	4	4	4	4
	7 µl – 70 µM	4	6	4	4
	10 µl- 100µ M				

(Table 3.1.2). Heavy metal sensitivity of luminous bacterial isolates VB6, VB9, VB23 and DN1W

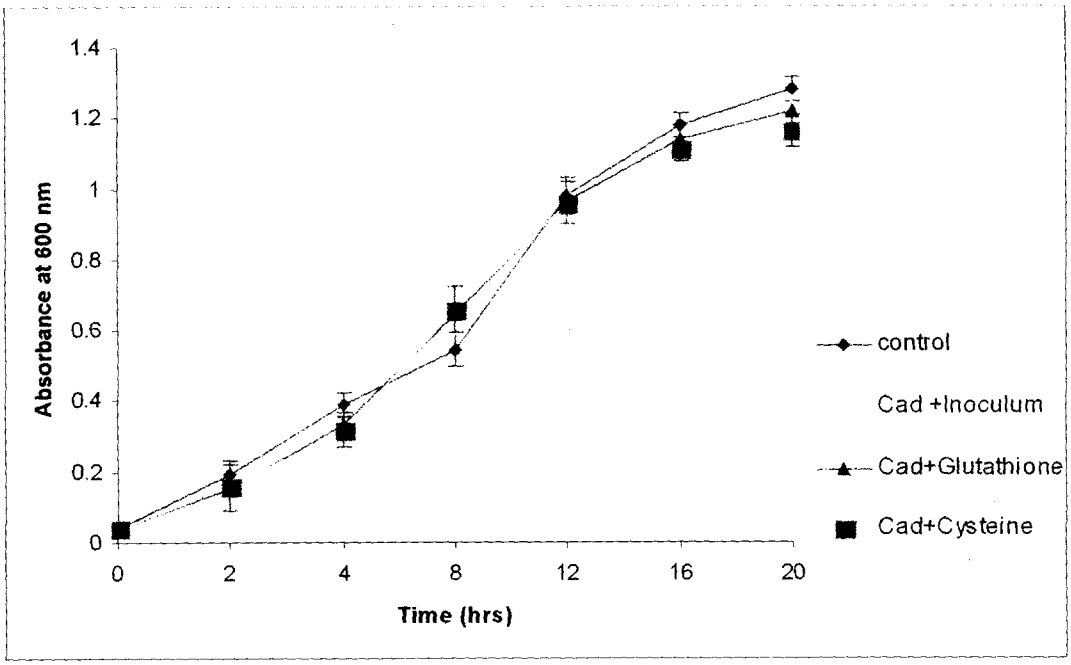


A

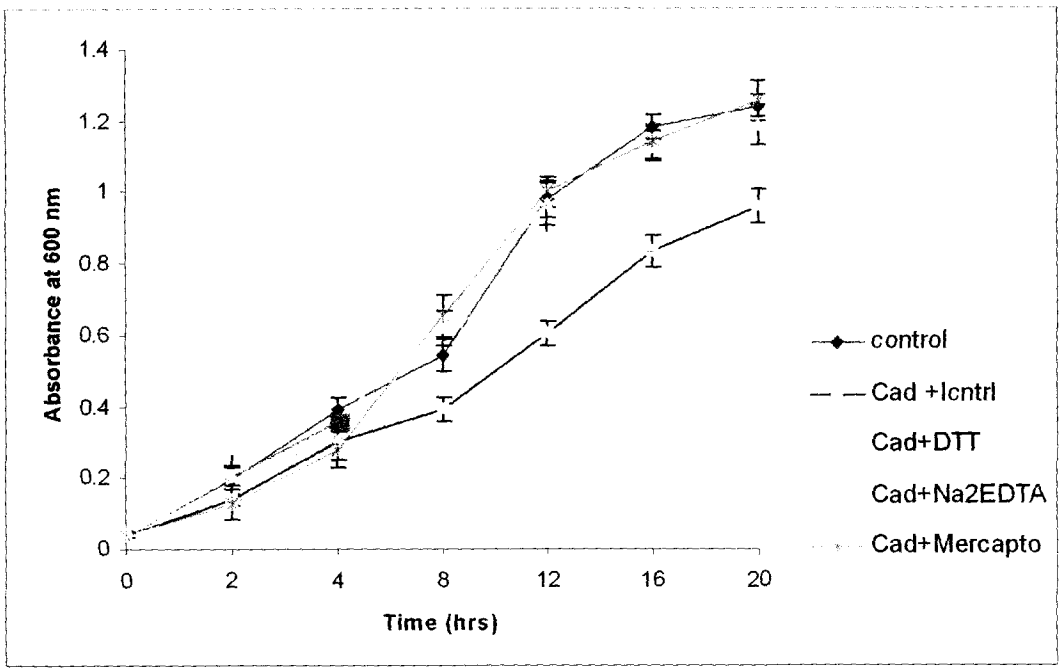


B

(Fig 3.1.11 A and 3.1.11 B). Ameliorative effect of chelating agents and thiol compounds on (Hg^{2+}) toxicity to luminous *Vibrio harveyi* strain VB23

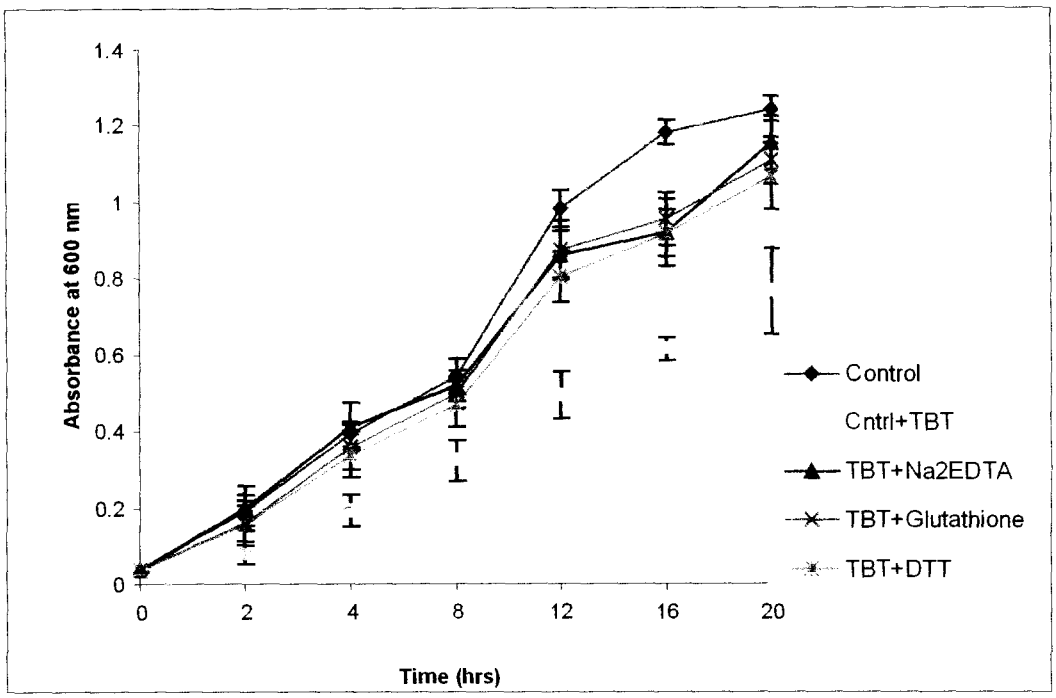


A



B

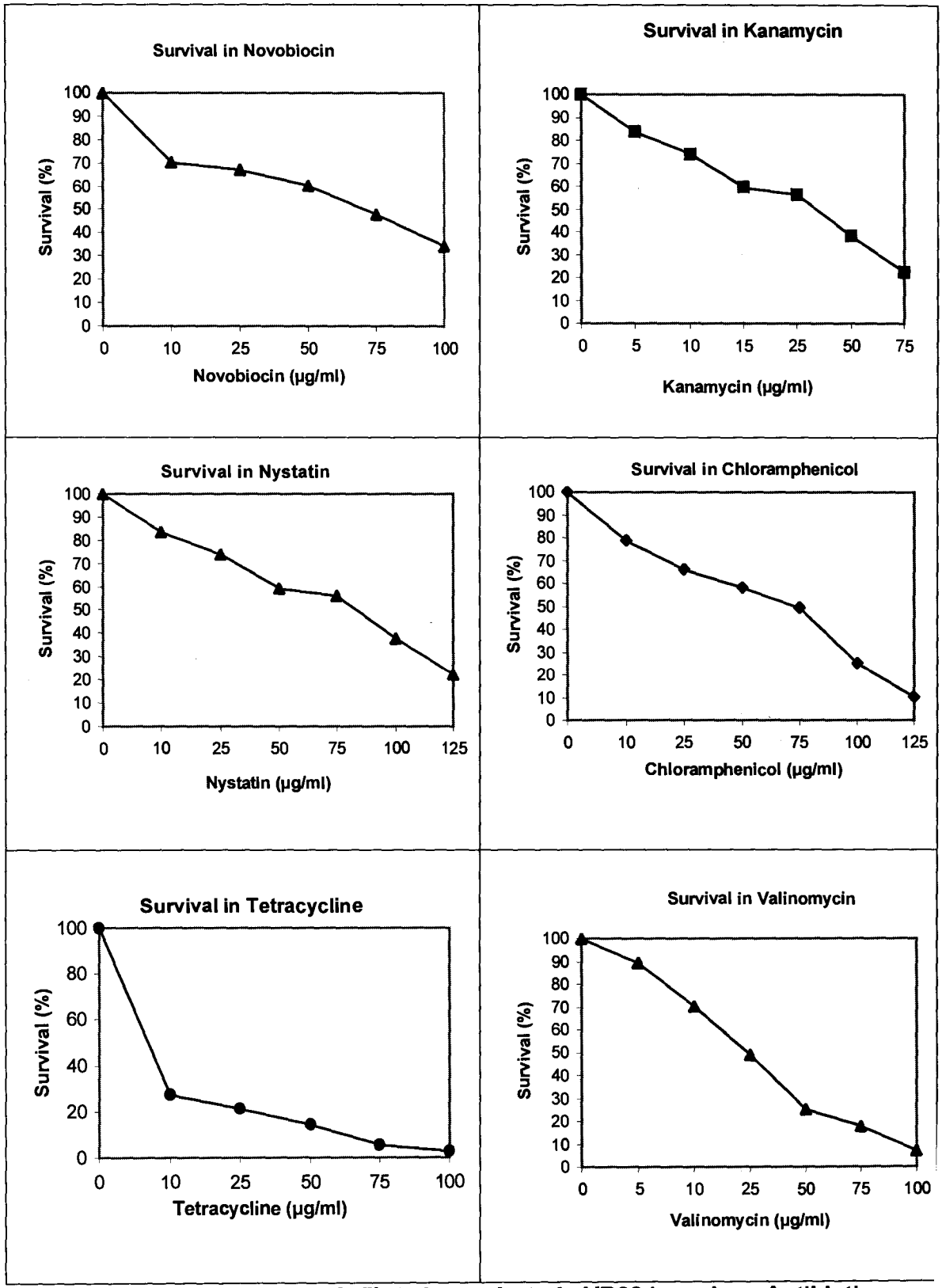
(Fig 3.1.12 A and3.1.12 B). Ameliorative effect of chelating agents and thiol compounds on (Cd^{2+}) toxicity to luminous *Vibrio harveyi* strain VB23.



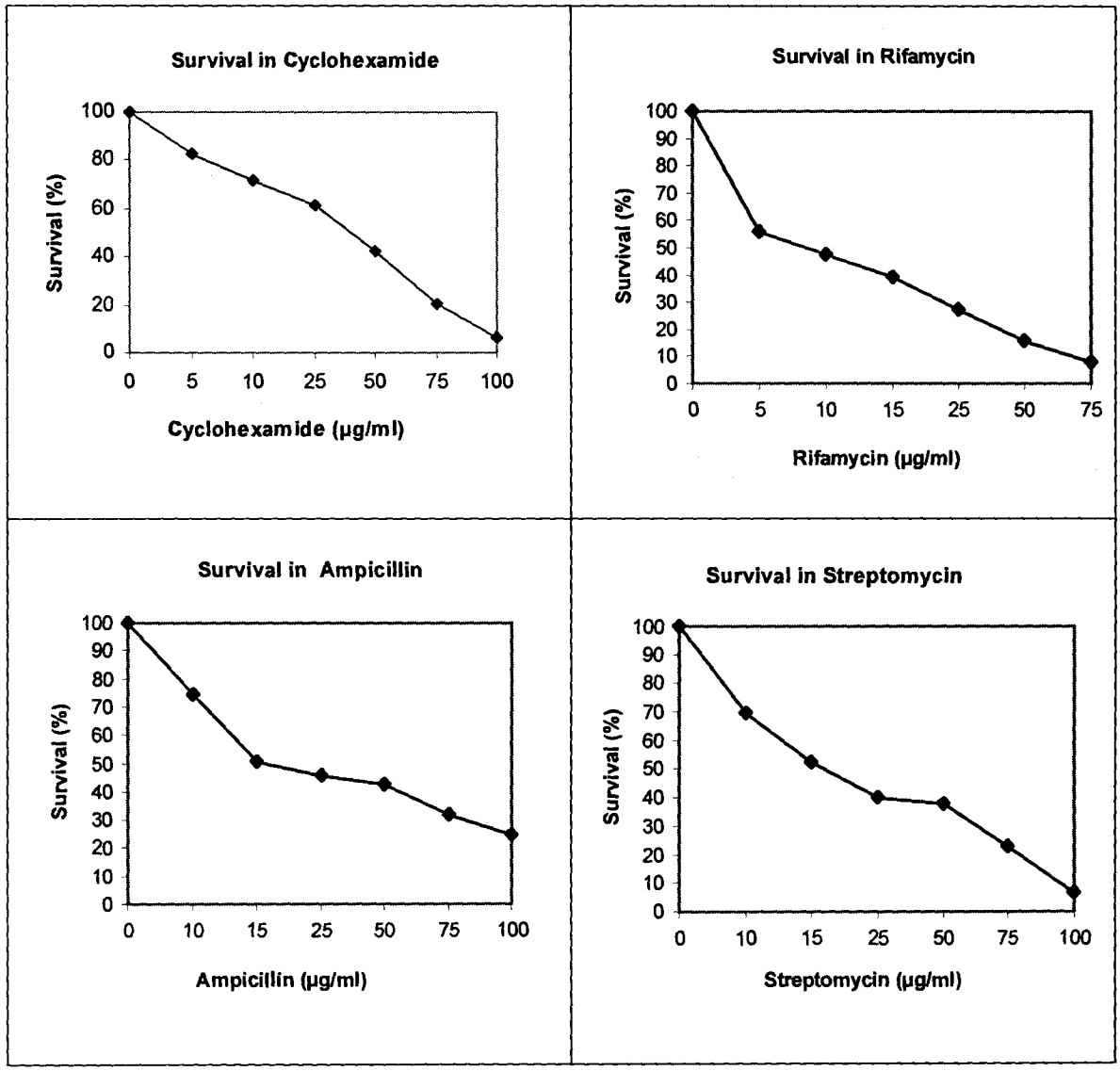
(Fig 3.1. 13). Ameliorative effect of chelating agents and thiol compounds on (TBT) toxicity of luminous *Vibrio harveyi* strain VB23

S.No.	Antibiotics	MIC values in µg/ml					
	Protein and DNA inhibitors	**VB6**	**Vb9**	**Vb23**	**BR9**	**DN1W**	**VB02**
1	Chloramphenicol	100	75	100	100	100	100
2	Erythromycin	75	50	75	75	75	75
3	Nalidixic acid	75	100	100	50	100	100
4	Streptomycin	50	25	50	50	50	50
5	Neomycin	75	50	75	50	50	50
	β-lactam antibiotics						
6	Ampicillin	200	75	100	75	100	100
7	Penicillin	150	200	150	150	150	200
8	Carbenicillin	100	150	150	150	150	150
9	Amoxicillin	150	100	100	100	100	100
10	Cephalothin	100	100	150	100	100	100

(Table 3.1.3). MIC values of heavy metals and Organo-metals in MSM Broth + 2%NaCl +0.2%Glucose for various luminous bacterial isolates



(Fig 3.1.14 A). Percent survival of *Vibrio harveyi* strain VB23 in various Antibiotics



B

(Fig 3.1.14 B). Percent survival of *Vibrio harveyi* strain VB23 in various Antibiotics

Broad range Antibiotics	ATCC *E. coli* 117755	VB6	VB23	VB9	VB30	VB50	BR9	DNIW	VB1
Kanamycin (K) 15μg	S	R	R	S	S	R	R	R	S
Ampicillin (A) 100μg	R	R	R	R	R	R	R	R	R
Tetracyclin (T) 25mcg	S	S	S	S	S	R	S	S	S
Chloramphenicol (C) 30μg	S	R	R	R	R	R	R	R	R
Gentamycin (G) 30μg	R	R	R	R	R	R	R	R	R
Co-trimoxazole (Co) 25 μg	S	R	R	R	S	S	R	R	R
Amikacin (Ak) 30μg	R	R	R	R	R	R	R	R	R
Nalidixic acid (Na) 30μg	S	R	R	R	R	R	R	S	R
Cephalothin (Cp) 30μg	R	R	R	R	R	R	R	R	R
Mecillinam (Mc) 33μg	R	R	R	R	R	R	R	R	R
Ciprofloxacin (Cp) 10μg	S	R	R	R	R	R	R	R	R
Erythromycin (E) 15μg	R	R	R	R	R	R	R	R	R
Cephalothin (Cf) 100 μg	R	R	R	R	R	R	R	R	R
Amoxycillin (Am) 30μg	S	R	R	R	R	R	R	R	R
Novobiocin (Nv) 30μg	S	R	R	R	S	S	R	R	R
Nitrofuran (Nf) 300μg	S	R	R	S	S	S	R	R	R
Trimethoprim (Tr) 5μg	R	R	R	R	R	R	R	R	R
Polymyxin-B (P) 300μg	R	R	R	R	R	R	R	R	R
Vancomycin (Va) 30μg	R	R	R	R	R	R	R	R	R
Streptomycin (S) 100μg	S	S	S	S	R	R	R	S	R
Rifamycin (Rf) 10μg	S	R	R	R	R	S	S	S	R
Norflaxacin (Nr) 10 μg	R	R	R	R	R	R	R	R	R

(Table 3.1.4). Antibiotic Sensitivity of Luminous isolates from Marine and Estuarine habitats of Goa

Measurement of Bioluminescence

Impact of heavy metals, organo metals antibiotics and mutagens on Bioluminescence (RLI)

The percent relative light intensity (RLI) for control culture (without metals) was considered as 100 %. RLI scan of luminescent bacteria using spectroflourimeter exhibits 3 significant peaks at 468, 482 and 492 nm in the blue –green region have been considered for comparison. % Relative light intensity (RLI) in presence of inorganic metals such as Hg (II) (5 µM, 10 µM), Cd (II) and (25 µM, Cd 50 µM) was declined immediately after the addition to the culture. Significant induction of light was observed as 179.29 %, 202.79 %, 173.42% and 174.94 % respectively (Fig 3.2.1 and Table 3.2.1). It is important to note that enhanced luminescence in *Vibrio harveyi* VB23 at a high cell density may ensure, more efficient DNA repair in response to potentially increased concentrations of heavy metals viz. mercury and cadmium (Czyz et al. 2000). Similar response is noticed in *Vibrio harveyi* VB23 when exposed to Hg (II) and Cd (II). Therefore it is assumed that enhanced luminescence in Vibrio harveyi facilitates an efficient DNA repair.

To check the regulating effect of chelating agent Na_2EDTA, non-inhibitory concentration of chelating agent i.e. Na_2EDTA (10 µM) was added along with $HgCl_2$ (5µM). When cells grown in presence of only Hg (5 µM), light induction of 33.92 % was observed after 120 mins of incubation, whereas in Na_2EDTA supplemented culture showed induction of 9.74 %. Interestingly when both are present in combination 26.18 % induction was observed at 150 mins (Fig 3.2.2 and Table 3.2.2).

The relative light intensity (RLI) in presence of As (III) (5 and 10 mM) was induced as 2 % and 13.4 % respectively, whereas at As (V) (5 and 10 mM) caused induction of 6.2 % and 10.94 % respectively, immediately after the addition of above metals. After 30 mins of incubation, a sharp decline in the relative light intensity (RLI) was observed till 120 mins. The decline of RLI at 120 mins was observed as 88.18 %, 138.1% for As (III) and 78.18 % and 88.66 % for As (V) as compared to the control. Cells exposed to Sn (II) 5 mM, showed a slight increase in the RLI as compared to the control was observed (Fig 3.2.3 and Table 3.2.3).

The relative light intensity (RLI) of the culture presence of DBT (0.5 and 1 mM), TBT (0.5 and 1 mM) was induced immediately after their addition (Fig 3.2.4 and Table 3.2.4). Whereas light emission (RLI) declines sharply with increasing duration of incubation with organo-metals respectively within two hours of incubation with organo-metals (DBT and TBT: 0.5, 1 mM) a significant reduction in light emission was observed i.e. 66.64 %, 95.71 %, 55.68 % and 69 % respectively. This inhibitory response of metal and organo-metal may be attributed to impaired quorum sensing regulation in *Vibrio harveyi* VB23 (Czyz et al. 2000).

It is interesting to note that luminescent *Vibrio harveyi* VB23 can successfully be used a s a test organism in bioassay of several environmental pollutants viz. heavy metals, chemical mutagens, organo-metals, industrial waste disposals and radionucliedes. The bioluminescence phenomenon (emission of light) is the most sensitive parameter of these microorganisms for quick detection of toxic and mutagenic (DNA damaging) environmental pollutants generated by anthropogenic activities. Ulitzur et al. (1986) have shown that the genotoxic agents can be detected at ng.ml-1 level suing intact and highly luminescent cells. Schiewe et al. (1985) has reviewed various short-term biological assays that have been employed to screen for the presence of hazardous environmental pollutants in the marine ecosystem. Knowing that bacterial bioluminescence is very sensitive to toxic pollutants, several investigations have tested its application for determining the toxicity of chemicals (Ramaiah and Chandramohan 1993). Among most of these studies reported so far, symbiotic luminous bacterium *Photobacterium phosphoreum* has been employed for these bioassays. As of now there are hardly any reports on free-living luminous bacterium *Vibrio harveyi.* The main application of marine luminous bacteria as biological indicators of chemical toxicity is based on bioluminescence inhibition by chemical compounds of various classes. The sensitivity of such biotests is determined by properties of bacterial strains used as biological indicators.

The light emission in presence of antibiotics viz. (Chloramphenicol and Rifamycin) was induced immediately after the addition, whereas a sharp decline was observed for cyclohexamide and Kanamycin (Fig 3.2.5 and Table 3.2.5). Induction was observed for Chloramphenicol and Rifamycin till 120 mins and lateron light emission was declined.

The decline of RLI was continued from 30- 120 mins in presence of Kanamycin and Cyclohexamide. The decline of RLI at 120 mins was observed as 20.77 % and 30.53 % respectively. In our previous results (Fig 3.2.6 and Table 3.2.6). When Hg (II) and Chloramphenicol were added separately, light emission was induced, whereas when Kanamycin was added, the light emission was declined, so we have combined Hg (II) with both the antibiotics separately to observe the effect on light emission. When cells are grown in chloramphenicol (25 µg/ml) and $HgCl_2$ (5 µM), light induction of 23.33 % was observed at 150 mins, whereas in presence of Kanamcyin (10 µg/ml) and $HgCl_2$ (5 µM), light induction of 14.46 % was observed. Although kanamycin (10 µg/ml) inhibits light emission (30.53 %) when added separately. This clearly indicates that these antibiotics (protein translation inhibitors) used when combined with $HgCl_2$ 5 µM), cause synergistic induction. The mechanism of such response is not reported in bioluminescent bacteria so far.

Mutagenic pollution of the natural environment is undoubtedly a serious problem, which needs an immediate detection by an efficient biological system (bioindicator). Since chemical methods for detection of chemical mutagens are expensive and time consuming, we need a less expensive, highly sensitive and quick assay for detection of toxic mutagenic chemicals. Phenomenon of bacterial bioluminescence is employed in a number of mutagenicity and toxicity tests since it is a very sensitive responsive parameter of luminescent bacteria (Ulitzur et al. 1980; Bulich and Isenberg, 1981). For testing the effect of chemical mutagens on the light emission we have chosen, *Vibrio harveyi* VB23 a luminous free-living bacterium which is predominantly found in diverse marine environments (Ruby and Morin 1979). Treatment of luminescent bacteria with mutagenic agents causes induction of light emission even in wild-type strains (Czyz et al. 2002).

The (RLI) in presence of chemical mutagens such as (AcOr, EthBr and NTG) was declined immediately after their addition. Whereas an induction in the RLI was observed only for AcOr (10 µg/ml) and NTG (25 µg/ml). After 120 mins the light emission was inhibited incase of NTG (25 µg/ml) and RLI for AcrOr (10 µg/ml) showed a relative induction till 150 mins. EthBr (10 µg/ml) showed decline in the light emission up to 120 mins (Fig 3.2.7 and Table 3.2.7). In case of sodium azide (25, 50 µg/ml) and 5-Bromouracil (10, 25 µg/ml), the RLI was induced immediately after the addition, but later

on the RLI was declined in all the concentrations up to 120 mins (Fig 3.2.8 and Table 3.2.8). Apart from heavy metal Hg (II) and Cd (II) treatment, luminescence of *Vibrio harveyi* VB23 tested in this study was also stimulated by chemical mutagens AcrOr 10 µg/ml) and NTG (25 µg/ml) (Table 3.2.7 and Figure 3.2.7). Acridine compounds and ethidium bromide are known to exert several biological effects in microbes, i.e. curing of plasmids, induction of frameshift mutations, inhibition of DNA repair, and inhibition of cell growth (Miller, 1992). Induction of bacterial luminescence after treatment of cultures with DNA-damaging or DNA synthesis-inhibiting agents has also been demonstrated previously (Ulitzur and Weiser 1981). The mutagenic agents through their interactions with DNA may cause configuration changes of the double helix resulting in repression of transcription of the *lux* operon (Ulitzur and Weiser 1981). The induction of the luminescence in luminous bacteria may be initiated either by a specific autoinducer, acting by binding the repressor or indirectly by altering the DNA configuration, enabling the initiation of uncontrolled transcription of the luciferase cistron (Ulitzur and Weiser 1981). A new mutagenicity assay has recently been developed by Czyz et al. (2000) in which a series of genetically modified strains of marine bacterium *V. harveyi* is used.

When the cells were irradiated with UV radiation (254 nm, 15 watts UV irradiation lamp) with increasing time of exposure to UV light, a significant light induction was observed. The RLI at 0 mins was considered as 100 %. After 5 and 10 mins of UV exposure, decline in RLI was observed as 26.5 % and 10.22 % respectively. The induction in RLI was observed after 15, 20, 25, 30 and 40 mins exposure as 5.28 %, 38 %, 44.92% and 54.14 % respectively (Fig 3.2.9 and Table 3.2.9). The induction of light emission was observed in Hg (II), Chloramphenicol (25 µg/ml) and UV light when added separately. Whereas the light emission was declined when Kanamycin was added separately. So we have initially treated the cells with antibiotics for 10 mins and then treated with UV light for 15 and 30 mins to observe the effect on light emission in a combination. When control cells along chloramphenicol (25 µg/ml) and Kanamycin (10 µg/ml) were exposed for 10 mins and then exposed to UV light for 15 and 30 mins, induction of RLI as 2.98 % and 1.66 % was observed respectively for the above treatments (Fig 3.2.10 and Table 3.2.10). These results, together with previous reports by (Czyz et al. 2000), clarify that the UV-mediated induction of light emission in *V. harveyi* may operate independently of quorum sensing. Induction of UV light was extensively studied in *E. coli* by Rames et al. (1997)

The UV-mediated induction of luminescence could be also due to formation of free radicals or by reduction of H_2O_2, generated by UV. Thus UV-irradiation leads to generation of active oxygen species (free radicals), which enhances the luminescence in the bacteria (Zhang et al.1997).

The (RLI) in presence of luciferase substrate Decanal (0.05, 0.1, 0.2 and 0.5 mM) declined immediately after the addition. Decanal with (0.05 mM) concentration caused good induction of RLI as compared to control (Fig 3.2.11 and Table 3.2.11). As decanal is a substrate for luciferase, induction of RLI was observed concomitantly.

Growth and Bioluminescence in (metals, organo-metals

Growth and Bioluminescence by serial transfer in presence of heavy metals and organo-metals was determined in GBM agar plates with increasing concentrations of metals and organo-metals. During serial subcultures, growth was subsequently noticed in all the concentrations of heavy metals and organometals as only the sub lethal concentrations were used. The visual luminescence persists only till 1^{st} subculture in presence of toxic metals like Hg, Cd, Cr, TBT and DBT, whereas in presence of $SnCl_2$, AsO_2 and AsO_4, visual luminescence was observed up to 3 consecutive subcultures. As compared to TBT, and DBT, Hg tends to be more toxic to luminescence (Table 3.2.12). When luminescent bacterial cells grown in liquid media supplemented with metal and organo-metals such as mercury, cadmium and chromate, TBT and DBT the luminescence was declined within 4 hours of incubation. However, when the same above metals and organo-metals present in the solid medium, all the bacterial colonies showed prolonged luminescence. It is intriguing to note that toxic metals and organo-metals may possibly inhibit the luminescence by blocking the luciferase proteins responsible for light emission (Ulitzur et al. 1980; Ulitzur and Weiser 1981).

Treatments	0 mins	30mins	60 mins	90 mins	120 mins
	Wavelength - Intensity	Wavelength - Intensity	Wavelength - Intensity	Wavelength - Intensity	Wavelength - Intensity
	nm- RLI	nm- RLI	nm- RLI	nm- RLI	nm- RLI
Control	468 – 155.547 482 – 138.974	468—278.832 482—253.816	468—333.596 482—304.998	468—376.753 482—343.170	468—440.648 482—404.337
$HgCl_2$ 5 µM	468 –147.286 482—132.784 492—131.814	468—366.335 482—339.540 492---337.734	468—474.050 482—436.671 492---434.906	468—585.102 482—542.274 492—540.168	468—679.104 482—632.605 492—631.605
$HgCl_2$ 7 µM	468—148.751 482—134.512 492—133.683	468---366.012 482—338.802 492—336.746	468—495.130 482—455.943 492—450.628	468---608.563 482—564.356 492—559.487	468—723.536 482—674.444
$CdCl_2$ 25 µM	468 –144.040 482—129.406 492—128.851	468—346.049 482—317.861 492—315.905	468—447.373 482—413.259	468—540.941 482—499.720	468—657.916 482—609.599
$CdCl_2$ 50 µM	468—144.040 482—129.406 492—128.851	468—342.731 482—314.504 492—312.215	468—461.993 482—426.537	468—545.142 482—503.886	468—660.033 482—611.214

(Table 3.2.1). Relative light intensities of *Vibrio harveyi* strain VB23 in presence of heavy metals Hg^{2+} and Cd^{2+}

(Table 3.2.2). Relative light intensities of *Vibrio harveyi* strain VB23 in presence of heavy metal Hg^{2+} and Chelating agent

Treatments	0 mins	30mins	60 mins	90 mins	120 mins	150 mins
	Wavelength - Intensity	Wavelength - Intensity	Wavelength - Intensity	Wavelength - Intensity	Wavelength - Intensity	Wavelength - Intensity
	nm- RLI	nm- RLI	nm- RLI	nm- RLI	nm- RLI	nm- RLI
Control	468 – 259.426 482 – 236.953 492 --234.569	468—269.582 482—245.331	468—291.131 482—265.385 492—263.095	468—313.907 482—285.170	468—307.740 482—280.349	468—326.323 482—293.822 492—291.223
Control +$HgCl_2$ 5 µM	468 –257.607 482—234.166 492—231.745	468—294.338 482—265.471	468—356.847 482—322.394	468—373.147 482—337.063 492—332.603	468—390.381 482—349.903	468—405.788 482—364.490
Control +Chelating agents 10 µM	468—254.319 482—232.501 492—231.531	468—281.387 482—256.432	468—293.839 482—267.621 492—265.326	468—316.430 482—289.451 492—287.732	468—326.446 482—298.935	468—345.656 482—315.791
Control +$HgCl_2$ 5 µM+ Chelating agents 10 µM	468—268.158 482—242.793	468—306.802 482—277.206	468—359.040 482—322.924	468—381.126 482—343.334 492—339.857	468—387.440 482—349.565 492—345.619	468—407.943 482—367.455 492—362.639

(Table 3.2.3). Relative light intensities of *Vibrio harveyi* strain VB23 in presence of heavy metals Sn (II), As (III) and As (V).

Treatments	0 mins	30mins	60 mins	90 mins	120 mins
	Wavelength - Intensity	Wavelength - Intensity	Wavelength - Intensity	Wavelength - Intensity	Wavelength - Intensity
	nm- RLI	nm- RLI	nm- RLI	nm- RLI	nm- RLI
Control	468 – 155.547 482 – 138.974	468—278.832 482—253.816	468—333.596 482—304.998	468—376.753 482—343.170	468—440.648 482—404.337
$SnCl_2$ 5 mM	468---158.422 482---141.904 492---139.529	468—279.095 482—255.334 492---253.153	468---341.011 482---311.441 492---308.546	468—404.086 482—369.896	468—448.771 482—441.337
AsO_2 5 mM	468 –159.627 482—143.156 492—141.873	468---235.990 482—216.109 492—214.387	468—254.586 482—231.886 492---230.042	468---279.273 482—253.619	468—311.539 482—282.889
AsO_2 10 mM	468—176.734 482—159.363 492—157.546	468—239.891 482—216.625 492—214.873	468—259.403 482—234.935 492—232.499	468—260.675 482—234.613	468—256.570 482—231.465
AsO_4 5 mM	468 –146.049 482—132.520 492—131.334	468—207.485 482—190.992	468—251.056 482—229.422	468—288.894 482—266.690	468—299.532 482—276.221
AsO_4 10 mM	468—138.531 482—125.086 492—124.226	468---191.768 482---176.004	468—226.730 482—207.651 492---206.307	468---242.569 482---221.595 492---221.197	468---273.770 482---251.230

(Table 3.2.4). Relative light intensities of *Vibrio harveyi* strain VB23 in presence of Organo-metals (TBT and DBT)

Treatments	0 mins	30mins	60 mins	90 mins	120 mins
	Wavelength -Intensity	Wavelength - Intensity	Wavelength - Intensity	Wavelength - Intensity	Wavelength - Intensity
	nm- RLI	nm- RLI	nm- RLI	nm- RLI	nm- RLI
Control	468 –155.547 482 –138.947	468—278.832 482—253.816	468—333.596 482—304.596	468—376.753 482—343.170	468—440.648 482—404.337
DBTC 0.5 mM	468 –204.196 482—182.768 492—180.451	468—310.581 482—286.536 492---260.154	468—348.784 482—319.142 492---269.853	468—403.385 482---367.629 492—311.984	468—441.155 482—400.782 492—344.179
DBTC 1mM	468—309.413 482—280.048	468—426.103 482—392.602	468—465.942 482---427.752	468—527.853 482—486.296	468—580.372 482—535.074
TBTC 0.5mM	521---281.674 468 –256.740 482---255.006	468---432.754 482—397.172	468—493.671 482—452.483 492---447.029	492---481.027 468---553.774 482—567.542	468—632.640 482—580.051 492---573.260
TBTC 1mM	468—372.085 482---341.153	468—603.557 482—557.087	468—680.136 482—630.895	468—725.108 482—669.993 492---665.993	468---797.324 482---742.032 492---738.662

(Table 3.2.5). Relative light intensities of *Vibrio harveyi* strain VB23 in presence of antibiotics

Treatments	0 mins	30mins	60 mins	90 mins	120 mins
	Wavelength - Intensity	Wavelength - Intensity	Wavelength - Intensity	Wavelength - Intensity	Wavelength - Intensity
	nm- RLI	nm- RLI	nm- RLI	nm- RLI	nm- RLI
Control	468 –126.551 482 – 116.149	468—138.213 482—127.612	468—145.256 482—127.329	468—152.080 482—129.552	468—164.077 482—149.509
Chloramphenicol 25 µg/ml	468 –146.474 482—130.816 492—128.930 514---110.080	468—180.066 482—160.542 492---161.446 522---141.070	468—190.589 482—171.882 492---168.781 514---148.402	468—194.590 482---175.455 492—149.746	468—203.371 482—183.143 492—180.732 519---155.518
Rifamycin 5 µg/ml	468—137.878 482—124.665 492---125.664	468—251.217 482—229.741 492---229.997	468—300.860 482—273.954 492---272.909	468—354.389 482—323.745 519---275.458	468—417.825 482—384.474
Kanamycin 10 µg/ml	521---108.300 468 –119.690 482---110.559	520---199.021 468---131.985 482—121.360	521---234.138 468—134.751 482—124.411	468---136.584 482—126.221	468—138.699 482—128.231
Cyclohexamide 10 µg/ml	468—125.470 482—116.559	468—127.075 482—117.119	468—126.743 482—116.350	468—124.269 482—114.234	468---133.156 482---122.560

(Table 3.2.6). Relative light intensities of *Vibrio harveyi* strain VB23 in presence of antibiotics and Hg^{2+}

Treatments	0 mins	30mins	60 mins	90 mins	120 mins	150 mins
	Wavelength - Intensity	Wavelength - Intensity	Wavelength - Intensity	Wavelength - Intensity	Wavelength - Intensity	Wavelength - Intensity
	nm- RLI	nm- RLI	nm- RLI	nm- RLI	nm- RLI	nm- RLI
Control	468 – 259.426 482 – 236.953 492 --234.569	468—269.582 482—245.331	468—291.131 482—265.385 492—263.095	468—313.907 482—285.170	468—307.740 482—280.349	468—326.323 482—293.822 492—291.223
Control +Kanamycin 10µg/ml	468 –248.418 482—226.417	468—260.576 482—236.275 492—235.410	468—279.894 482—255.351	468—284.433 482—258.282	468—299.912 482—272.738	468—312.469 482—285.414 492—283.244
Control + Chloramphenicol 25µg/ml	468—246.718 482—223.958	468—256.059 482—231.844 492—230.271	468—258.762 482—235.061	468—263.077 482—229.964 492—227.753	468—254.419 482—239.908 492—237.493	468—256.561 482—232.392
Control +$HgCl_2$ 7µg/ml	468 –257.607 482—234.166 492—231.745	468—294.338 482—265.471	468—356.847 482—322.394	468—373.147 482—337.063 492—332.603	468—390.381 482—349.903	468—405.788 482—364.490
Control + Kanamycin 10µg/ml +$HgCl_2$- 7µg/ml	468—263.706 482—241.416	468—301.427 482—272.705 492—270.914	468—367.420 482—330.659 492—326.942	468—374.116 482—335.780	468—376.058 482—337.797	468—391.010 482—351.332
Control + Chloramphenicol 25µg/ml+$HgCl_2$ 7µg/ml	468—285.141 482—257.657	468—331.590 482—299.753	468—377.542 482—340.816 492—336.897	468—385.569 482—347.267 492—343.178	468—402.120 482—362.259	468—400.348 482—359.804

(Table 3.2.7). Relative light intensities of *Vibrio harveyi* strain VB23 in presence of Chemical mutagens

Treatments	0 mins	30mins	60 mins	90 mins	120 mins	150 mins
	Wavelength - Intensity	Wavelength - Intensity	Wavelength - Intensity	Wavelength - Intensity	Wavelength - Intensity	Wavelength - Intensity
	nm- RLI	nm- RLI	nm- RLI	nm- RLI	nm- RLI	nm- RLI
Control	468 –126.551 482 – 116.149	468—138.213 482—127.612	468—145.256 482—127.329	468—152.080 482—129.552	468—164.077 482—149.509	468—170.348 482—149.637
Acridine Orange 10 µg/ml	433 –50.458 533—183.794	468—83.557 482—61.946 534--316.517	468—100.709 482—74.853 534—316.517	468—126.098 482—97.475 534---368.446	468—164.571 482—135.764 492—444.887	468—166.528 482—134.764 492—442.887
Ethidium Bromide 10 µg/ml	468—64.681 482—55.750 492—68.962	468---66.012 482—38.802 492—36.746	468—52.190 482—46.101 492—59.705	468---53.113 482—46.152 492—59.705	468—59.230 482—52.410 545—69.662	468—62.681 482—54.840 545—69.758
Nitrosoguanidine 10 µg/ml	468 --115.768 482—109.406	468—124.913 482—115.151	468—129.528 482—119.811	468—135.165 482—125.163	468—148.829 482—137.590	468—154.272 482—142.510
Nitrosoguanidine 25 µg/ml	468—130.087 482—120.133	468—145.515 482—134.618	468—156.561 482—145.024	468—166.104 482—154.297	468—173.371 482—160.660	468—157.259 482—145.324

(Table 3.2.8). Relative light intensities of *Vibrio harveyi* strain VB23 in presence of Chemical mutagens

Treatments	0 mins	30mins	60 mins	90 mins	120 mins
	Wavelength -Intensity	Wavelength-Intensity	Wavelength - Intensity	Wavelength - Intensity	Wavelength - Intensity
	nm- RLI	nm- RLI	nm- RLI	nm- RLI	nm- RLI
Control	468 – 288.010 482 – 260.982 492 –258.429	468—288.900 482—262.884 492—261.774	468—309.444 482—282.459 492—280.934	468—322.636 482—293.711 492—291.295	468—340.126 482—310.709 492—308.444
Sodium Azide 10 µg/ml	468 –355.415 482—326.035	468—334.599 482—306.093	468—349.943 482—321.540 492—381.832	468—366.243 482—335.764 492—334.910	468—377.599 482—343.667 492—344.398
Sodium Azide 25 µg/ml	468—330.160 482—303.986 492—302.053	468—314.023 482—287.210 492—286.543	468—321.022 482—292.981	468—345.779 482—316.812 492—314.147	468—387.489 482—353.392 492—348.733
Sodium Azide 50 µg/ml	468 –339.597 482—312.595 492—311.583	468—335.283 482—307.665 492—306.186	468—352.205 482—324.161 492---322.429	468—377.147 482—341.506	468—384.673 482—353.513 492—352.792
5-BromoUracil 10 µg/ml	468—335.429 482—309.113 492—306.858	468—330.275 482—330.126 492—301.246	468—347.376 482—317.444 492—316.086	468—373.267 482—341.506 492—339.817	468—363.475 482—334.599 492—329.737
5-BromoUracil 25 µg/ml	468—317.983 482—292.279 492—291.485	468—315.230 482—288.078 492—286.412	468—327.449 482—300.270 492—298.610	468—369.533 482—337.062 492—335.866	468—363.489 482—333.521 492—329.737
5-BromoUracil 50 µg/ml	468-- 332.954 482—304.625 492—303.005	468—338.882 482—310.695 492—308.494	468—352.230 482—322.572 492---321.393	468—379.053 482—346.697	468—398.254 482—363.616 492—362.198

(Table 3.2.9). Relative light intensities of *Vibrio harveyi* strain VB23 Irradiated by UV light

Control (0 mins) Wavelength - Intensity	5 mins Wavelength - Intensity	10mins Wavelength - Intensity	15 mins Wavelength - Intensity	20 mins Wavelength - Intensity	25 mins Wavelength - Intensity	30 mins Wavelength - Intensity	40 mins Wavelength - Intensity
nm- RLI	nm- RLI	nm- RLI	nm- RLI	nm- RLI	nm- RLI	nm- RLI	nm- RLI
468 – 546.518	468 – 409.456	468—490.668	468—575.378	468—692.067	468—754.264	468—792.058	468—842.448
482 – 503.892	482 – 372.523	482—451.022	482—528.932	482—644.894	482—696.551	482—741.780	482—799.010
492 –501.753	492 --234.569	492---448.540		492---643.474			492---802.427

(Table 3.2.10). Relative light intensities of *Vibrio harveyi* strain VB23 in presence of antibiotics and UV exposure.

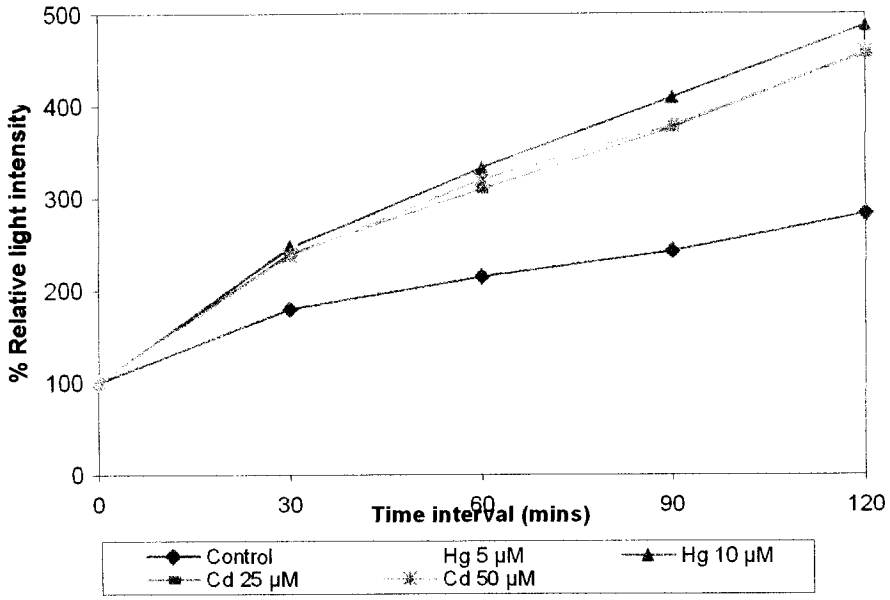
Treatments	Wavelength -Intensity	Wavelength -Intensity	Wavelength - Intensity
	nm- RLI	nm- RLI	nm- RLI
Control (no treatment)	468 – 331.926 482 – 307.642 492 –308.290	468—341.794 482—314.803 492---314.957	468—350.461 482—323.517 492—323.901
Control +Kanamycin 10µg/ml 10mins (Antibiotics treated, 15, 30 min UV treated	468 –-319.131 482—296.183 492—295.119	468—323.882 482—299.631 492---298.537	468—346.480 482—321.303 492---320.786
Control + Chloramphenicol 25µg/ml 10mins (Antibiotics treated, 15, 30 min UV treated	468—307.961 482—283.429 492—283.241	468—307.928 482—283.928 492---284.417	468—330.280 482—304.188

Treatments	0 mins	30mins	60 mins	90 mins	120 mins	150 mins	180 mins
	Wavelength - Intensity	Wavelength - Intensity	Wavelength - Intensity	Wavelength - Intensity	Wavelength - Intensity	Wavelength - Intensity	Wavelength - Intensity
	nm- RLI	nm- RLI	nm- RLI	nm- RLI	nm- RLI	nm- RLI	nm- RLI
Control	468 – 247.394 482 – 230.460 492 –231.488	468—274.599 482—256.074 492—256.603	468—283.940 482—255.154 492—255.360	468—290.234 482—270.261 492—270.180	468—315.806 482—294.232 492—295.235	468—333.544 482—310.057 492—311.208	468—377.379 482—351.961 492—353.453
0.05 mM Decanal	468 –219.979 482—205.722 492—207.524	468—251.322 482—234.657 492—235.638	468—280.155 482—261.584 492—215.331	468—306.072 482—286.061 492—286.541	468—334.042 482—310.197 492—311.755	468—350.206 482—326.028 492—327.949	468—396.068 482—367.827 492—370.327
0.1 mM Decanal	468—233.898 482—218.586 492—220.496	468—265.753 482—246.114 492—247.735	468—282.773 482—263.145 492—263.660	468—302.025 482—279.922 492—280.992	468—329.505 482—306.875 492—308.542	468—377.875 482—354.288 492—355.613 526—289.183	468—389.647 482—363.903 492—366.247
0.2 mM Decanal	468 –231.407 482—215.956 492—217.094	468—266.200 482—247.717 492—248.855	468—291.022 482—271.130 492—272.297	468—314.209 482—241.919 492—242.640	468—340.423 482—316.823 492—318.593	468—423.671 482—390.595 492—390.347	468—396.700 482—369.881 492—372.877
0.5 mM Decanal	468—228.485 482—213.614 492—214.103	468—257.527 482—238.606 492—238.593	468—284.208 482—264.874 492—265.877	468—296.671 482—276.535 492—278.153	468—330.368 482—308.064 492—307.844	468—333.366 482—310.310 492—307.844	468--372..713 482—346.628 492—327.844

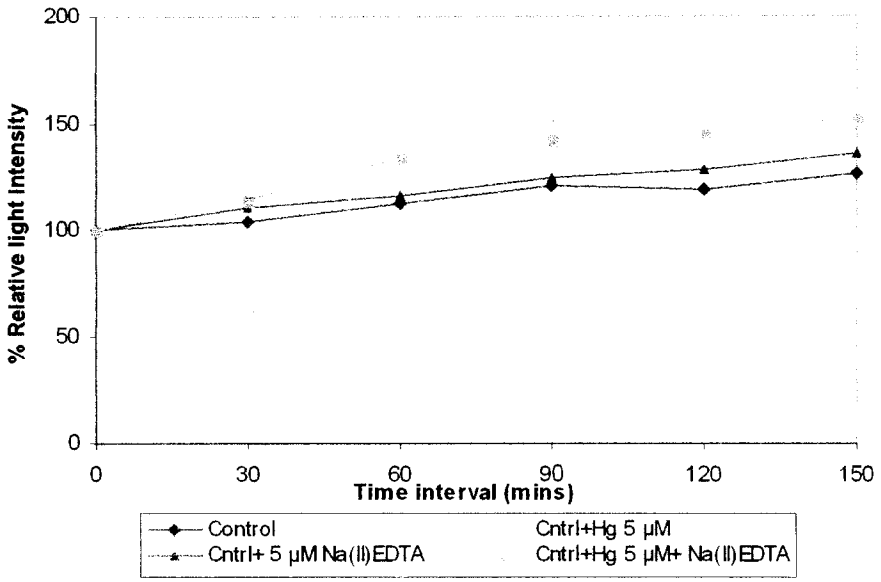
(Table 3.2.11). Relative light intensities of *Vibrio harveyi* strain VB23 in presence of n-Decanal

Table. 3.2.12). Growth and Luminescence of *Vibrio harveyi* VB23 in the presence of heavy metals and organometals

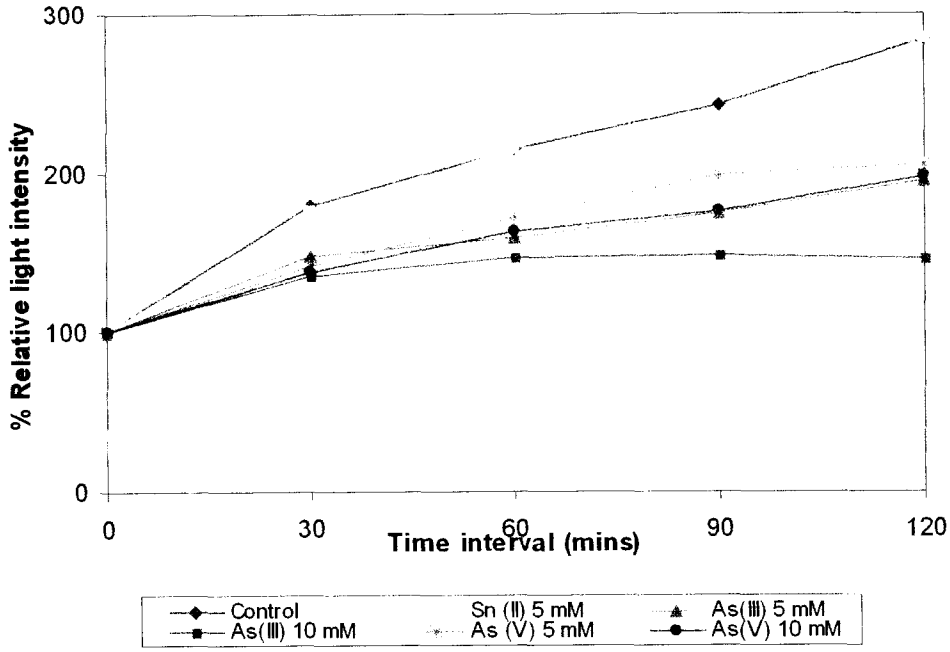
Heavymetals/ Organo-metals	Serial sub culturing on GBMA								Growth-G Luminescence-L
	1	2	3	4	5	6	7	8	
Heavy metals	+	+	+	+	+	+	+	+	G
$HgCl_2$ –5 µM	L	--	--	--	--	--	--	--	L
	+	+	+	+	+	+	+	+	G
$HgCl_2$—7 µM	L	--	--	--	--	--	--	--	L
	+	+	+	+	+	+	+	+	G
$CdCl_2$—50 µM	L	--	--	--	--	--	--	--	L
	+	+	+	+	+	+	+	+	G
$CdCl_2$—100 µM	L	--	--	--	--	--	--	--	L
$SnCl_2$—5 mM	+	+	+	+	+	+	+	+	G
	L	L	L	--	--	--	--	--	L
CrO_4 –100 µM	+	+	+	+	+	+	--	--	G
	L	L	--	--	--	--	--	--	L
AsO_2 – 5 mM	+	+	+	+	--	--	--	--	G
	L	L	L	--	--	--	--	--	L
AsO_4----10mM	+	+	+	--	--	--	--	--	G
	L	L	L	--	--	--	--	--	L
Organo-metals									
TBTC -- 0.5 mM	+	+	+	+	+	+	+	+	G
	L	--	--	--	--	--	--	--	L
TBTC – 1 mM	+	+	+	+	+	+	+	+	G
	L	--	--	--	--	--	--	--	L
DBTC - -0.5 mM	+	+	+	+	+	+	+	+	G
	L	--	--	--	--	--	--	--	L
DBTC ---1 mM	+	+	+	+	+	+	--	--	G
	L	--	--	--	--	--	--	--	L



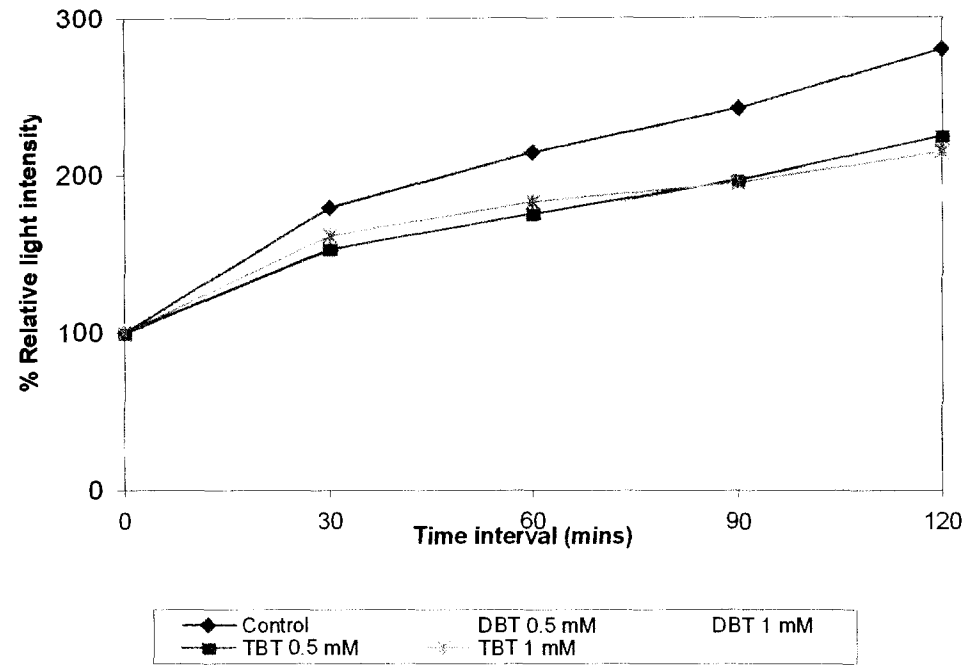
(Fig. 3.2.1). % Relative light Intensity of *Vibrio harveyi* VB23 in Hg(II) and Cd(II)



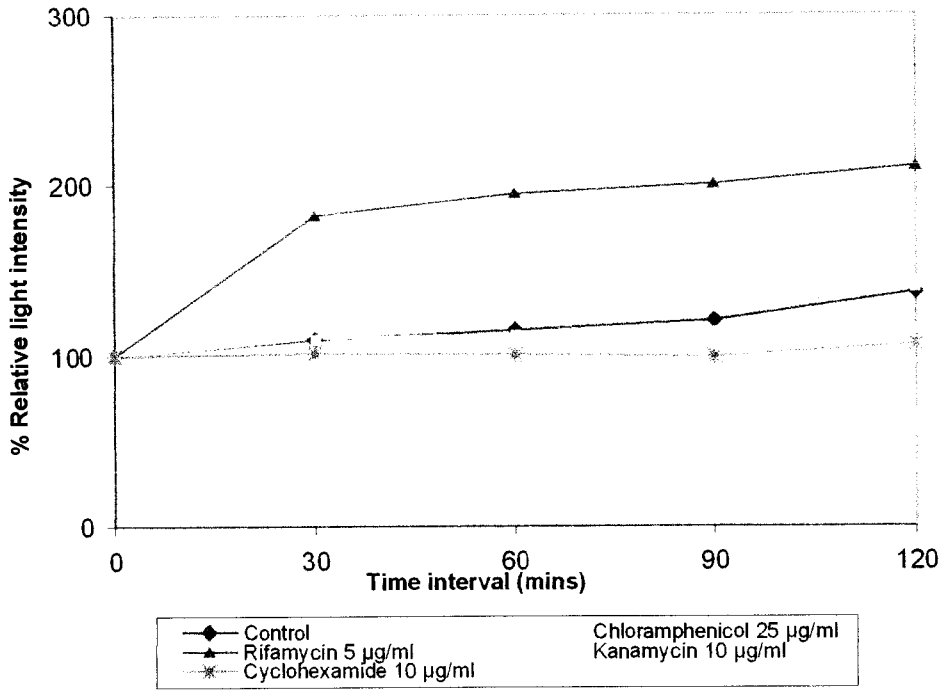
(Fig. 3.2.2). % Relative light Intensity of *Vibrio harveyi* VB23 in Hg(II) and Chelating agent Na_2EDTA



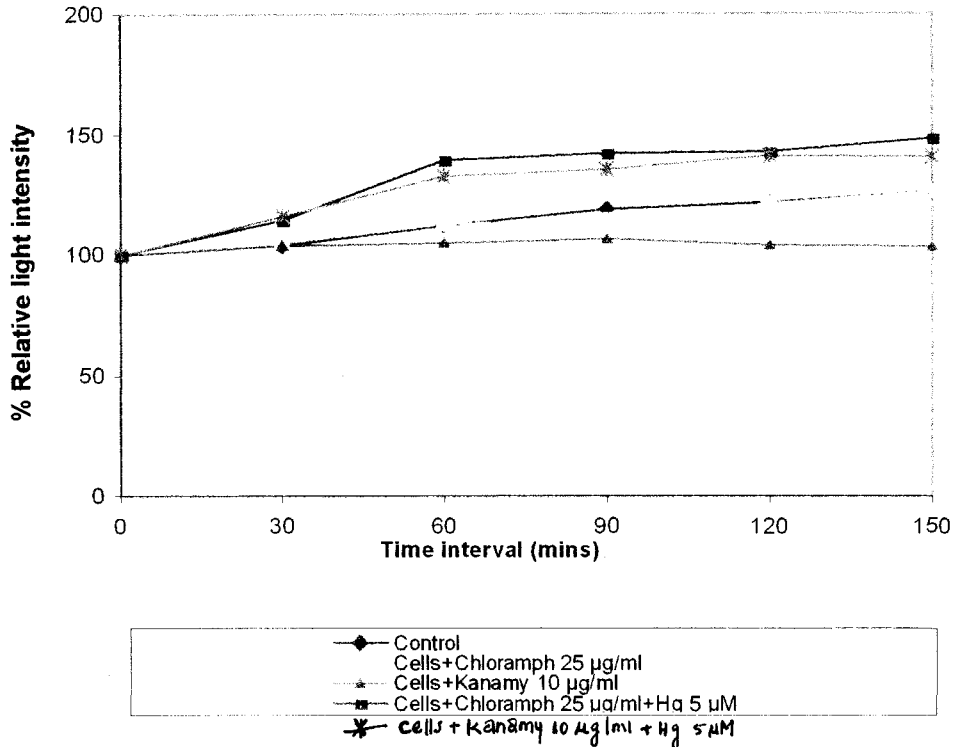
(Fig. 3.2.3). % Relative light Intensity of *Vibrio harveyi* VB23 in Sn(II), As(III) and As(V).



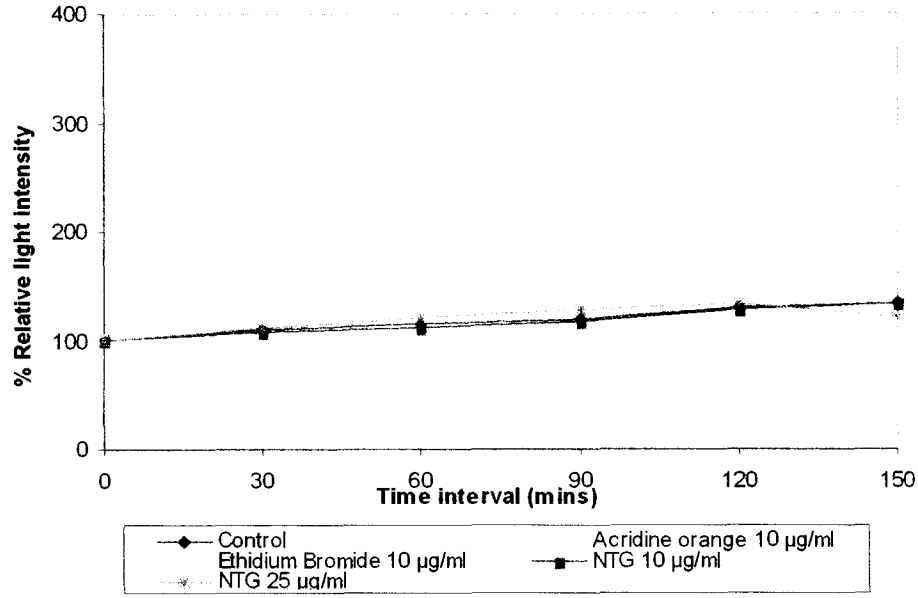
(Fig. 3.2.4). % Relative light Intensity of *Vibrio harveyi* VB23 in TBT and DBT



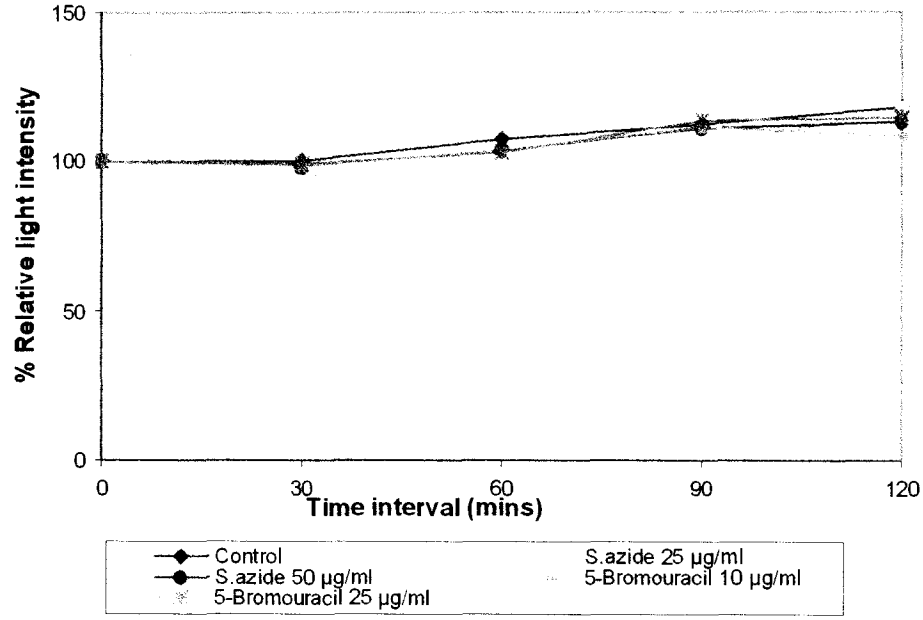
(Fig. 3.2.5)% Relative light Intensity of *Vibrio harveyi* VB23 in antibiotics



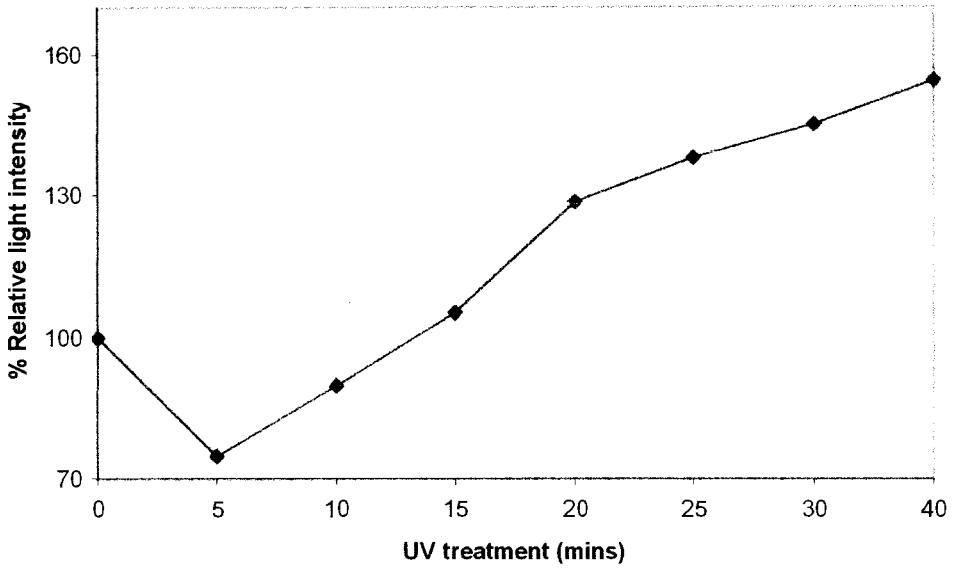
(Fig. 3.2.6)% Relative light Intensity of *Vibrio harveyi* VB23 in antibiotics and Hg^{2+}



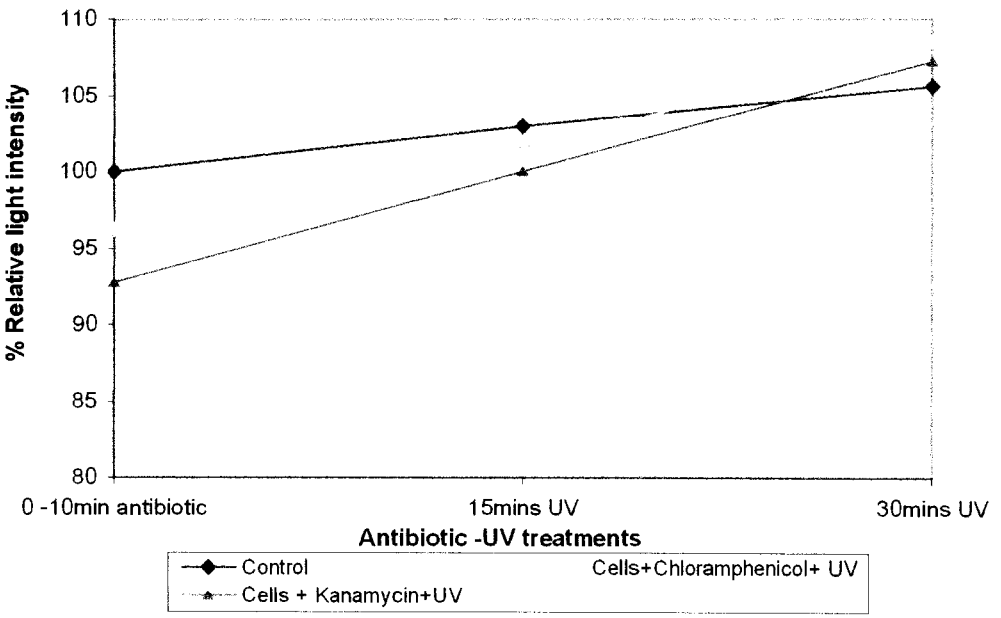
(Fig. 3.2.7). % Relative light Intensity of *Vibrio harveyi* VB23 in chemical mutagens



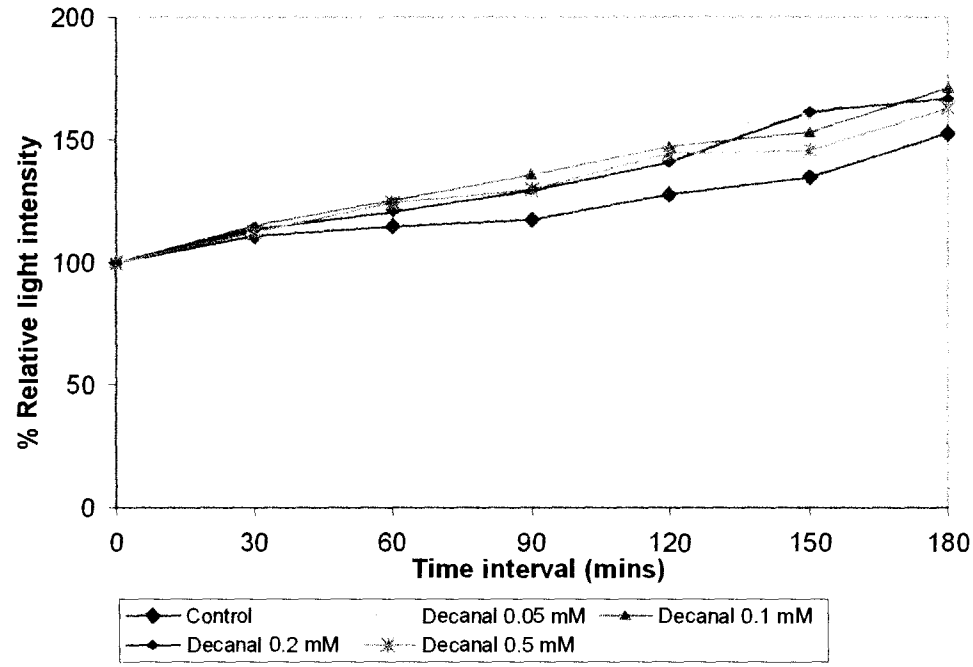
(Fig. 3.2.8). % Relative light Intensity of *Vibrio harveyi* VB23 in Sodiumazide and 5-BU



(Fig. 3.2.9). % Relative light Intensity of *Vibrio harveyi* VB23 under UV light



(Fig. 3.2.10). % Relative light Intensity of *Vibrio harveyi* VB23 in antibiotics and UV exposure.



(Fig. 3.2.11). % Relative light Intensity of *Vibrio harveyi* VB23 in n-Decanal.

Protein Profiles

Induction of heavy metal stress proteins

The microbial stress response is a set of conserved and unique biochemical mechanisms that an organism activates or induces under adverse conditions, specifically for the protection of vital cellular components, protein and enzymes (Higham et al. 1984; Gadd, 2000). The impact of stresses viz osmolarity shock, heat shock, cold shock, oxidative stress and heavy metal stress has already been reported in cyanobacteria and few marine bacteria (VanBogelen et al. 1987; Bhagwat and Apte, 1989; Jakob, et al. 1993; Lupi et al. 1995; Segal et al. 1996). Interestingly few bacterial strains like *Psudedomonas putida* and *Vibrio alginolyticus* are also known to synthesize metal induced low molecular weight cysteine rich metallothionein like proteins, which play an important role in metal sequestration of cadmium and copper respectively (Pazirandeh, 1998).

Microorganisms inherently adapt to adverse environmental conditions such as heavy metal stress by activating several resistance mechanisms. One of these important stress

responsive proteins in response to a diverse range of stresses that provide nonspecific protection, in contrast to stress-specific proteins and referred as universal or general stress response (Nystrom and Neidhardt, 1992; Bernhardt et al.1997). Microbial response to toxic heavy metals is governed by several factors viz. growth conditions (phase), bioavailability of metals to the cells and presence of metal complexing ligands. The abiotic factors include the physico-chemical characteristics of the environment viz. pH, salinity, temperature, and redox potential. Environmental bacterial strains are known to induce prompt toxic responses when cells are exposed to non-permissive environment such as high levels of heavy metals. The cell membrane is the first major barrio for entry of hydrophilic metal ions into the cell interior involving membrane proteins. These membrane proteins serve as one of the resistance mechanism for microbial cells (Rouch et al. 1995). This is because S-H related metabolic processes are strongly inhibited after these heavy metals are taken into the cells (Wakatsuki, 1995). Three prominent types of modifications occur under stress conditions: (i) synthesis of several proteins declines, under lethal doses (ii) synthesis of certain specific proteins was selectively enhanced, and (iii) synthesis of a new set of proteins are induced *de novo* (VanBogelen et al. 1987; Lupi et al. 1995).

Although, there are several reports on metal induced metallothionein like proteins in cyanobacteria and marine bacteria, there is no such report in bioluminescent bacteria, inspite of their predominance in metal contaminated environment. The luminous bacterial strain, VB23 exposed to test heavy metal and organo-metals viz. cadmium, mercury, arsenite, arsenate, arsenite, arsenate, TBT, DBT separately, the strain interestingly exhibited the induction of several novel stress proteins, which is evident by one-dimensional SDS-PAGE analysis of protein samples. Comparably arsenate and inorganic tin were less effective for stress protein expression as compared to other toxic heavy metals, arsenite, mercury, Cadmium TBT and DBT. The concentrations of As (V) and Sn (II) used for induction studies may not be sufficient to stimulate stress response at a translational level, possibly due to less availability of these inducers. Some of these induced stress proteins showed different level of expression, whereas few proteins were found to be unique when cells were exposed to mercury. Metal stress protein synthesis occurred even at concentrations that had no effect on growth. Synthesis of certain

proteins was significantly enhanced and few proteins were selectively induced *de novo* during heavy metal stress. In order to examine whether proteins were induced throughout the exposure period, a detailed time course experiment was performed by exposing cells to mercury concentrations of 2, 5 and 7 μM Hg^{2+}. It is important to note that cells exposed to 2, 5 and 7 μM $Hg2^{+}$ induced a unique novel mercury stress protein of 54 kDa during mid logarithmic phase (12-16 hrs) of growth.

The luminous strain *Vibrio harveyi* VB23 shows prominent profile of several mercury induced proteins. Stress caused significant changes in the protein profile, when cells were exposed to the concentration above 2 μM Hg (II). More changes occurred at concentrations of 2-5 μM as compared to 7 μM $HgCl_2$. When the VB23 cells were exposed to 2 μM and 5 μM mercury, similar and unique protein induction pattern was observed as 15 kDa, 20 kDa and 23 kDa and 54 kDa were present. Proteins of 50 kDa and 52 kDa were exclusively induced by 5 μM $HgCl_2$ (Fig's 3.3.1,3.3.2 and 3.3.3). It is interesting to note that when the cells were exposed to 7 μM $HgCl_2$, the level of synthesis of certain proteins were apparently reduced. 54 kDa and 32 kDa proteins were also induced at 7 μM Hg (II), whereas the set of induced proteins observed at low concentrations of Hg^{2+} were not seen at 7 μM $HgCl_2$ (Fig. 3.3.3). These mercury induced proteins may be involved in the mercury resistance in *Vibrio harveyi* VB23 and this must be the first report.

When cells are exposed to Cd (II) 25μM, a 54 kDa protein was induced. Cd (II) 50 μM, induced 20 kDa, 52 kDa, 70 kDa and 78 kDa proteins. The similar proteins are present in control at basal level, but over expression of these proteins occurs when cells are exposed to Cd (II) 50 μM (Fig 3.3.4). Induction of 36 kDa and 38 kDa proteins was observed when the cells were exposed to As (III) 5 mM, whereas none of the stress proteins were induced at As (III) 2.5 mM. Likewise, stress proteins were not induced when cells were exposed to 2.5 and 5 mM concentrations of As (V). It is interesting to note that when the cells were exposed to 5 mM of As (V), the levels of certain protein synthesis were apparently declined as compared to the controls at base level. (Fig 3.3.1 and 3.3.2).

Induction of two novel proteins of 32 kDa and 40 kDa size occurred at DBT 0.5 mM and 0.75 mM. When the cells wee exposed to TBT 0.5 mM only 50 kDa protein was induced. It

is interesting to note that a constitutive protein of 58 kDa protein was expressed several fold at DBT 0.5 mM, 0.75 mM and 0.5 mM TBT as compared to the control (Fig 3.3.5).

Since ethanol is used as a diluent for organotins, TBT and DBT, we have also checked the differential expression of protein profile of luminous bacteria in presence of ethanol. Ethyl alcohol at 0.25-1.5 % levels induces the synthesis of certain specific stress proteins. 54 kDa protein was induced in all the concentrations of ethyl alcohol; 36 kDa protein was induced only at 1.5, 1.25, 1 and 0.75 % of ethyl alcohol, whereas 42 kDa protein was induced at 1.25, 1 and 0.75 % of ethyl alcohol. Interestingly, the level of 32, 40 and 48 kDa proteins reduced due to ethanol stress, but the same set of proteins were present in the control, without ethanol (Fig 3.3.6). In comparison to controls, the relative level of proteins of 75 kDa, 58 kDa and 20 kDa increased and remained high when ethyl alcohol was present.

There have been few reports on universal stress proteins produced following exposure to pollutants, by Blom et al. (1992). They showed that when *E. coli* was exposed to selected pollutants unique and condition-specific proteins were induced. Studies on prolonged chemical stresses have progressed in environmental organisms, showing that new proteins are synthesized after 3 hours of chemical stress in *E. coli* (Blom et al. 1992). S-H related metabolic processes are strongly inhibited after mercury is taken into bacterial cells (Wakatsuki, 1995). TBT resistant *Vibrio* spp also exhibited synthesis of two polypeptides of 30 kDa and 12 kDa when cells are gown in presence of 125 µM TBT (Fukagawa et al.1992). The isolation of bacterial cadmium binding proteins from *P. putida* and cysteine-rich protein from *P. cepacia* grown in Au (I) thiolate may have a common role in detoxification (Higham and Sadler, 1984). All the test heavy metal and organo-metals significantly affected the growth pattern and protein profiles of *Vibrio harveyi* VB23. The unique response of bioluminescent cells to metal and organo-metal stress results in such response of several induced proteins in luminous *Vibrio harveyi* isolates. Bioluminescent *Vibrio harveyi* can be used as unique and sensitive marker for detecting the bioavailability of specific potentially toxic metal elements eg. Hg, Cd, As (III), TBT and DBT. The stress responses of high osmolarity, high temperature, high oxidative and toxic heavy metals have already been reported in cyanobacteria and few other marine bacteria (VanBogelen et al. 1987; Bhagwat and Apte, 1989; Blom et al. 1992; Jakob, et al. 1993; Lupi et al.

1995; Segal et al. 1996). However, hardly any reports are available on heavy metal stress induced proteins of bacteria from marine environment. Thus these reports on metal and organo-metal induced proteins in luminescent bacterium, *Vibrio harveyi* VB23 are first of its kind and very important in environmental monitoring of toxic heavy metals.

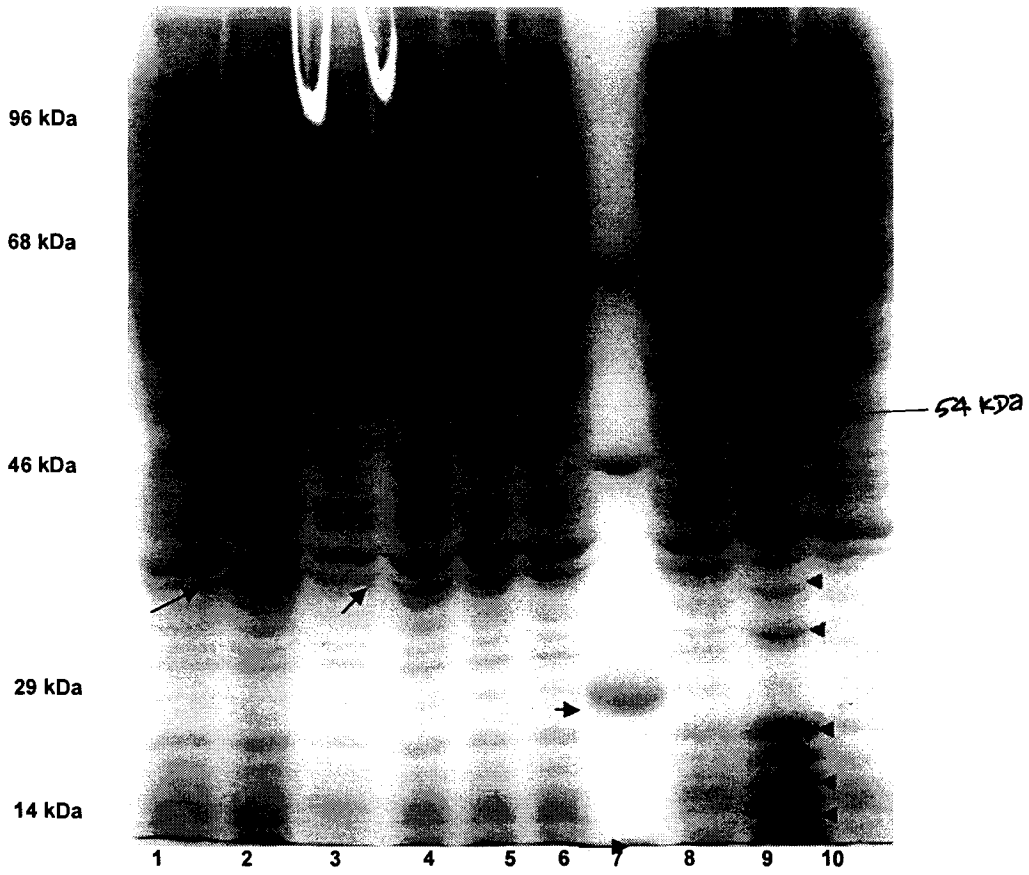


Fig 3.3.1. SDS-PAGE (gel electrophoresis) of heavy metal induced stress proteins

Lanes 1 As (V)-2 mM, Lane 2 As (V)-5 mM; Lane 3, As (III) –5 mM, Lane 4, As (III) –2.5 mM Lanes 5-6, Cd-25 µM, 50 µM, Lane 7- Protein molecular weight marker, Lanes 8 Control without heavy metals, Lane 9 Hg (II) 5, Lane 10, 2 µM Hg (II) (arrow indicates stress proteins)

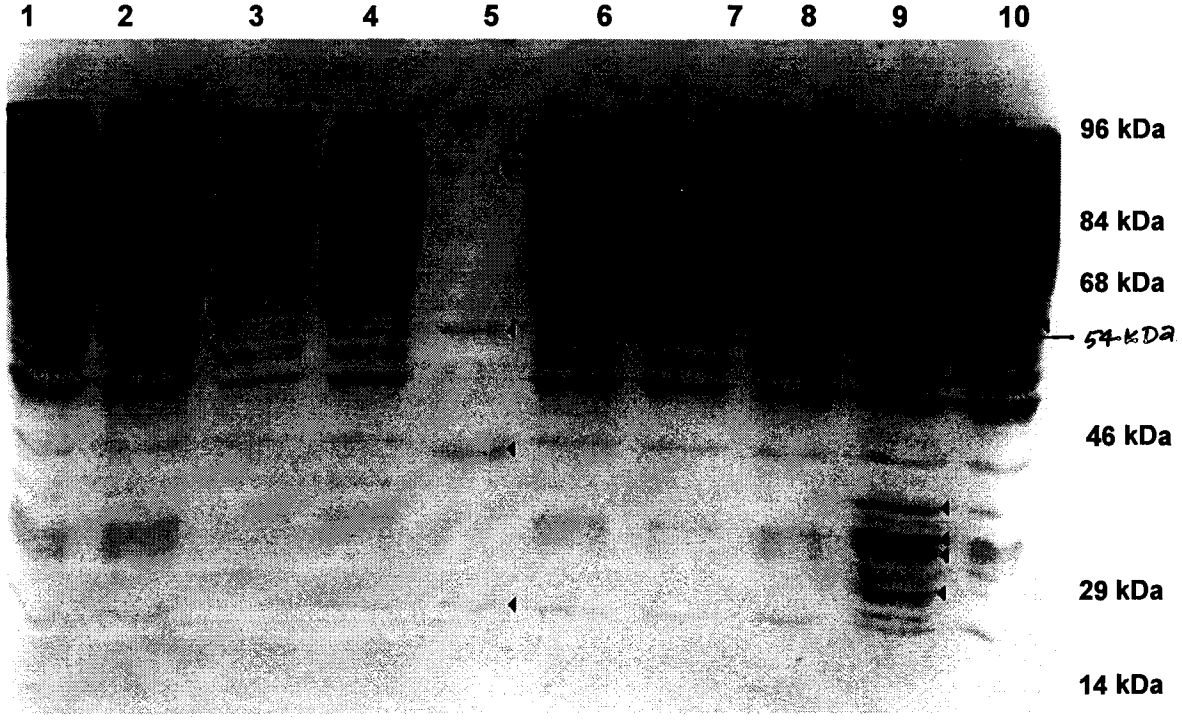


Fig 3.3.2. SDS-PAGE(gel electrophoresis) of heavy metal induced stress proteins

Lanes 1 As (V)-2 mM, Lane 2, As (V)-5 mM; Lane 3, As (III) –5 mM, Lane 4, As (III) – 2.5 Lane 5- Protein molecular weight marker, Lanes 6 , Control without heavy metals, Lane 7, Cd (II) -10 µM, Lane 8, Cd (II) 25 µM , Lane -9 Hg (II) 5 µM and Lane 10 Hg (II) 2 µM) , (Arrow indicates stress proteins)

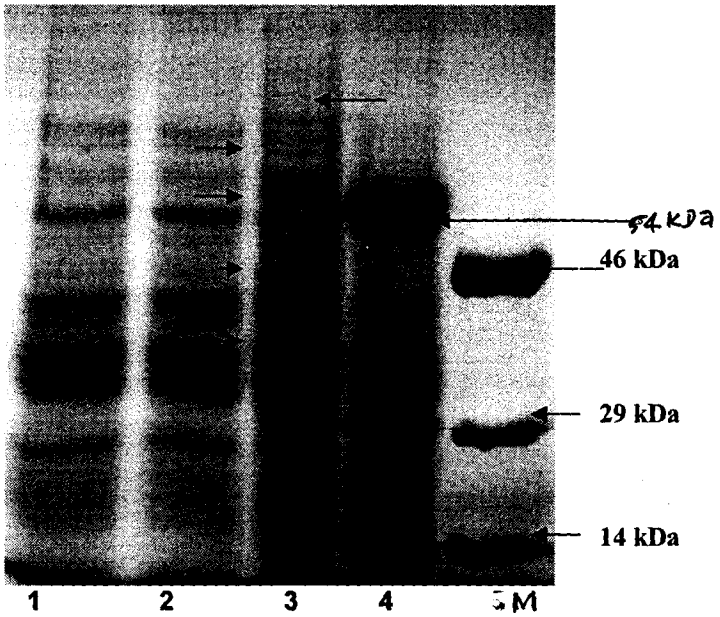


Fig 3.3.3 **(Lane1 control without metals, Lane , 2 Hg µM –Lane 3 Hg 5µM and Lane 4, 7 µM Hg, Lane M , protein molecular weight marker(arrow indicate** stress proteins).

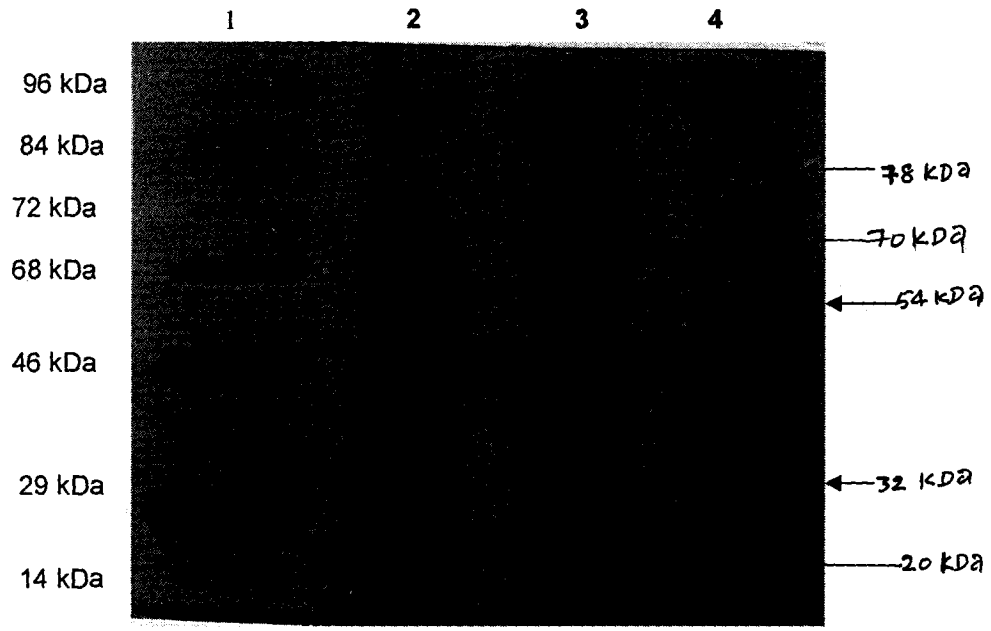


Fig. 3.3.4. Lane 1, MWM (marker) Lane 2, control without metals, Lane 3, 25 µM Cd and Lane 4, 50 µM Cd) (arrow indicates Stress proteins)

Fig 3.3.3 and Fig 3.3.4. SDS-PAGE(gel electrophoresis)of heavy metal induced stress proteins

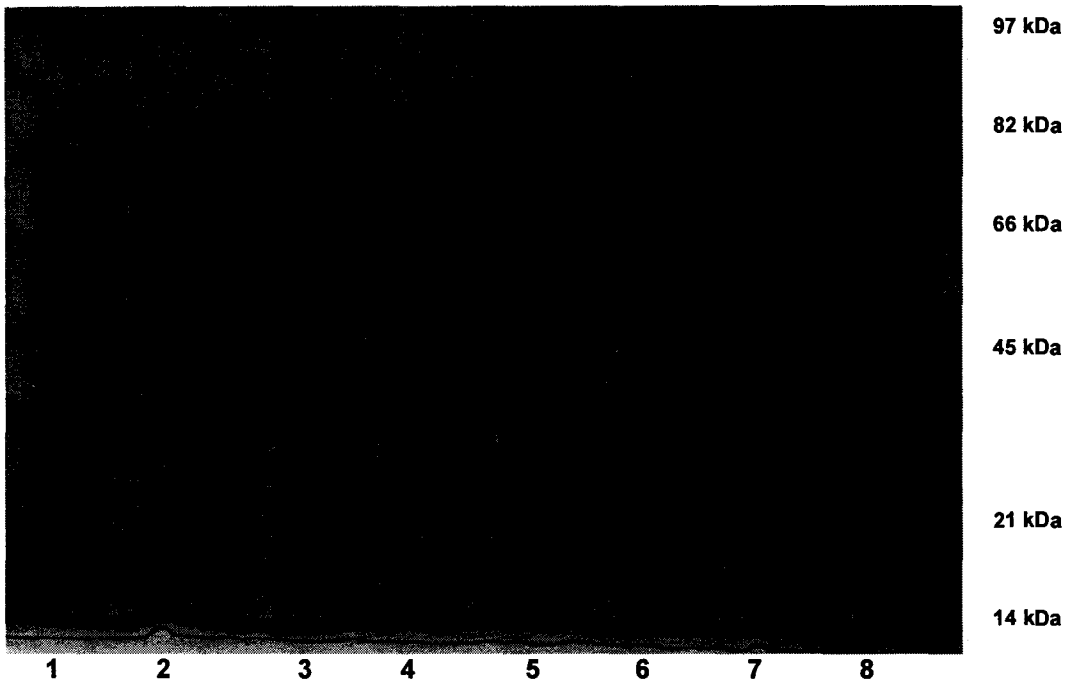


Fig 3.3.6. SDS-PAGE(gel electrophoresis)of ethanol induced stress proteins

Lane 1. 0.25 % Ethanol

Lane 2 0.5 % Ethanol,

Lane 3 0.75 % Ethanol,

Lane 4 1 % Ethanol,

Lane 5 1.25 % Ethanol,

Lane 6 1.5 % Ethanol,

Lane 7 Control without ethanol

Lane 8 MWM, -Protein molecular weight marker.

Arrows indicate stress proteins

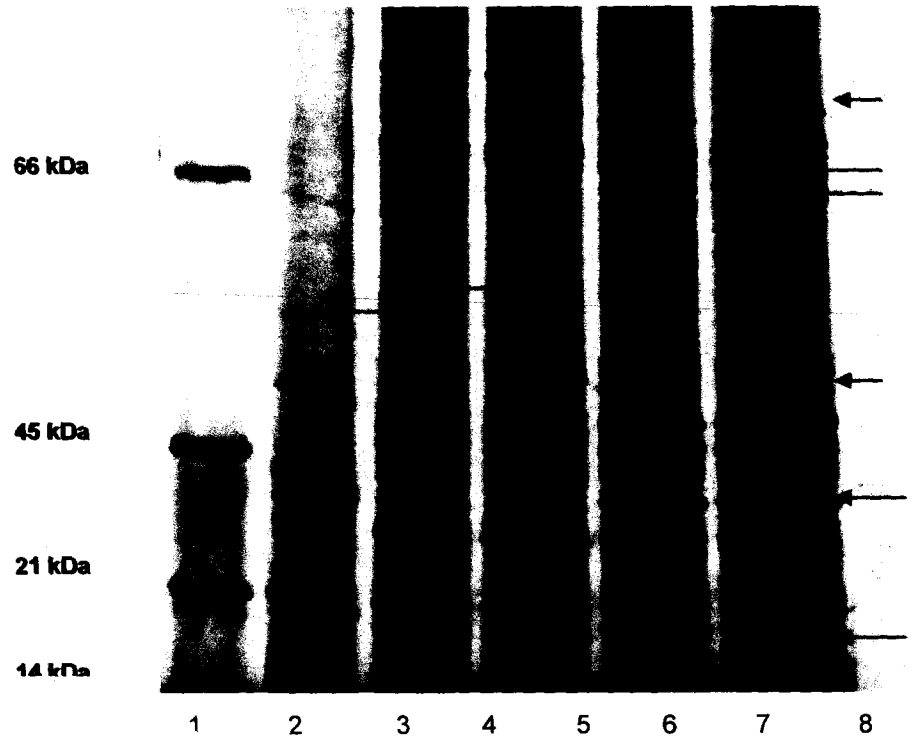


Fig 2.3.5. SDS-PAGE analysis of proteins for strain of *Vibrio harveyi* VB23 following addition of organ-metals tributyltin chloride and dibutyltin chloride

Lane 1 Protein molecular weight marker
Lane 2 NTG treated sample
Lane 3 Control without organo-metals
Lane 4 Tributyltin chloride 0.5 mM
Lane 5 Dibutyltin chloride 0.5 mM
Lane 6 Dibutyltin chloride 1 mM
Stress proteins (⟶

Fig 2.3.5. Induction of stress proteins in presence of organo-metals (TBT and DBT)

Exopolysaccharide (EPS) Characterization

Growth and EPS production of *Vibrio harveyi* VB23

EPS production of *Vibrio harveyi* VB23 clearly indicates a strong correlation with growth behavior. The rate of EPS production in batch culture was highest between the late log phase and stationary phase of growth i.e. (30-32 hrs), as the yields of the batch culture were 24.3 mg/ml (wet weight) and 3.2 mg/ml (dry weight) respectively. Most exopolysaccharides are produced both in the exponential and stationary phases (Uhlinger and White 1983). Whereas an exception has been noted for another non-marine pseudomonad, which produces exopolysaccharide only in the stationary phase (Williams and Wimpenny 1977). The level of EPS recorded was 12 μg ml^{-1} at 6 hr, which increased up to 29.8 μg.ml^{-1} after 24 hrs of incubation and followed by the decline of EPS production after 32 hrs. When the cultures were centrifuged (15,000 x g, 30 min), the supernatants from the isolates were viscous and formed stringy precipitates with cold ethanol. Various methods including high-speed cold centrifugation (Decho, 1990), mild alkali, EDTA, NaCl (Bhosle et al. 1995) have been reported for extraction of EPS from microbial cultures. However we found fairly effective extraction of exopolysacchardies by cold ethanol precipitation and incubation at 4 °C for 24 hrs.

Alcian Blue staining and SEM analysis of the exopolymer

The localization of the soluble and bound carbohydrates was visualized microscopically using alcian blue to stain the anionic sugars (Fig. 3.4.6.). Microscopic observation of cells stained with alcian blue showed that carboxylated and sulphated polysaccharides were present in the EPS produced by luminous *Vibrio harveyi* VB23 (Crayton et al.1982). The S.E.M. micrograph (Fig.3.4.7) revealed that the polymer surface morphology is a porous structure with small pore-size distribution. The small pore structure may also be responsible for the compactness and stability of the polymer (Kumar et al. 2004).

Effect of Carbon and nitrogen sources on EPS production

Vibrio harveyi showed maximum growth when the medium was supplemented with 1 % glucose and 1.5 % NaCl, however only 0.4 % glucose was used for the growth studies, thereafter the EPS production did not increase, although the cell growth increased up to 2 % glucose. Glucose was most suitable carbon sources amongst the six carbon sources

examined for bacterial growth and EPS production (Fig.3.4.3). Ammonium chloride was most suitable source nitrogen followed by peptone, Yeast extract, NH_4NO_3, $(NH_4)_2$ SO_4 and malt extract respectively (Fig.3.4.4). *Vibrio harveyi* VB23 showed maximum growth when the medium was supplemented with 1 % glucose and 1.5 % NaCl and 0.02 % Nitrogen source in the media. Whereas, with the increasing concentrations of nitrogen (0.05, 0.1, 0.2 and 0.5 %), the cell density was decreased. Most bacteria use carbohydrates as a carbon and energy source and amino acids or an ammonium salt as a nitrogen source (Sutherland, 1982). The composition of EPS and the chemical and physical properties of these biopolymers can vary greatly (Decho, 1990), but it is generally independent of the carbon substrate (Sutherland, 1982). Uptake of substrate is one of the first limitations on EPS production, and the presence of a carbohydrate substrate such as glucose results in optimal EPS yields (Sutherland, 1979). Marine strain *Hahella chejuensis* produced the highest EPS yield in batch culture, when grown on sucrose (Ko et al. 2000). Marine bacteria may also produce EPS during growth in seawater alone (Decho, 1990) and during carbon limitation as many species can make use of non-sugar sources for EPS biosynthesis (Sutherland, 1979).

Effect of heavy metals and organometals on EPS production

Vibrio harveyi VB23 showed maximum growth when the medium was supplemented with 0.4 % glucose, 1.5 % NaCl and 150 µM $CdCl_2$ with compared to the control and other heavy metals such as Hg, AsO_2, AsO_4 and Sn, organometals such as TBT and DBT. The yield of the exopolymer was comparatively higher (27.96 $\mu g.ml^{-1}$) when the media was supplemented with 150 µM $CdCl_2$, whereas 26.1 $\mu g.ml^{-1}$ in control (without metals), 17.78 $\mu g.ml^{-1}$ in arsenite, 19.04 $\mu g.ml^{-1}$ in arsenate, 20.42 $\mu g.ml^{-1}$ in Sn, 23.0 $\mu g.ml^{-1}$ in TBT and 25.14 $\mu g.ml^{-1}$ in DBT respectively (Fig.3.4.5). These results indicated an important role for cell wall components such as proteins in metal binding in complex biofilm systems (Spaeth et al., 1998). These findings were confirmed in a study that showed heavy metals were bound by cellular sorption as well as extracellularly by polymeric substances such as polysaccharides in bacterial biofilms and microbial flocs (Wuertz et al., 2000). Interaction of bacterial exopolymers with metals. It is unequivocally accepted that microbial exopolymers, including lipopolysaccharides (LPS), play an important role in the process of cell attachment to metal surfaces (Beech and Gaylarde, 1989). Several classes of

polymeric molecules participate in EPS/metal interactions by formation of salt bridges with carboxyl groups on acidic polymers, i.e. polysaccharides containing uronic acids, and by forming weak electrostatic bonds with hydroxyl groups on polymers containing neutral carbohydrates. A large number of metals have been reported to cross-link polysaccharides (Geesey and Jang 1990).

Emulsification activity

The emulsification activity of the exopolymer is determined by its strength in retaining the emulsion breaks rapidly within an initial incubation of 30min.The absorbance reading after 30 and 60 mins gives a fairly good indication of the stability of the emulsion. The dialyzed fraction of the exopolymer produced by *Vibrio harveyi* retained 40.42 % and 34.04 % of the emulsification activity after 30 mins and 60 mins respectively. But the non-dialyzed fraction of the exopolymer produced 36.36 % and 9.0 % after 30 and 60 mins. The stability of the emulsion by exopolymer from *Vibrio harveyi* VB23 is comparable to those reported earlier in *Pseudomonas* spp. (Rosenberg et al 1979; Royan et al.1999). The EPS excreted by *Vibrio harveyi* is highly surface active, which is probably due to an uronic-acid containing polymer (Geesey and Jang 1990) (Table 3.4.2).

Chemical analysis

Chemical analyses of the *Vibrio harveyi* VB23 bacterial exopolymers shows gross differences in composition of the exopolysaccharide. The chemical analysis of EPS showed that the exopolymer composed the following macromolecules as neutral sugars 211.71 µg/mg, proteins 186 µg/mg., uronic acids 114.62 µg/mg, and methyl pentoses 32 µg/mg (Table.3.4.1). The sulphate contents were below detection limits. Among the two strains reported during initial screening, *Vibrio harveyi* VB23 showed the highest exopolymer production with reference to yield (µg/ml). Fazio et al. (1982) have previously reported that exopolymer from the marine bacterium contains high quantities of galacturonic acid.

Analysis of exopolysaccharides by Gas Chromatography

The sugar composition of the EPS, analyzed using gas chromatography shows the percentage relative contribution of hexoses (galactose, 10.08 %; glucose, 3.6 %),

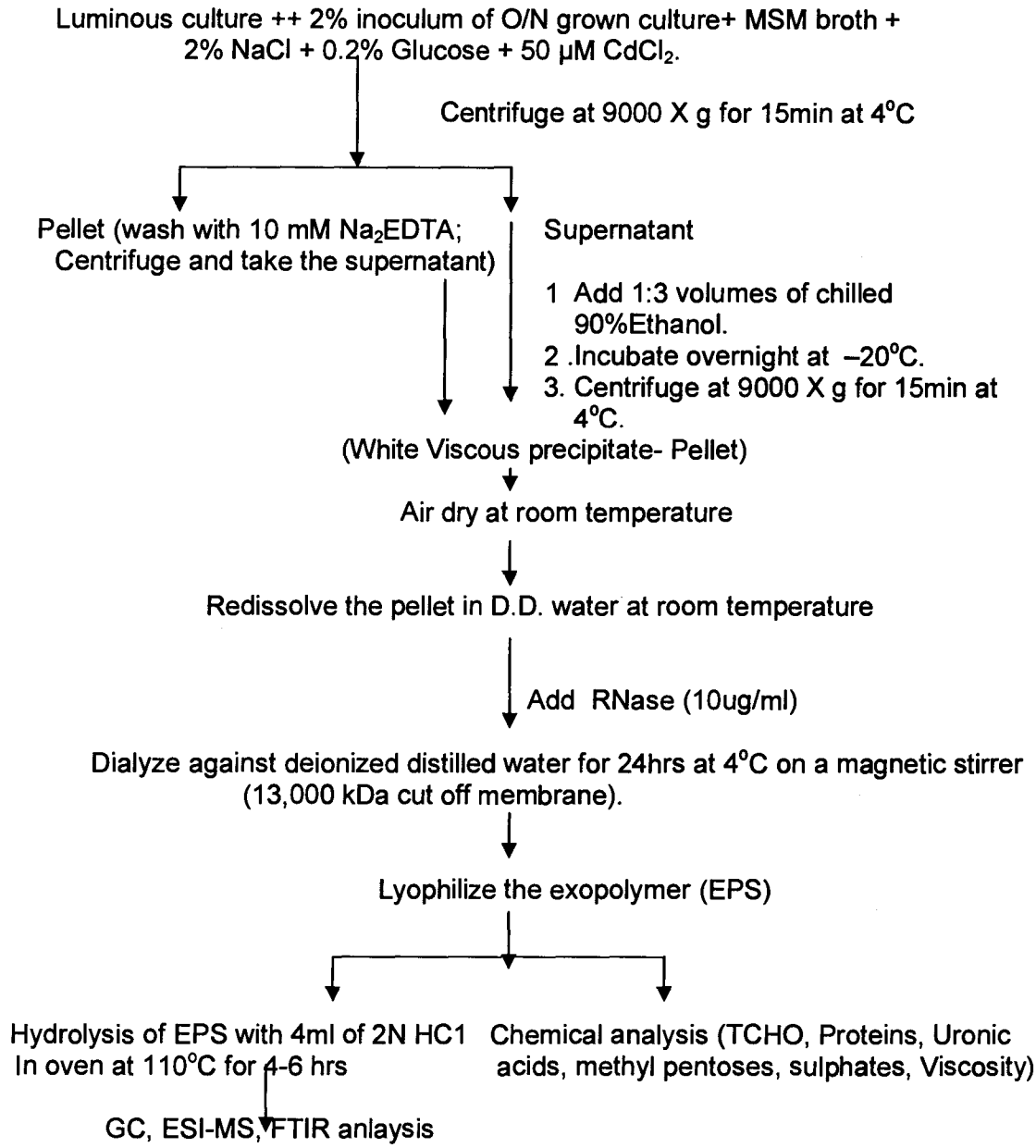
deoxyhexoses (rhamnose, 0.7 %; fucose, 0.15 %) and pentoses (ribose, 0.2 %; arabinose, 0.3 %; xylose, 0.45 %; mannose, 1.56 %) (Fig.3.4.8). Glucose and galactose were the most abundant neutral sugars in differential amounts. The composition of EPS may also vary according to the growth phase of the bacteria (Christensen et al., 1985). Although culture conditions generally do not affect the types of monosaccharides in an EPS, they do affect the functional properties of the polysaccharide such as molecular weight, conformation, and monosaccharide ratios (Arias et al., 2003). In natural systems where nutrients levels close to the bacterial cell may vary considerably, shifts in the physiological state of the cell probably result in variable EPS compositions (Geesey, 1982). Most EPS's produced by marine bacteria are heteropolysaccharides consisting of 3 or 4 different monosaccharides arranged in groups of 10 or less to form repeating units (Decho, 1990).

ESI-Mass spectra of the exopolymer

Exopolysaccharide (EPS) of *Vibrio harveyi* VB23 are analyzed using negative ion electrospray ionization mass spectrometry (ESI-MS). The characteristic fragmented ions provided unambiguous assignment of the molecular weights of the complex luminous bacterial polysaccharide VB23. The mass spectra obtained for polysaccharide repeating units generated through chemical hydrolysis and in-source fragmentation were directly compared, both in positive and negative ion modes (Li et al. 2004). The ESI mass spectrum from VB23 exopolymer corresponds using the formulae: [Mass - No: of water molecules/ each sugar moiety + H]. The exopolymer contains a sharp peak of m/z, 631 corresponds to an oligosaccharide having a composition of (1 Rha +1 Fuc + 1Glu +1 D-Gal. Uronic acid), whereas a peak of m/z, 543 corresponds to (1 Rib + 1Ara + 1 Glu +1 D-Gal. Uronic acid). A peak of m/z, 455 corresponds to (1 Rib + 1 Rha + 1 D-Gal. Uronic acid), likewise a significant sharp peak of m/z, 439 corresponds to (1 Rha + 1 D-Gal. Uronic acid + 1 Deoxyribose), a peak of m/z 398 corresponds to (1 Rib + 1Ara + 1 Xyl). A peak of m/z, 381 corresponds to (1 Rib + 1 Rha + 1 Deoxyribose), a peak of m/z, 323 corresponds to (1 Rha + 1 D-Gal. Uronic acid), a peak of m/z, 309 corresponds to (1 Rib + 1 Deoxyribose), a peak of m/z, 180 corresponds to (1 Glu/ 1 Gal/ 1 Man/ 1 Ino), a peak of m/z, 164 corresponds to (1 Rha/ 1 Fuc) respectively. (Fig 3.4.10). A similar observation of the exopolymers of *P. nudum* and *P. bifurcatum* were reported, but the polysaccharide

had a glycosylamine-terminated oligosaccharide composed of one Rha, one AceA, one Gal, one Ara, three 6-deoxy methyl hexoses, and one O-acetyl residue (Li et al. 2004). Spectrophotometric chemical analysis of the exopolymers in Vibrio spp. are reported by Bholse et al. (1995), whereas no reports are available on ESI-MS, GC and FTIR characterization from luminous bacteria.

Fig 3.4.1. Schematic representation for isolation and purification of EPS produced by marine luminous bacterial strains.



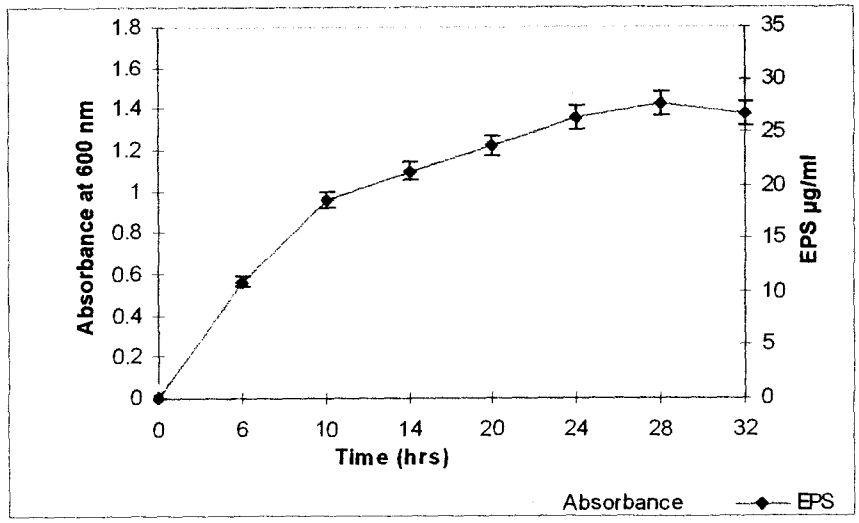


Fig 3.4.2. Growth and EPS production of *V. harveyi*. Culture conditions were: medium MSM broth+1.5% NaCl+ 0.2%glucose, temperature 28°C, pH 7.0 and agitator speed 160 rpm.

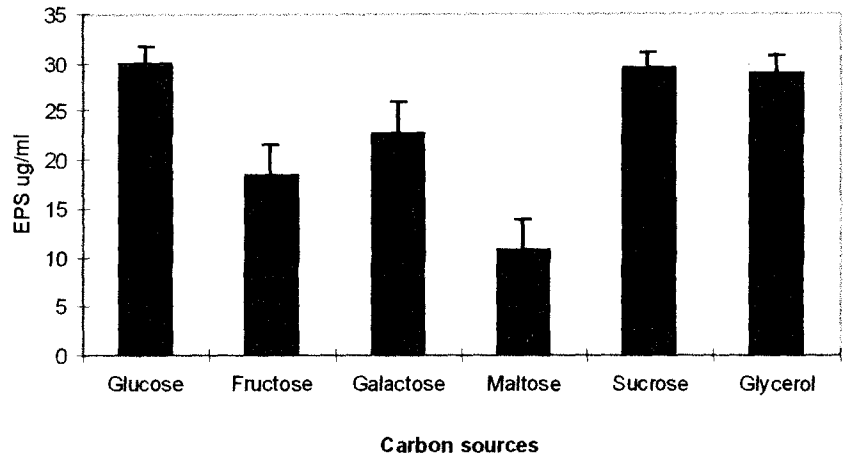


Fig. 3.4.3 Effect of various Carbon sources on the growth an production of EPS (exopolymer) by marine luminous bacterial strain VB23.

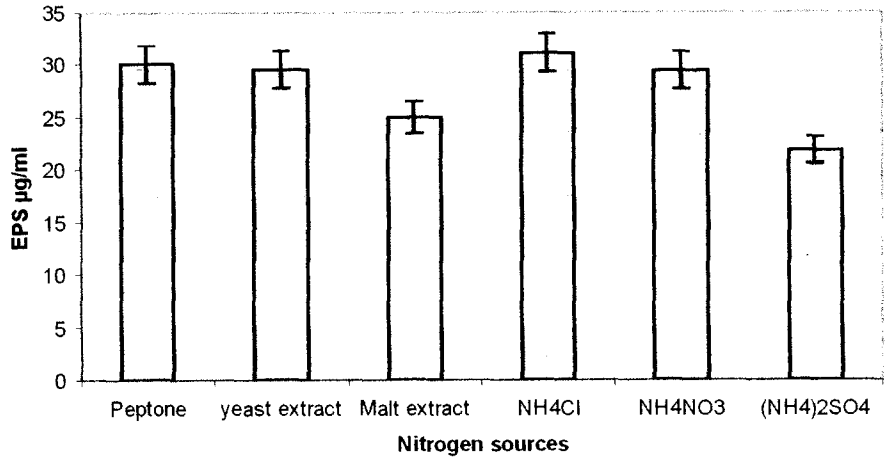


Fig 3.4.4 Effect of various Nitrogen sources on the growth and production of EPS (exopolymer) by marine luminous bacterial strain VB23.

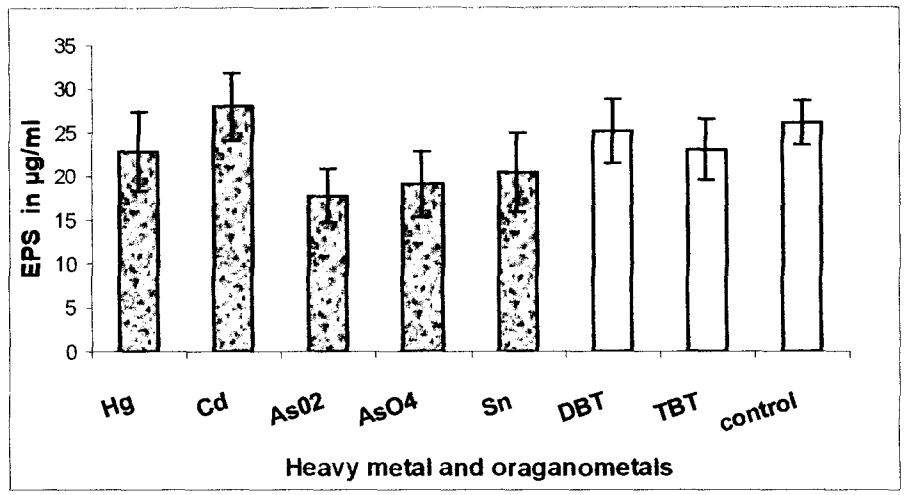


Fig 3.4.5 Exopolymer (EPS) production of luminous bacterial strain VB23 in the presence of heavy metals and oragno metals.

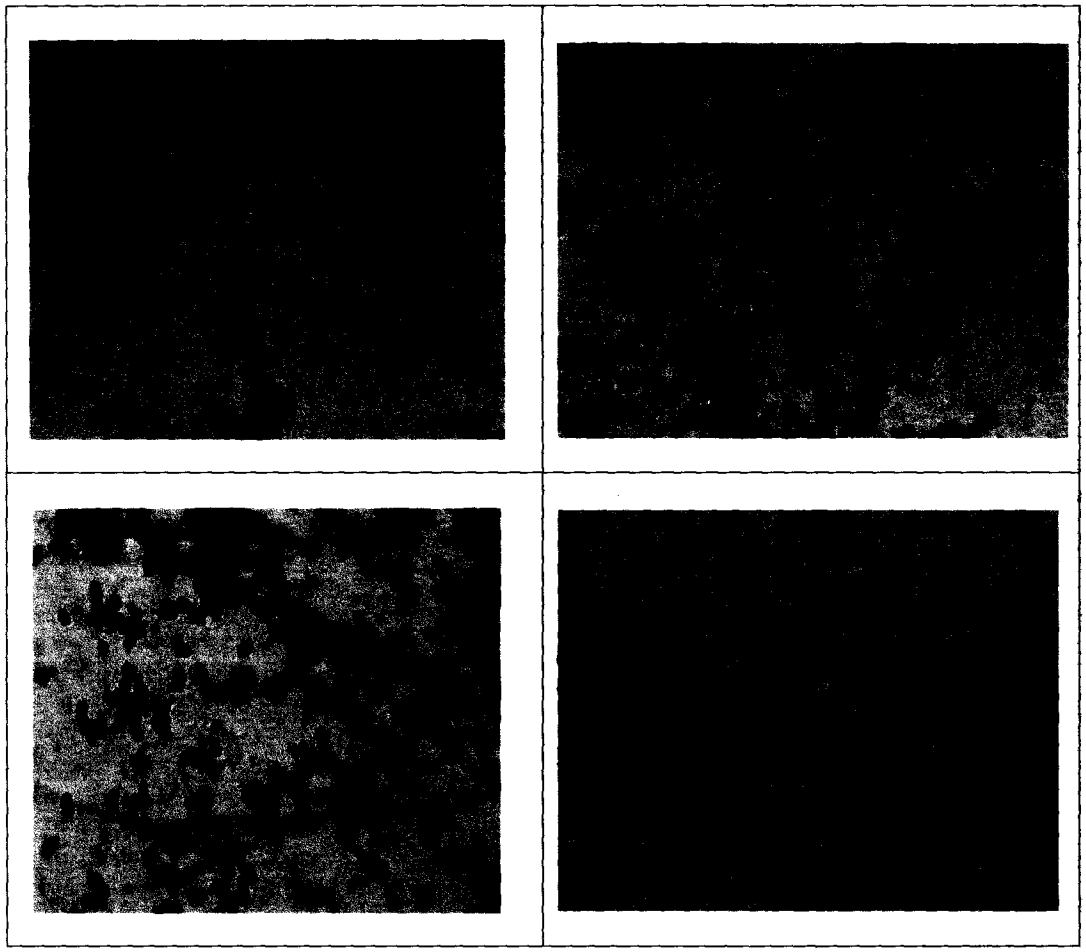


Fig 3.4.6. Images of Alcian blue staining of luminous bacterial strain VB23 exopolymer (Uronic acids (pH 2.5) and Sulphates (pH 0.5))

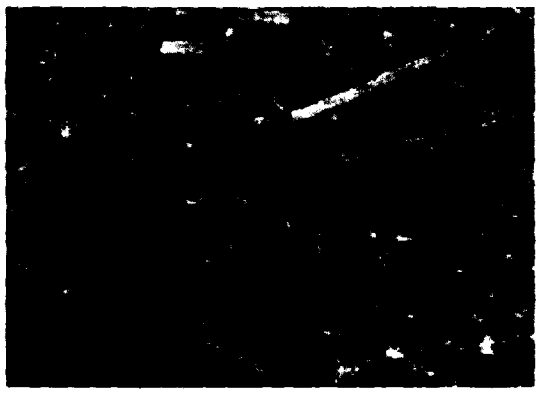


Fig. 3.4.7. Scanning electron microscopy image of the exopolymer

Microorganism	TCHO	Proteins	Uronicacids	Sulphates	Methyl Pentoses	References
V.harveyi (VB23)	211.71	186	114.62	---	32	present study
Vibrio sp.	510.9	17.93	143.89	---	ND	Majumdar et al. (1999)
Alteromonas Infernus	570	40	420	88	ND	Guezenne et al. (1998)
A. macleodi	420	40	380	50	ND	Rougaune et al. (1998)
Marinobacter	168.9	438.9	175.8	26.9	ND	Bhaskar (2003)

Table 3.4.1. Chemical composition of exopolymer (µg/mg) isolated from luminous *Vibrio harveyi* VB23 in comparison to other bacterial isolates

Exopolymer	Incubation time (mins)	Control O.D	Sample at A_{540}nm	% Emulsifying activity
EPS (VB23)				
Dialyzed, lyophilized	0	0.00	0.47	100%
	30	0.00	0.19	40.42%
	60	0.00	0.16	34.04%
EPS (VB23)				
Non-dialyzed	0	0.00	0.22	100%
	30	0.00	0.08	36.36%
	60	0.00	0.02	9.0%

Hexadecane, 0·5 ml, was added to 0·5 ml EPS (1 mg ml^{-1}) diluted to 2 ml with phosphate-buffered saline (PBS), vortexed for 1 min and the absorbance monitored at 540 nm. A control was run with 2 ml PBS without EPS.

Table 3.4.2 Emulsifying activity of the exopolymer VB6 and VB23

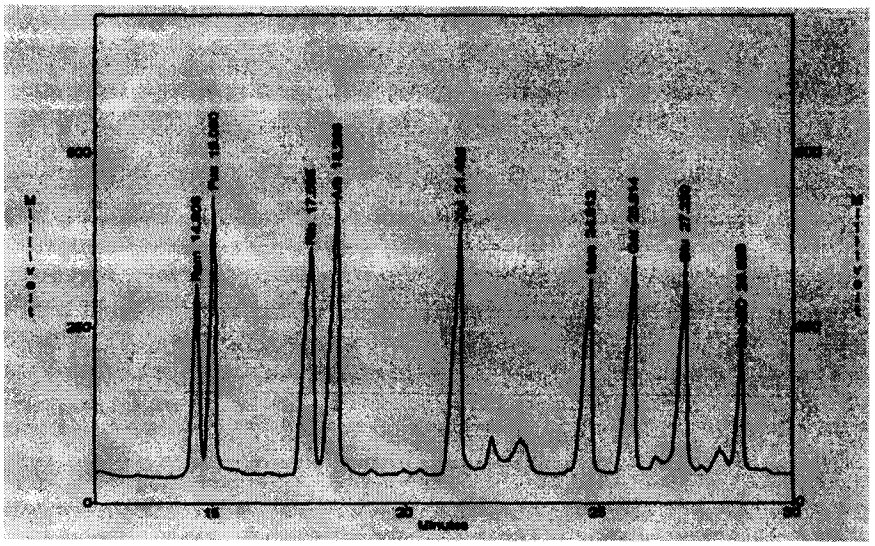


Fig. 3.4.8 A. Gas chromatogram of the standard: Inositol, as internal standard

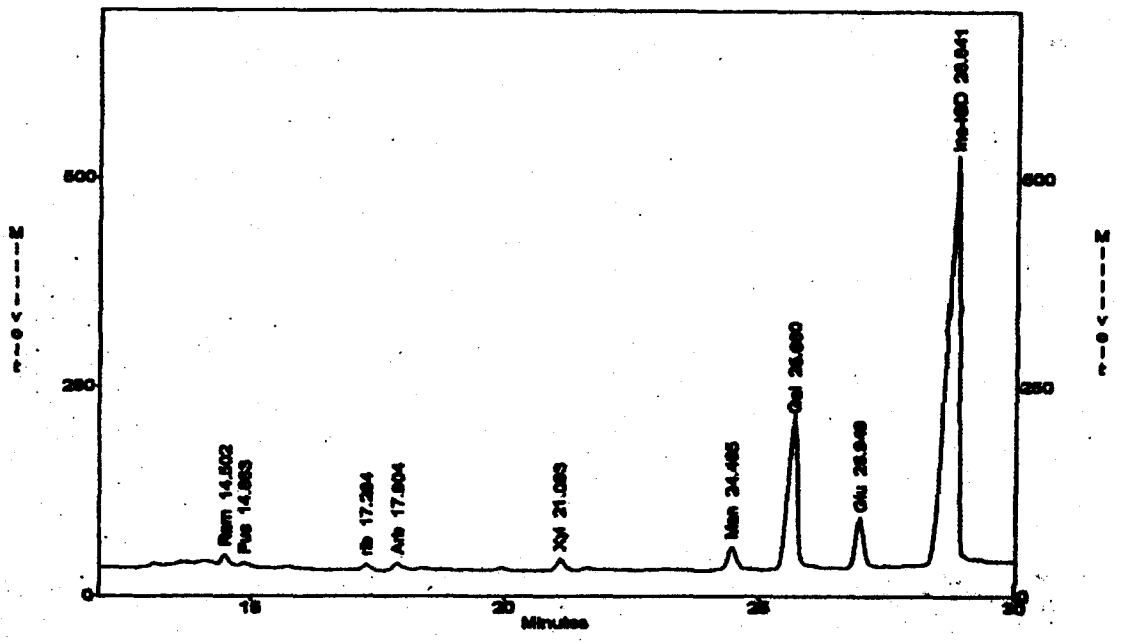
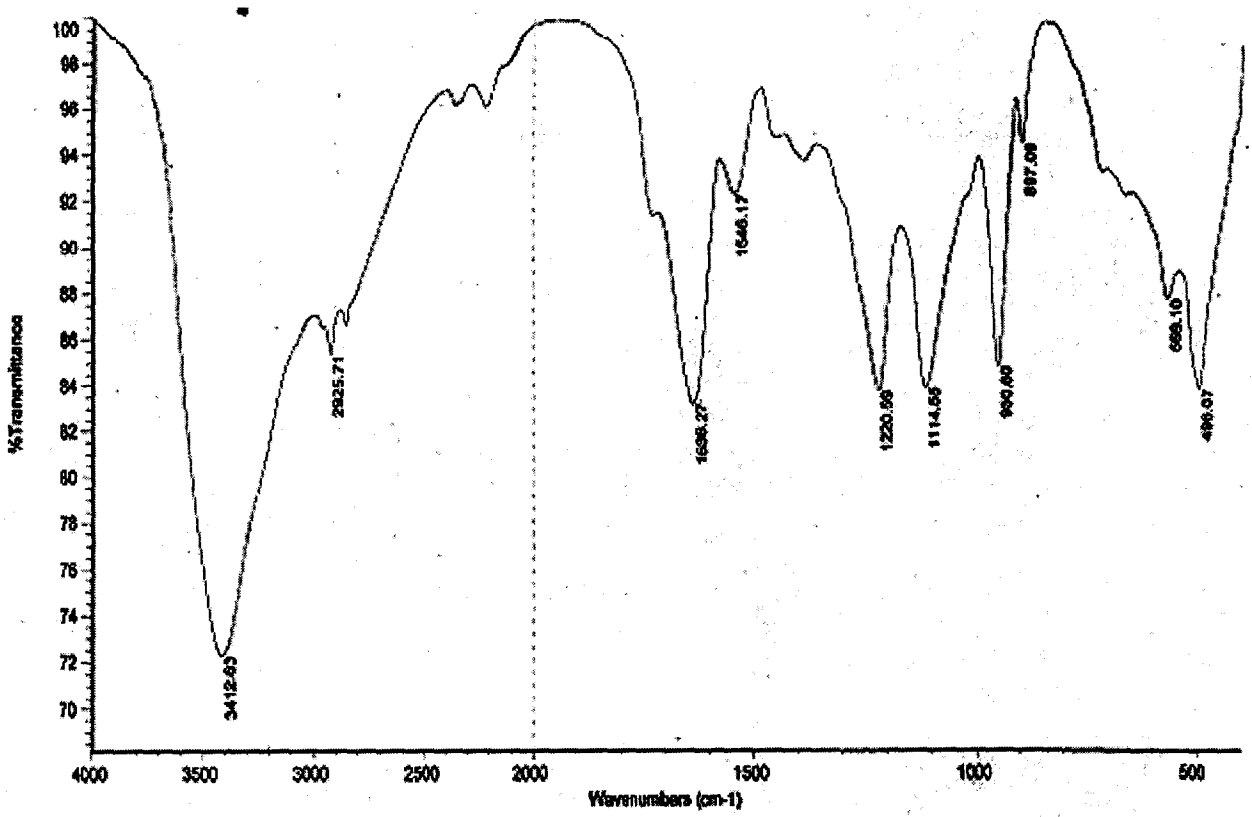


Fig. 3.4.8 B. Gas chromatogram of alditol acetate derivative of hydrolyzed exopolysaccharide from *Vibrio harveyi* VB23

Exopolymer	Wavenumber (cm^{-1})	Functional groups	
Vibrio harveyi (VB23)	890	ß-glycosidic linkages	-C-H-
	1220.59	asymmetric carbon	-C-O-
	1546.17	amide (protein)	$-CONH_2-$
	1636.27	carboxylic acids	-COOH-
	2925.71	methyl	$-CH_3$
	3412.63	hydroxyl	-OH-

Table 3.4.3. FTIR–functional groups of the exopolymer produced by VB23



(Fig 3.4.2. FTIR spectrum of the exopolysaccharide produced by *Vibrio harveyi* VB23

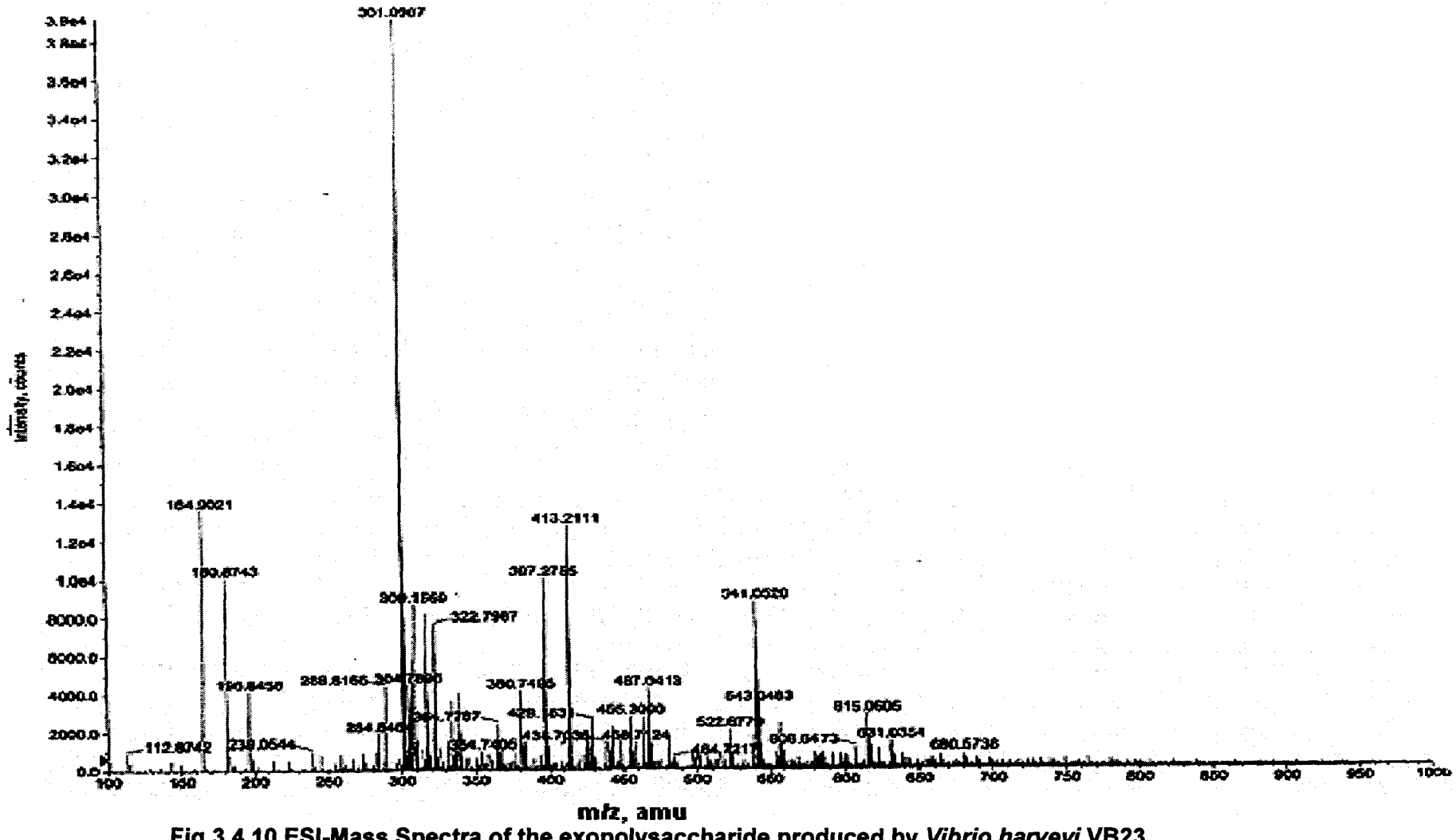


Fig.3.4.10 ESI-Mass Spectra of the exopolysaccharide produced by *Vibrio harveyi* VB23

Pigment studies

Induction of VB23 pigments in presence of metal and organo-metals

The pigments of luminous bacterium *Vibrio harveyi* VB23 pigments extracted with various organic solvents. Methanol (100 %) was found to be the most efficient solvent for extracting the VB23 pigment, followed by acetone, ethyl acetate, ethanol, chloroform and pigment was not extracted by tetahydrofuran (**Table 3.5.1).** Extraction and purification of the luminous bacterial pigment resulted in a yellow water-insoluble powder that was observed under long-wavelength of UV light.

FTIR spectra of the VB23 pigment

The FTIR spectrum of the *Vibrio harveyi* VB23 pigment isolated in methanol revealed characteristic functional groups of broad stretching at 1638 cm^{-1} characteristic for peptide of a protein (amide II group) group. Further, a stretching peak noticed at 1739 cm^{-1} revealed the presence of ester group, a peak at 1456 cm^{-1} and 2844 cm^{-1} could be assigned to the presence of C-H (Symmetry) of $>CH_2$ (methylene group). Specifically, the peaks at 770 cm^{-1} shows the monosubstitution on the aromatic ring. The major FTIR functional groups present in the VB23 pigment are peptide, ester and methylene groups on an aromatic ring (Fig.3.5.6). Overall the structure of the test organism's pigment corresponds to an aromatic polar compound, which is easily soluble in organic solvent methanol (100%).

UV Vis and HPLC spectra of the VB23 pigment

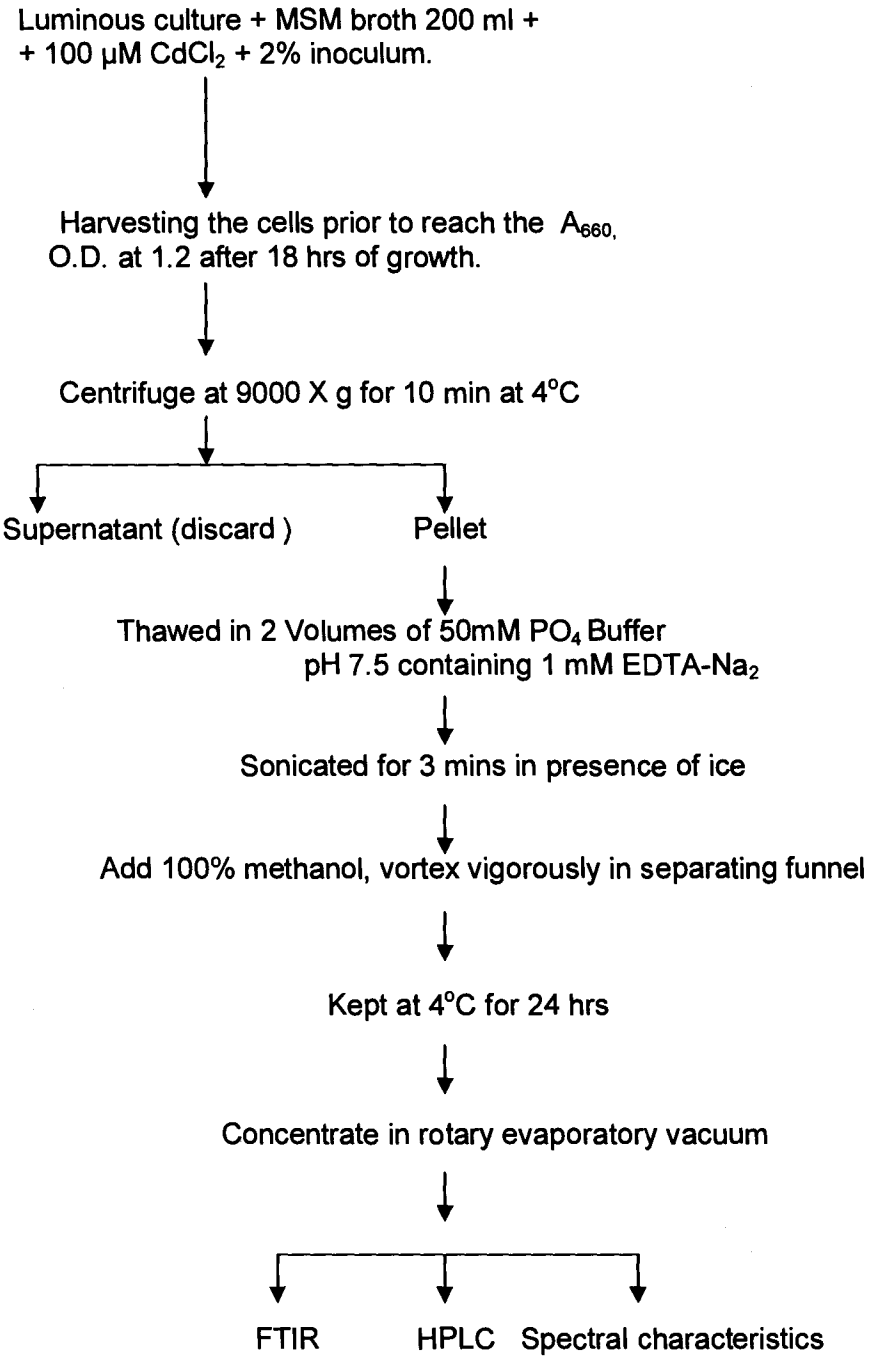
The UV-visible light absorption spectrum of the pigment extracted from control was dissolved in methanol showed major peaks, at 229.5 and 194 nm, whereas the absorption spectrum of the pigment extracted from cells exposed to 5 µM Hg, showed a peak shift at 237 nm and two peak shifts were observed at 238.5 nm and 600 nm respectively, when the cells were exposed to at 7 µM Hg (Fig 3.5.2. A). The bacterial cells grown in presence of Cd 50 µM and 100 µM, showed a peak shift at 235.5 nm, 240.5 nm and 286 nm (Fig 3.5.2. B). This also indicates that enhancement of pigment concentration under the stress of heavy metals. In presence of oxyanionic metals such as As (III) and As (V), peaks shifts

at 214 nm, 231.5 nm, 240 nm, and 280 nm were observed (Fig 3.5.3. A), but in presence of inorganic tin Sn (II) 2 peaks were observed at 235.5 nm and 600 nm respectively (Fig 3.5.2. A). The absorption spectrum of the pigment in presence of organo-metals such as TBT and DBT showed two peaks ranging from 212.5 nm and 601 nm respectively along with the enhancement in the concentration of pigment synthesis (Fig 3.5.4 A & B). Six major pigment components were separated on HPLC with a retention factors ranging from 2.002 to 14.727 (Fig 3.5.5). Based on these data and other changes in the IR and UV-Vis spectral pattern, the bacterial pigment of VB23 has spectral characteristics similar to the antenna lumazine protein (LumP) of *Photobacterium leiognathi* (Daubner et al 1987; Petushkov et al. 1996). These results clearly demonstrate that toxic metals and organo-metals tested caused enhanced synthesis of pigments in *Vibrio harveyi* VB23, which may be involved in biochemical resistance against toxic metals and organo-metals possibly through intracellular sequestration.

Organic solvents	**Concentration %**	**Pigment extraction**
Methanol	100	+++
Methanol	50	++
Chloroform	100	++
Chloroform	50	++
Ethylacetate	100	++
Acetone	100	++
Ethanol	100	+
Diethylether	100	+

Table 3.5.1. Extraction of luminous bacterial stress pigments by different organic solvents

Fig. 3.5.1. Schematic representation for isolation of Pigment produced by marine luminous bacterial strains.



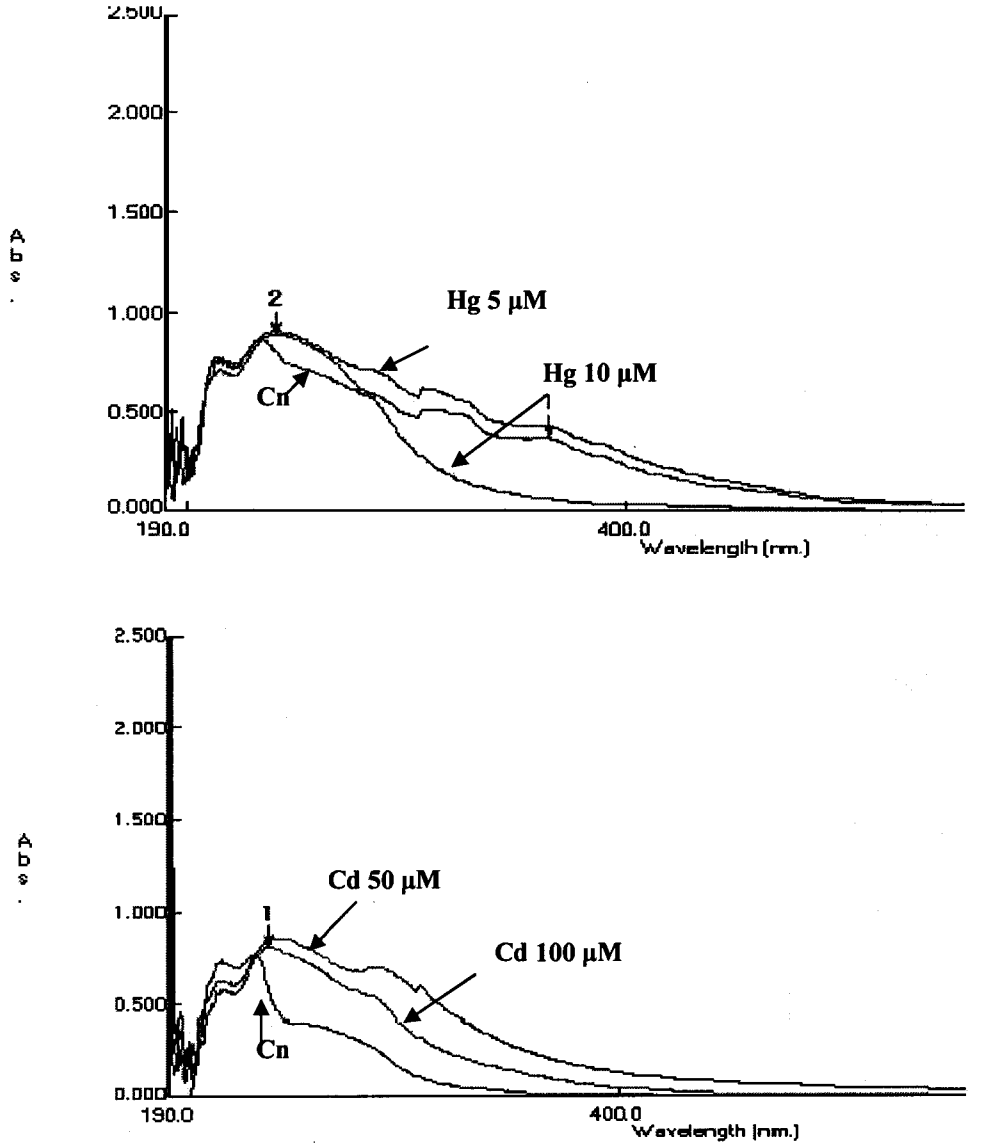


Fig. 3.5. 2 Spectral scan of stress pigments of *V. harveyi* VB23 grown in presence of heavy metals (A), $HgCl_2$ (B), $CdCl_2$ (Cn –Control, Hg and Cd –heavy metals)

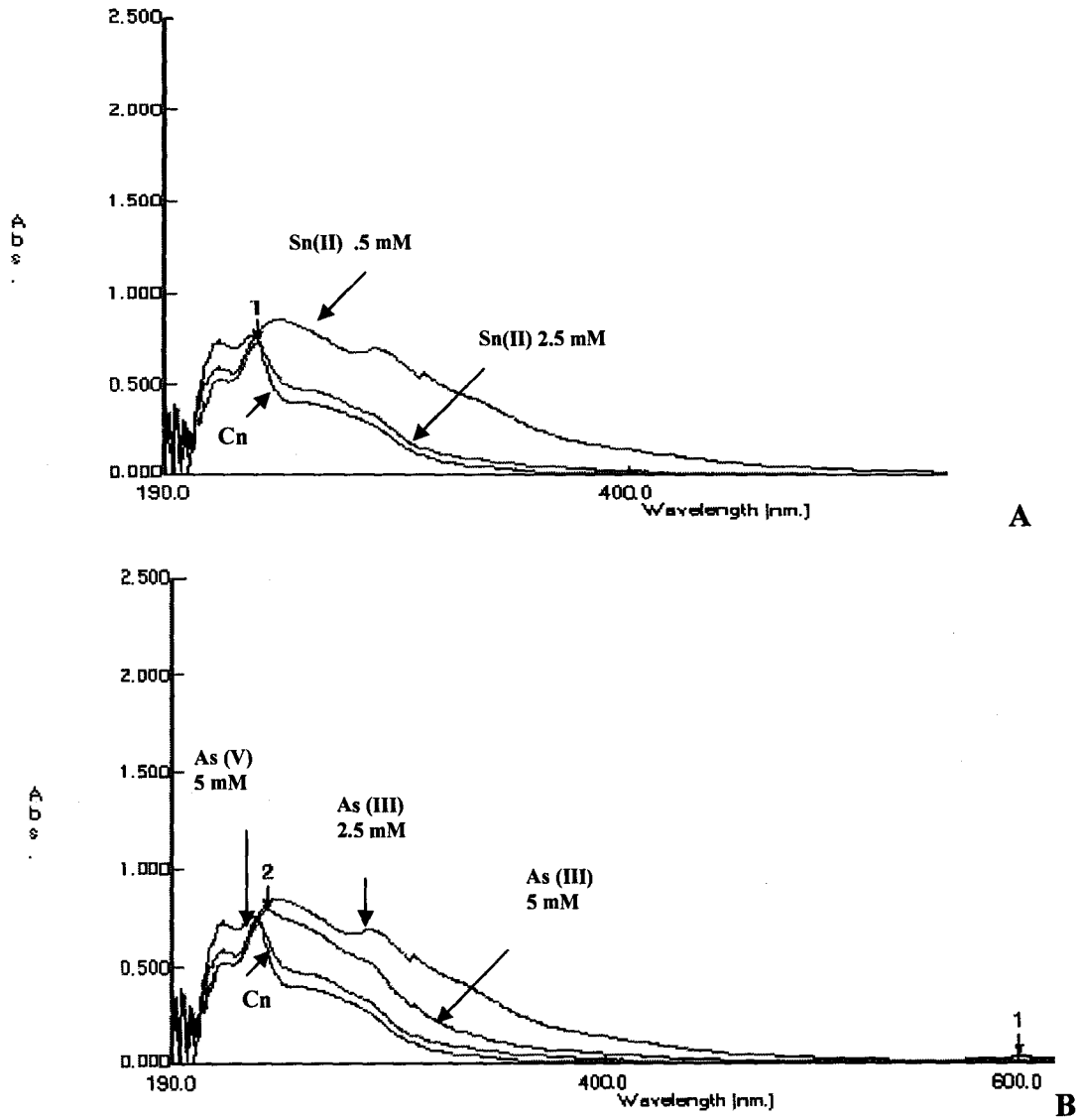


Fig 3.5. 3. Spectral scan of stress induced pigments grown in presence of heavy metal. (A). $SnCl_2$,(B) As (III) and As (V) . (Cn –Control, As(III) and As(V)–heavy metals)

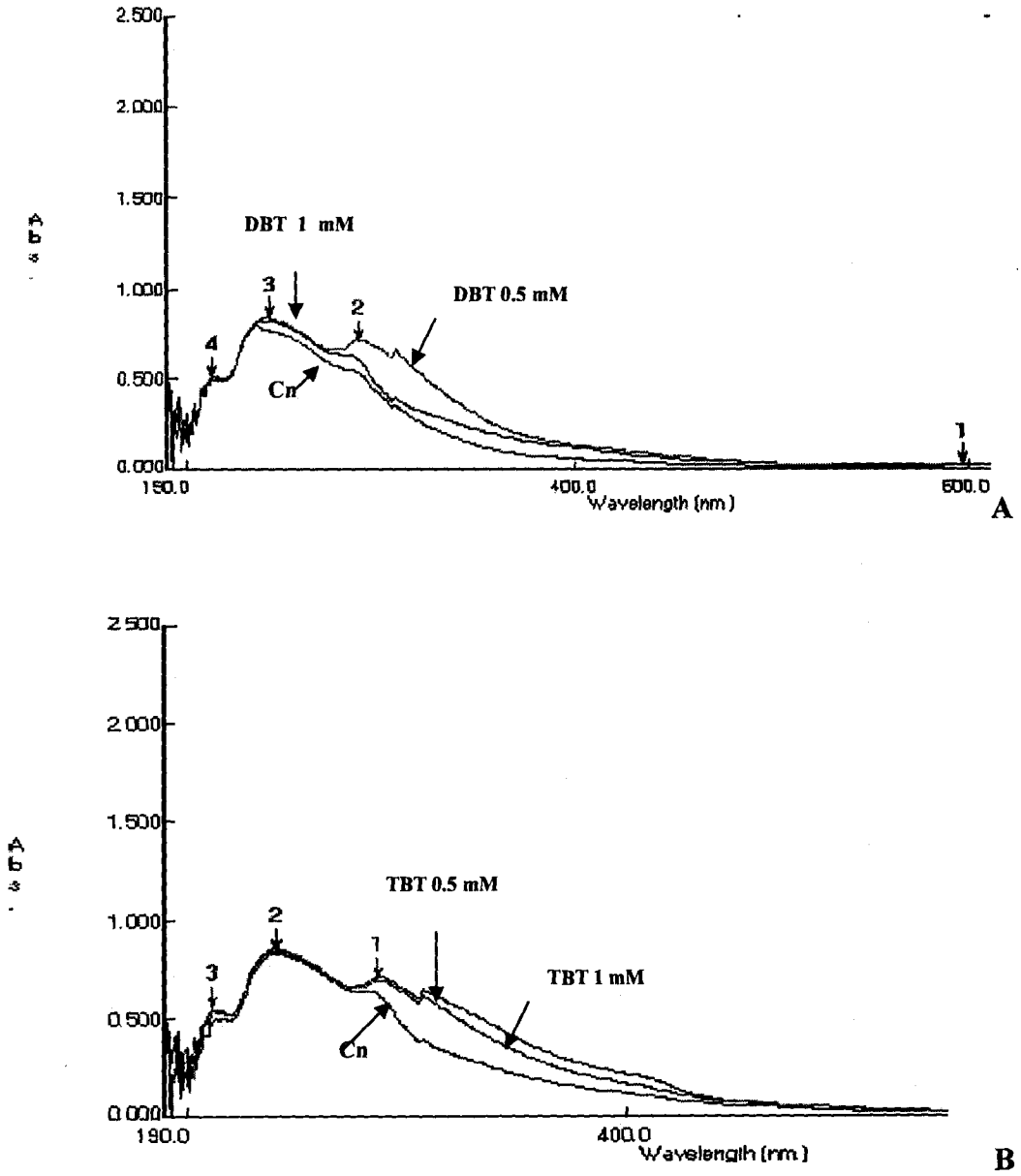


Fig 3.5. 4. Spectral scan of stress induced pigments grown in presence of heavy metal (A), DBTC (B) TBTC. (Cn –Control, DBT and TBT–organo- metals)

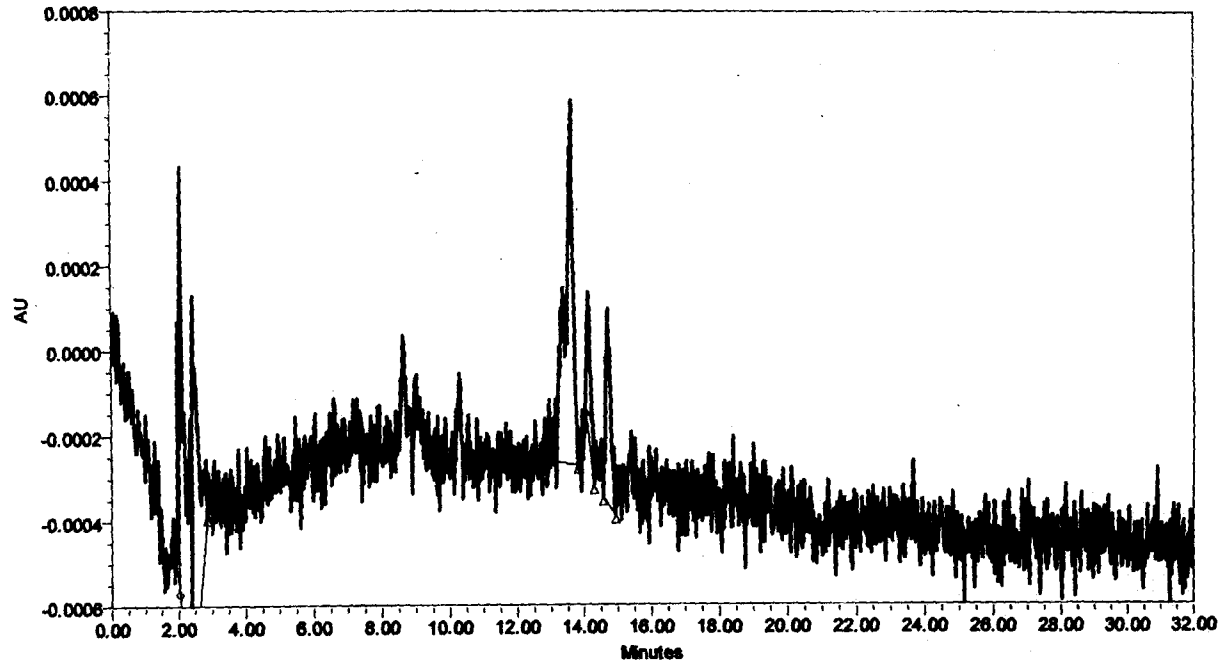


Fig 3.5.5. HPLC spectra of the pigment produced by VB23

Peak Results

	Name	RT	Area	Height	Amount_1 ug
1	1	2.002	1929	565	
2	2	2.087	11703	1011	
3	3	2.451	10885	888	
4	4	13.630	14643	799	
5	5	14.157	2673	304	
6	6	14.727	4679	436	

HPLC peak results for the pigment VB23

Pigment	Wavenumber (cm^{-1})	Functional groups	
Vibrio harveyi (VB23)	770	aromatic ring mono substituted	
	1456	methylene	- CH_2
	1638	amide (protein)	-$CONH_2$-
	1739	Ester group	-CH_3
	2844	methylene	- CH_2
	3423.63	hydroxyl	-OH-

Table 3.5.2. FTIR –functional groups of the pigment produced by VB23

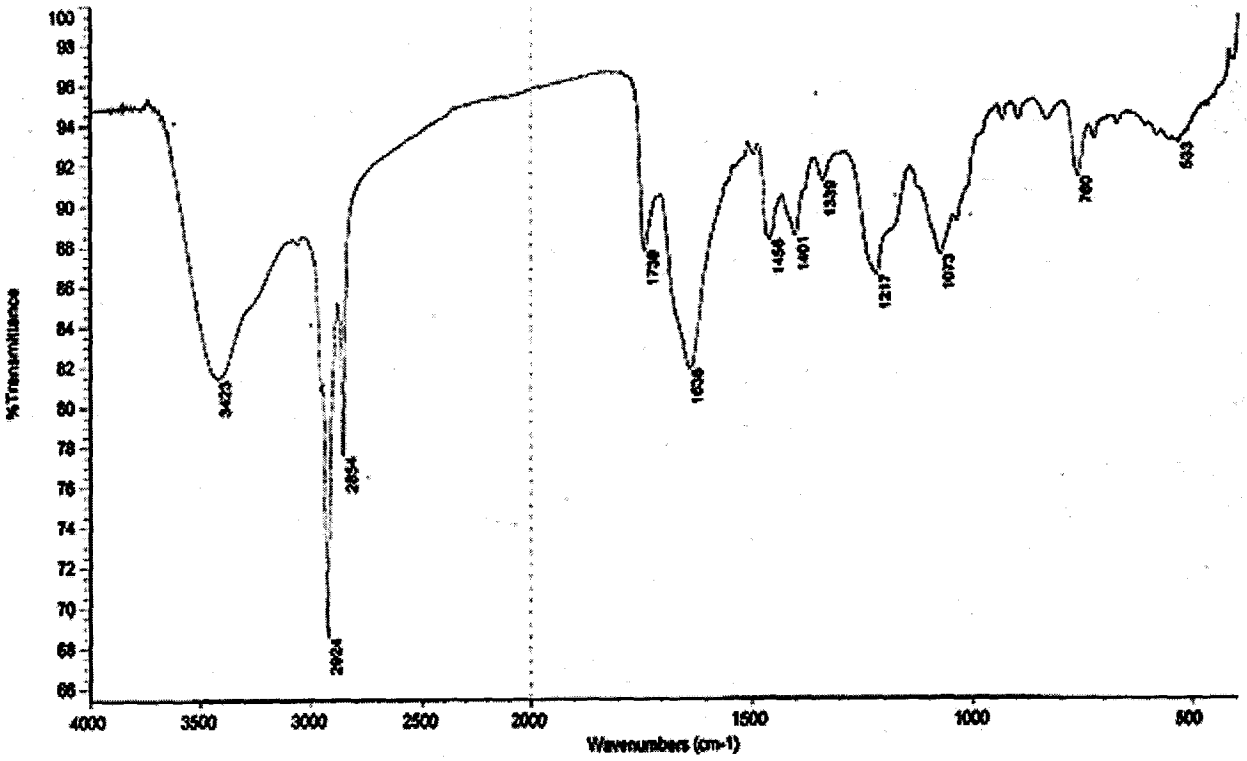


Fig 3.5.6. FTIR spectra of the pigment produced by VB23

References

Abraham, T. J., Manley, R., Palaniappan, R. and Dhevendaran, K. 1997. Pathogenicity and antibiotic sensitivity of luminous *Vibrio harveyi* isolated from diseased penaeid shrimp. J. Aquacult. Trop. 12:1–8.

Abu, G.O., Weiner, R.M., Rice, J. and Colwell, R.R. 1991. Properties of an extracellular adhesive polymer from the marine bacterium, *Shewanella colwelliana*. Biofouling. 3: 69-84.

Alsina, M. and Blanch, A. R. 1994. A set of keys for the biochemical identification of environmental *Vibrio* species. J. Appl. Bacteriol 76: 79–85.

Arias, S., del Moral, A., Ferrer, M.R., Tallon, R., Quesada, E. and Bejar, V. 2003. Mauran, an exopolysaccharide produced by the halophilic bacterium *Halomonas maura*, with a novel composition and interesting properties for biotechnology. Extremophiles. 7: 319–326.

Barkay, T. 1987. Adaptation of aquatic microbial communities to Hg^{2+} stress. Appl Environ Microbiol 53:2725–2732.

Barkay, T, Gillman, M. and Liebert, C. 1990. Genes encoding mercuric reductases from selected gram-negative aquatic bacteria have a low degree of homology with *merA* of Transposon 501. Appl. Environ. Microbiol. 56(6):1695-1701.

Baumann, P.S. and Schubert, R.H.W. 1984. The family II Vibrionaceae Veron. In: Bergey's Manual of Systematic Bacteriology, Vol.1 (Ed. Krieg N.R.), pp. 515-538.Williams and Williams, Baltimore, MD, USA.

Beech, I. B. and Gaylarde, C.C. 1989. Adhesion of *Desulfovibrio desulfuricans* and *Pseudomonas fluorescens* to mild steel surfaces. J. Appl. Bacteriol. 67: 201–207.

Belliveau, B.H., P.T.S. Wong and J.T. Trevors. 1987. Lead, tin and multiple antibiotic resistance *Pseudomonas* spp. isolated from polluted sediment. Toxicity Assessment 2: 377-386.

Bernhardt, J., Volker, U., Volker, A., Antelmann, H., Schmid, R., Mach, H. and Hecker, M 1997. Specific and general stress proteins in *Bacillus substilis* - a two-dimensional protein electrophoresis study. Microbiol. 143: 999- 1017.

Bhaskar, P.V. 2003. Studies on some aspects of marine microbial exopolysaccharides. Ph.D. Thesis, Goa University.

Bhosle, N. B., Sawant, S. S., Garg, A. and Wagh, A. B. 1995. Isolation and partial chemical analysis of exopolysaccharides from the marine fouling diatom *Navicula subinflata*. Bot. Mar. 38: 103-110.

Cerventes, C, Chavez, K. and Vaca, S. 1991. Mechanism of bacterial resistance to heavy metals. Rev Latinoam Microbiol.33: 61-70.

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

Chaudhury, P. and Kumar, R. 1996. Association of metal tolerance with multiple antibiotic resistance of enteropathogenic organisms isolated from coastal region of Deltaic Sunderbans. Ind. J. Medical Research 104:148-151.

Christensen, B. E, Kjosbakken, J, Smidsrod, O. 1985. Partial chemical and physical characterization of two extracellular polysaccharides produced by marine, periphytic *Pseudomonas* sp. strain NCMB 2021. Appl Environ Microbiol. 50: 837–845.

Collins, Y., Vonne, E. and Stotzky, G. 1989. Factors Affecting the Toxicity of Heavy Metals to Microbes. Beveridge, Terrance J. and R.J. Doyle. pp-(31-90). Eds. *Metal Ions and Bacteria*. New York: John Wiley & Sons, Inc. New York.

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

Corpe, W. A. 1970. An acid polysaccharide produced by primary film forming bacteria. Dev Ind Microbiol 16, 249–255.

Crayton, M. A. 1982. A comparative cytochemical study of volvocacean matrix polysaccharides. J. Phycol, 18: 336-344.

Czyz, A., Jasiecki, J., Bogdan, A., Szpilewska, H. and Wegrzyn, G. 2000. Genetically modified *Vibrio harveyi* strains as potential bioindicators of mutagenic pollution of marine environments. Appl. Environ. Microbiol. 66: 599–605.

Czyz, A., Plata, K., Wegrzyn, G. 2002. Induction of light emission by luminescent bacteria treated with UV light and chemical mutagens. J. Appl Genetics, 43, 377-389.

Daubner, S. C, Astorga, A. M, Leisman, G. B. and Baldwin, T. O. 1987. Yellow light emission of *Vibrio fischeri* strain Y-1: purification and characterization of the energy-accepting yellow fluorescent protein. Proc. Natl. Acad. Sci. U.S.A. 84:8912–16.

Decho, A.W. 1990. Microbial exopolymer secretion in ocean environment, their roles in food webs and marine processes. Ocean Marine Biol Ann Reviews. 28: 73–153.

De Vicente, A., Aviles, M., Codina, J.C., Borrego, J.J and Romero, P. 1990. Resistance to antibiotic and heavy metals of *Pseudomonas aeruginosa* isolated from natural waters. J. Appl. Bacteriol. 68: 625–632.

Dioro, C., Cai, J., Marmor, J., Shinder, R., and Dubow, M.S. 1995. An *Escherichia coli* chromosomal *ars* operon homolog is functional in arsenic detoxification and is conserved in gram-negative bacteria. J. Bacteriol 177(8): 2050-2056.

Dische, Z. 1962. Color reactions of hexuronic acids. Methods Carbohy Chem. 1: 497-501.

Dische, Z. and Shettles, L.B. 1948. A specific colour reaction of methyl pentoses and a spectrophotometric micro method for their determination. Journal of Biological Chemistry 175: 595-603.

Dressler, C., Kues, U., Nies, D. and Friedrich, B. 1991. Determinants encoding resistance to several heavy metals in newly isolated copper-resistant bacteria. Appl. Environ. Microbiol. 57: 3079–3085.

Dubey, S.K. and Rai, L.C. 1989. Toxicity of chromium and tin to *Anabaena doliolum.* Biol. of Metals. 2: 55-60.

Dubois, M., Gilles, K. A., Hamilton, J. K., Rebers, P.A., and Smith, F. 1956. Colorimetric method for determination of sugars and related substances. Analytical Chemistry 28: 350–356.

Fazio, S. A., Uhlinger, D. J., Parker, J. H. and. White, D. C. 1982. Estimations of uronic acids as quantitative measures of extracellular and cell wall polysaccharide polymers from environmental samples. Appl. Environ. Microbiol 43: 1151-1159.

Ford, T, Maki, J. and Mitchell, R. 1995. Metal-microbe Interactions, In: Gaylarde, Christine C. and Videla, Hector A. pp. 1–23. eds. Bioextraction and Biodeterioration of Metals. Cambridge University Press; Boston.

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

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

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

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

Geesey, G.G. 1982. Microbial exopolymers: ecological and economic considerations. Am Soc Microbiol News 48, 9–14.

Geesey, G.G. and Jang, L. 1990. Extracellular polymers for metal binding. In: Ehrlich, H.L., Brierley, and C.L. pp. 223–247. (Eds.), Microbial Mineral Recovery. McGraw-Hill, New York.

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

Gadd, G.M. 2000. Microbial interaction with tributyltin compounds: detoxification, accumulation and environmental fate. Sci. Total. Environ. 21. 119-127.

Guezennec, J., Ortega-Morales, O., Raguenes, G. and Geesey, G. 1998. Bacterial colonization of artificial substrate in the vicinity of deep – sea hydrothermal vents. FEMS Microbiol. Ecol 26: 89–99.

Heng, L.Y, Jusoh, K, Ling, C.H.M. and Idris M. 2004. Toxicity of single and combinations of lead and cadmium to the cyanobacteria *Anabaena flos-aquae*. Bull Environ Contam Toxicol. 72: 373–379

Hideomi, N., Ishikawa, T., Yasunaga, S., Kondo, I., and Mitsuhasi, S. 1977. Frequency of heavy-metal resistance in bacteria from inpatients in Japan. Nature: 266:165–167.

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

Horitsu, H., Yamamoto, K., Wachi, S., Kawai, K. and Fukuchi, A. 1986. Plasmid-determined cadmium resistance in *Pseudomonas putida* GAM-1 isolated from soil. J Bacteriol 165, 334–335.

Hughes, M., Martin N. and Poole, R. K. 1989. Metals and Microorganisms, NY: Chapman and Hall. New York.

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

Jude, F. C., Arpin, C., Brache, C., Capdepuy, M., Caumette, P. and Quentin, C. 2004. TbtABM, a multidrug efflux pump associated with tributyltin resistance in *Pseudomonas stutzeri*. FEMS Microbiol. Lett. 232: 7- 14.

Kachur, A.V, Koch, C. J. and Biaglow J. E. 1998. Mechanism of copper catalyzed oxidation of glutathione. Free Radic Res 28: 259–269.

Kennedy, A.F.D and Sutherland, I.W. 1987. Analysis of bacterial exopolysaccharides. Biotechnol Appl Biochem 9:12–19.

Khazaeli, M.B. and Mitra, R.S. 1981. Cadmium-binding component in *Escherichia coli* during accommodation to low levels of this ion. Appl. Environ. Microbiol 41: 46–50.

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

Ko, S.H., Lee, H.S., Park, S.H, Lee, and H.K. 2000. Optimal conditions for the production of exopolysaccharide by marine microorganism *Hahella chenjuensis*. Biotechnol Bioproc Eng 5: 181–185.

Kumar, C.G., Joo, H. S., Choi, J.W., Koo, Y.W., Chang, C.S. 2004. Purification and characterization of an extracellular polysaccharide from haloalkalophilic *Bacillus* sp. I-450. Enzyme and Microbial Technology 34: 673–681.

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

Li, J, Yie, J., Foo, R.W.T, Ling, J.M.L., Xu H, and Woo, N.Y.S. 1998. Antibiotic resistance and plasmid profiles of *Vibrio* isolates from cultured silver sea bream, *Sparus sarba*. Mar Pollut Bull. 39:245–9.

Li, J., Yie, J., Foo, R.W.T., Ling, J. M. L., Xu, H. and Woo, N.Y.S. 1999. Antibiotic resistance and plasmid profiles of *Vibrio* isolates from cultured *Sparus sarba*. Mar. Poll. Bull. 39: 245-249.

Li, J., Wang, Z and Altman, E. 2004. n-source fragmentation and analysis of polysaccharides by capillary electrophoresis/mass spectrometry. J. Mass Spectrometry.39: 505 – 513.

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

Lupi, C.G., Colangelo, T. and. Mason, C.A. 1995. Two-dimensional gel electrophoresis analysis of the response of *Pseudomonas putida* KT2442 to 2-chlorophenol. Appl. Environ. Microbiol. 61(8): 2863-2872.

Mahler, I., Levinson, H.S, Wang, Y. and Halvorson, H.O. 1986. Cadmium and mercury-resistant *Bacillus* strains from a salt marsh and from Boston Harbor. Appl. Environ. Microbiol. 52: 1293–1298.

Majumdar, I., D'Souza, F. and Bhosle, N.B. 1999. Microbial exopolysaccharides: effect on corrosion and partial chemical characterization. J. Indian Instit. Science 79, 539-550.

Malik, A. and Ahmad, M. 1994. Incidence of drug and metal resistance in *E. coli* strains from sewage water and soil. Chem. Environ. Res. 3:3-11.

Mao, R., Tang, J. and Swanson, B.G. 2001. Water holding capacity and microstructure of gellan gums. Carbohydr Polym. 46:365-71.

McArthur, J.V. and Tuckfield, R.C. 2000. Spatial patterns in antibiotic resistance among stream bacteria: effects of industrial pollution. Appl. Environ. Microbiol. 66(9): 3722-3726.

Muller K.A., Rasmussen, L.D., Sorensen, S.J. 2001. Adaptation of the bacteria community to mercury contamination. FEMS Microbiol Lett 204:49–53

Nandivada, L.S, Amyes, S.G.B. 1990. Plasmid mediated ß-lactam resistance in pathogenic Gram negative bacteria isolated in South India. J. Antimicrob. Chemother. 26: 279-290.

Nies, D. H. 1992. *CzcR and CzcD*, gene products affecting regulation of resistance to cobalt, zinc, and cadmium (*czc* system) in *Alcaligenes eutrophus.* J. Bacteriol. 174: 8102-8110.

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

Nyström, T. and Neidhardt, F.C. 1992. Cloning, mapping and nucleotide sequencing of a gene encoding a universal stress protein in *Escherichia coli.* Mol. Microbiol. 6: 3187-3198.

Osborn, A. M., Bruce, K. D., Strike, P., and Ritchie, D. A. 1997. Distribution, diversity and evolution of the bacterial mercury resistance (*mer)* operon. FEMS Microbiol Rev 19:239–262.

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

Petushkov, V.N., van Stokkum, I. H. M., Gobets, B., Mourik, F., Lee, J., Grondelle, R.V. and Visser, A. J. W. G. 2003. Ultrafast Fluorescence Relaxation Spectroscopy of 6,7-Dimethyl-(8-ribityl)-lumazine and Riboflavin, Free and Bound to Antenna Proteins from Bioluminescent Bacteria. J. Phys. Chem. B 107: 10934-10939.

Rahman Khan, M.K. and Malik, A. 2001. Antibiotic resistance and detection of b-lactamase in bacterial strains of *Staphylococci* and *Escherichia coli* isolated from foodstuffs. World J. Microbiol. Biotechnol. 17: 863- 868.

Ramaiah, N. and Chandramohan, D. 1993. Ecological and laboratory studies on the role of luminous bacteria and their luminescence in coastal pollution surveillance. Mar Poll Bull 26:190–201.

Ramaiah, N., Kenkre, V.D., and Verlekar, X.N. 2002. Marine environmental pollution stress detection through direct viable counts of bacteria. Water Res. 36:2383–2393.

Rames, J., Chaloupecky, V., Sojkova, N. and Bencko, V. 1997. An attempt to demonstrate the increased resistance of selected bacterial strains during repeated exposure to UV radiation at 254 nm. Cent. Eur. J. Public. Health, 5: 30--31.

Ravel, J., Amoroso, J.M., Colwell, R.R., and Hill, R.T. 1998. Mercury-resistant actinomycetes form the Chesapeake Bay. FEMS Microbiol Lett 162:177–184.

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

Reccchia, G. D. and Hall, R. M. 1997. Origins of the mobile gene cassettes found in integrons. Trends Microbiol. 5:389–394.

Robinson, J. B. and Tuovinen, O. H. 1984. Mechanisms of microbial resistance and detoxification of mercury and organomercury compounds: physiological, biochemical, and genetic analyses. Microbiol. Rev. 48:95-124.

Rosenberg, E., Zuckerberg, A., Rubinovitz, C. and Gutnick, D.L. 1979. Emulsifier of *Arthrobacter RAG-1*: Isolation and emulsifying properties. Appl. Environ.Microbiol. 37: 402–408.

Rosen, B.P. 1996. Bacterial resistance to heavy metals and metalloids. J. Biol. Inorg. Chem .1:273–277.

Rouch, D.A., Lee, B.T.O., and Morby, A.P. 1995. Understanding cellular responses to toxic agents: a model for mechanism-choice in bacterial metal resistance. J. Indus. Microbiol. 14: 132-141.

Rougeaux, H., Pichon, R., Kervarec, N., Raguenes, G.H.C. and Guezennec, J.G. 1998. Novel bacterial exopolysaccharides from deep-sea hydrothermal vents. Carbohydrate Polymers 31: 237–242.

Royan, S., Parulekar, C and Mavinkurve, S .1999. Exopolysaccharides of *Pseudomonas mendocina* P2d. Lett. Appl Microbiol.1999, 29: 342–346.

Said, W. A. and Lewis, D. L. 1991. Quantitative assessment of the effects of metals on microbial degradation of organic chemicals. Appl.Environ. Microbiol. 57: 1498-1503.

Schiewe, M. H., Hawk, E.G., Actor, G. I. and Krahn, M. M. 1985. Use of a bacterial bioluminescence assay to assess toxicity of contaminated marine sediments. Can. J. Fish. Aqua. Sci. 42: 1244-1248.

Silver, S. and Walderhaug, M. 1992. Gene regulation of plasmid and chromosome-determined inorganic ion transport in bacteria. Microbiol. Rev. 56: 195–228.

Silver, S. 1996. Bacterial resistances to toxic metals-a review. Gene 179:9-19.

Silver, S. and Phung, L.T. 1996. Bacterial heavy metal resistance: new surprises. Annu. Rev. Microbiol. 50: 753-789

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

Spaeth, R., Flemming H.C, Wuertz, S. 1998. Sorption properties of biofilms. Water Sci Technol. 37: 207–210.

Sunda, W.G 1990. Trace metal interactions with marine phytoplankton. Biol. Oceanogr 6:411–442.

Sutherland, I. W. 1979. Microbial exopolysaccharides: control of synthesis and acetylation. In: Microbial Polysaccharides and Polysaccharases. pp 1–34. Berkeley RCW, eds. New York, Academic Press, NewYork.

Sutherland, I. W. 1982. Biosynthesis of microbial exopolysaccharides. Adv Microbial Phys 23, 79–150.

Suzuki, S. and Fukagawa, T. 1995. Tributyltin resistant bacteria: a summary of recent work. J. Indus. Microbiol. 14.154-158.

Terho, T. T. and Hartiala, K. 1971. Method for determination of the sulfate content of glycosaminoglycans. Analytical Biochemistry. 41: 471-476.

Uhlinger, D. J., and White. D. C.1983. Relationship between physiological status and formation of extracellular polysaccharide glycocalyx in *Pseudomonas atlantica*. Appl. Environ. Microbiol. 45: 64-70.

Ulitzur, S., Weiser, I. and Yannani, S. 1980. A new, sensitive and simple bioluminescence test for mutagenic compounds. Mutat. Res. 74: 113–24.

Ulitzur, S. and Weiser, I. 1981. Acridine dyes and other DNA intercalating agents induce the luminescence system of luminous bacteria and their dark variants. P. N. A. Scien. U.S.A. 78: 3338–3342.

Ulitzur, S. 1986. Bioluminescence test for genotoxic agents. Methods Enzymol. 133:264-274.

Van Bogelen, R. A., Kelley, P.M., and Neidhardt, F.C. 1987. Differential induction of heat shock, SOS, and oxidation stress regulons and accumulation of nucleotides in *Escherichia coli*. J. Bacteriol. 169: 26-32.

Wakatsuki, T. 1995. Metal oxidoreduction by microbial cells. J. Indus. Microbiol. 14: 169-177.

Wang, C.L., Maratukulam, P.D, Lum, A. M., Clark, D.S, Keasling, J. D. 2000. Metabolic engineering of an aerobic sulfate reduction pathway and its application to precipitation of cadmium on the cell surface. Appl. Environ Microbiol. 66: 4497–4502.

Williams, A.G. and Wimpenny, J.W.T. 1977. Exopolysaccharide production by *Pseudomonas* NICB 11264 grown in batch culture. J. Gen. Microbiol. 102: 255–263.

Wuertz, S., Muller, E., Spaeth, R., Pfleiderer, P. and Flemming, H.C. 2000. Detection of heavy metals in bacterial bofilms and microbial floes with the fluorescent complexing agent Newport Green. J. Ind Microbiol. Biotechnol. 24: 116–123.

Chapter IV

Molecular and genetic characterization of bioluminescent bacterial strains

Material and Methods

Screening of plasmids from luminous bacterial strains

Extraction of plasmid DNA

40 heavy metal resistant luminous bacterial isolates were screened for the presence of plasmids. Plasmid mini preps were done, using alkaline lysis method (Sambrook et al. 1989). A single bacterial colony was transferred into 10 ml of Luria Bertani broth supplemented with additional 1 % NaCl and incubated overnight in a incubator shaker (180 rpm) at 28±2°C. 1.5 ml of culture suspension, taken in a microfuge tube was centrifuged at 12,000 rpm for 5 mins at 4 °C. The supernatant was discarded leaving the bacterial pellet as dry as possible. The pellet was suspended in 100 µl of ice-cold glucose EDTA tris buffer (solution I) (Appendix G.4) by vortexing and microfuge tubes were subsequently kept in ice for 10 mins. 200 µl of freshly prepared (solution II) (Appendix G.4) was added and the contents were mixed by inverting the tube rapidly 4-5 times. The microfuge tubes were stored on ice for 10 min. Then 150 µl of ice-cold Potassium acetate (solution III) (Appendix G.4) was added and the microfuge tubes gently vortexed to disperse solution III through the viscous bacterial lysate. The microfuge tubes were stored on ice for 3-5 min, followed by harvesting at 12,000 rpm for 5 mins at 4 °C. The clean supernatant was transferred to a fresh microfuge tube. Plasmid DNA was precipitated with two volumes of cold ethanol at room temperature. The contents were gently mixed and by inversion and allowed to-stand for 2 mins on ice. The microfuge tubes were centrifuged at 12,000 rpm for 5 min at 4 °C. The supernatant was removed and the pellet of plasmid DNA was rinsed with 70% (v/v) chilled ethanol at 4°C. The supernatant was discarded and the pellet was allowed to dry in air for 10 mins by keeping microfuge tubes inverted on tissue paper. The dry pellet of plasmid DNA is dissolved in appropriate volume (50 µl) of TE buffer (pH 8.0) containing DNase free RNase (20 µg/ml) was stored at -20 °C until needed for gel electrophoresis analysis.

Agarose gel electrophoresis

Agarose gel electrophoresis of plasmid DNA samples was performed using horizontal slab gels BG-100, 10.5.8 cm apparatus (B. Genei) and 0.8 % agarose dissolved in 1X electrode buffer). Samples containing approximately 5 µg of plasmid DNA were mixed with 2-4 µl of 6X DNA loading buffer. These samples were briefly spun using an Eppendorf centrifuge before being loaded into the wells of agarose gel. Large-scale DNA gels were performed using a BG-200 apparatus (B. Genei), approximately 10x20 cm, capacity 100 ml of agarose gel solution with ethidium bromide for 2 hours (80 V, 1xTAE buffer). The electrophoresis is usually stopped when the dye front had run up to 2/3rd of the gel (Sambrook et al. 1989; Ausubel et al. 1992). The gel was viewed and photographed using a Gel documentation system (BioRad, U.S.A). 1 kbp DNA ladder (Promega) was used in gels in parallel with DNA samples.

Transformation of *E. coli* DH5α with plasmids

Plasmid DNA from four bacterial isolates VB6, VB23, BR9 and DN1W were used for transforming *E. coli* DH5α. A single bacterial colony of *E. coli* DH5α was inoculated with plasmids from a freshly grown plate in to a flask containing 10 ml LB medium and grown overnight at 28±2 °C. The overnight grown culture was inoculated in 100 ml Luria Bertani broth in a 500 ml Erlenmeyer flask. Inoculum density at this point should not be more than 0.1 O.D. at 600 nm. The culture was grown in an incubator shaker (160 rpm) at 28±2 °C. The optical density was observed after 3 hrs and then after every 30 mins till the O.D. at 600 nm reached to 0.55-0.6. The culture was chilled immediately on ice. The cells were harvested by centrifugation at 6000 rpm for 10 mins at 4 °C. The supernatant was discarded and the entire medium was drained by inverting the tubes.

The pellet was suspended in 2 ml of ice cold 0.1 M $CaCl_2$ for each 50 ml of original culture. The tubes were gently mixed by inverting and kept on ice until the cells gets resuspended completely. 100 µl of $CaCl_2$ treated competent cells for each transformation was taken in polypropylene falcon tubes 15 ml. Cells are mixed with 10 µl of plasmid DNA (1 µg) and tubes are incubated in ice for 30 mins. The tubes were transferred to

preheated water bath at 42 °C for 50 seconds. The tubes were transferred back to ice for another 2 minutes for ice quenching. 1 ml of fresh LB medium was added into each tube and incubated at 37 °C for 1 hr (Hanahan, 1985). This step allows the bacteria to recover and from heat shock.

Screening of Heavy metals and Organo-metal resistant clones (transformants)

0.1 ml of transformation mixture from respective polypropylene tubes was spread plated on to control LB agar plates supplemented with final concentrations of heavy metals viz. $HgCl_2$ (5 µM) $CdCl_2$ (50 µM), $SnCl_2$ (2.5 mM), $NaAsO_2$ (2.5 mM), $NaHAsO_4$ (5 mM) and organo-metals viz. Tributyltin Chloride (0.25 mM), Dibutyltin Chloride (0.5 mM). Transformation mixture (10 µl) was also plated on a LB gar plate without any metal and organo-metals, which served as control. All the metal and organometals used in transformation were of minimal inhibitory concentrations for the test organism *Vibrio harveyi* VB23. Plates were incubated for 24 hrs at 37 °C and the discrete colonies were observed.

Screening of antibiotic resistant clones (transformants)

Similarly 100 µl of transformation mixture was spread plated on control LB agar plates as well as LB agar plates supplemented with final concentrations of test antibiotics viz. Kanamycin (10 µg/ml), Ampicllin (25 µg/ml), Streptomycin (10 µg/ml), Chloramphenicol (25 µg/ml), Novobiocin (10 µg/ml), Tetracycline (10 µg/ml) and Rifamycin (5 µg/ml). All the antibiotics used in the transformation were of minimal inhibitory concentrations for the test organism *Vibrio harveyi* VB23. Host *E. coli* DH5α is sensitive to these antibiotics. Plates were incubated for 24 hrs at 37 °C and the discrete colonies were observed on respective selection (antibiotic plates).

MIC determination of different metals and organo-metals for transformants

The *E. coli* DH5α transformants containing plasmids from VB6, VB23, BR9 and DN1W were tested to determine the minimal inhibitory concentration (MIC) of metals and organo-metals according to the procedure followed by Malik and Ahmad (1994) and Forbes et al.

(1998). The experimental tubes were prepared by supplementing Mueller-Hinton broth with metal salts for cationic concentrations of Hg^{2+} (0, 0.001, 0.0025, 0.005, 0.007, 0.01 mM); Cd^{2+} (0, 0.01, 0.025, 0.05 0.07, 0.1, 0.15 mM); AsO_2 (0, 1, 2, 5, 7.5, and 10 mM) AsO4 (0, 1, 2, 5, 7.5, and 10 mM); $SnCl_2$ (0, 0.5, 1, 2, 5, and 7.5 mM) for and organo-metals viz. TBTC (0, 0.1 0.25, 0.5, 0.75 and 1 mM) DBTC (0, 0.1 0.25, 0.5, 0.75 and 1 mM) respectively. The metal salts used were $HgCl_2$, $CdCl_2$, $NaAsO_2$, $NaHAsO_4$, $SnCl_2$, and organo-metals were TBTC and DBTC. One milliliter of the test organism suspension's as inoculum (1×10^6 CFU/ml or O.D. of 0.25 at 600 nm) was added to each experimental tubes. The tubes were incubated for 24 hrs at 28±2 °C and visual turbidity was noted. The lowest concentration that inhibited growth compared with the control plate was defined as the minimal inhibitory concentration of the compound. A control tube was maintained under the same conditions to the test experimental tubes.

Extraction of plasmid DNA from transformed *E. coli* DH5α cells

After the transformation experiment, plasmid DNA was extracted from the screened *E. coli* DH5α transformants appearing on selection agar plates, using the method of Sambrook et al. (1989). These transformants possess plasmid DNA of strains VB6, VB23, BR9 and DN1W. The extracted plasmid DNA was purified and subjected to agarose gel electrophoresis. After electrophoresis, the gel was stained in ethidium bromide solution (0.5 µg/ ml) for 20 min. Then the gel was washed with milli-Q water and placed on an UV-transsilluminator and finally photographed by using BioRad gel documentation system. Similar experiment was performed to extract plasmid DNA from metal and organo-metal resistant *E. coli* DH5α transformants and analyzed by agarose gel electrophoresis.

Restriction digestion of plasmid DNA of transformants

5 µl of plasmid DNA of VB6, VB23, BR9 and DN1W extracted from *E. coli* transformants was taken in separate microfuge tubes. Restriction enzymes was added into four microfuge tubes containing plasmid DNA separately to a final concentration of buffer 1 µl of (10 X), 3 µl of sterilized milli-Q water was added to make up 10 µl as final volume of reaction mixture. The restriction enzymes 1 µl were added to get final concentration of 1U/

µl in the reaction mixture. Restriction enzymes *Hind* III, *EcoR* I, *Not* I, and *BamH* I, restriction enzymes (MBI fermentas) were used for digestion and by incubating at 37 °C for 90 mins according to the manufacturer's instructions. One control without any enzyme was also incubated at same temperature. Restriction digested DNA sample was analyzed on 1 % horizontal agarose gel electrophoresis carried out at 80 V for 90 mins and stained with ethidium bromide for 20 mins. 1 Kbp DNA ladder (MBI fermentas) was used as a standard DNA marker. Gel picture was recorded by Bio Rad Gel documentation system and size of DNA fragment was determined by comparison with DNA ladder.

Plasmid curing using *Vibrio harveyi* VB23

The plasmid curing was performed as described by Trevors (1986). Stock solution of acridine orange and ethidium bromide were prepared in milli-Q water, filter sterilized and kept in amber colour bottle in dark and SDS was stored at room temperature. Different concentrations of sodium dodecylsulphate (SDS) (0-5 %), ethidium bromide (EthBr) (0-400 µg/ml) and acridine orange ranging from (10-300 µg/ml) (Appendix G5) were added to Luria Bertani broth and overnight grown culture of bioluminescent bacterial culture (2%) was inoculated, and incubated at standard optimum conditions for 24 hrs. After incubation the optical density of the culture broth at 600 nm was determined and graph was plotted as concentration of sodium dodecylsulphate or acridine orange or Vs % survival (µg/ml). Control was considered as 100 %. LD_{50} values of 3 these curing agents for the test organism was determined form the plotted graph.

The bioluminescent bacterial culture VB23 was grown in LB broth supplemented with respective LD_{50} concentrations of curing agents for 5 generations by serial sub culturing at 30 °C and then plated on LB agar plates to obtain discrete isolated colonies. In order to correlate the loss of heavy metal resistance with loss of a specific plasmid, the discrete colonies were replicated on LB agar as well as LB agar with (5 µM $HgCl_2$, 50 µM $CdCl_2$ and 0.25 mM TBT) separately and incubated at 28±2°C for 24 hrs. Plates were observed to check colonies on control plate as well as metal /organo-metal treated agar plates. Presence of plasmid was checked by using the extraction method of Sambrook et al.

(1989). Plasmids were isolated from the treated cells and agarose gel electrophoresis was performed for the visualization of cured and uncured derivatives of plasmids.

PCR amplification of *merA* and *cadA* genes from plasmid DNA and genomic DNA

Isolation of total genomic and plasmid DNA.

Total genomic and plasmid DNA were isolated from various luminous bacterial isolates. The extraction protocol followed using the standard methods of Jones and Bartlett (1990) and Sambrook et al. (1989). (Appendix G.4 and G.5) All primers were at least 23 bases long to allow only specific binding to templates and had no self-complementary regions. The primers were synthesized from Integrated DNA technologies (IDL, U.S.A). The criteria used in the design of the *merA* and *cadA* primers included conservation of homologous sequences determined in multisequence alignments or inclusion of active sites for heavy metal binding.

Oligonucleotide primers and PCR conditions

Following oligonucleotide primers were used for PCR amplification of *mer*A gene (amplicon size 280 bp) encoding mercuric reductase enzyme.

***mer A* 1 F'-(5'-ACC ATC GGC GGC ACC TGC GT -3')**
***mer A* 5 R'-(5'- ACC ATC GTC AGG TAG GGG AAA AA - 3')** (Liebert et al. 1997)

PCR reaction mixtures contains of 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 15 mM $MgCl_2$, 20 nM each primer, 1 U/µl of *Taq* DNA polymerase per 25 µl, and 100 ng of plasmid and genomic DNA as template. The PCR conditions are as follows: Initial denaturation step at (94°C for 4 mins) followed 30 cycles of 94°C for 45 sec, 52 °C for 45 sec, and 72°C for 90 sec, along with final extension for 5 min at 72 °C. The 10 X diluted genomic DNA of *E. coli* was used as negative control.

PCR primers for amplification of Cadmium resistant gene primers, which codes for *czc cadA* gene.

***czc cadA* 1 F'- (5'- GTT TGA ACG TAT CAT TAG TTT C - 3')**
***czc cadA* 2 R'- (5'- GTA GCC ATC CGA AAT ATT CG -3')** (Trajanovska et al. 1997)

PCR reaction mixtures contains of 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 15 mM $MgCl_2$, 20 nM each primer, 1 U/µl of *Taq* DNA polymerase per 25 µl, and 100 ng of plasmid and genomic DNA as template. The PCR conditions are as follows: Initial denaturation step at (94°C for 4 mins) followed 30 cycles of 94°C for 60 sec, 46 °C for 45 sec, and 72 °C for 1 min. A final extension was done for 5 min at 72 °C. The 10 X diluted genomic DNA of *E. coli* was used as negative control.

Size determination of PCR products by agarose gel electrophoresis

The PCR products (5 µl) were analyzed on 1 % agarose gel containing (0.5 µg of ethidium bromide per ml), prepared in 1x Tris-borate-EDTA (TBE buffer) by gel electrophoresis at 80 V for 90 mins. The DNA bands were visualized on a UV transilluminator and the gel image was taken using gel documentation system.

Mutagenesis studies using *Vibrio harveyi* VB23

Determination of % killing curves using Nitrosoguanidine (NTG)

Overnight grown culture (2 %) of the test organism was inoculated in 5 ml of modified Luria Bertani broth supplemented with 1 % NaCl in a test tube and culture was incubated in an incubator orbital shaker at 160 rpm at 28± 2 °C, until a cell density of 3. 5 $x10^6$ cells/ml. The culture was centrifuged at 8000 rpm for 5 mins and the pellet was washed thrice in 5 ml of citrate buffer (pH 5.5). (Appendix G.7 and G.8). 5 ml of the cell suspension was measured into nine separate test tubes after suspending in citrate buffer (pH 5.5). Three sets of test tubes were kept as different concentrations of NTG i.e. 25 µg/ml, 50 µg/ml and 100 µg/ml. Required volume of NTG was added in to each test tube from the stock solution. The test tubes were placed in an environmental incubator shaker at (160 rpm) at 28± 2 °C for 120 mins. One set of tubes was withdrawn immediately for zero hour reading. At different time intervals, the culture was withdrawn, centrifuged, and washed once with phosphate buffer (pH. 7). Pellet from each sample was finally resuspended in 5 ml of phosphate buffer, immediately serial dilution was made and spread plated on to LB agar plates. The samples were withdrawn at the following time intervals 0, 15, 30, 45, 60, 90 and 120 mins. 0.1 ml aliquots of 10^{-3}, 10^{-4} and 10^{-5} dilutions

were plated for respective samples. The viable cell count was determined from the plates at each time interval. The graphs were plotted as % Survival against the time of exposure (mins) to NTG.

Determination of % killing (UV mutagenesis)

Quantitative UV survival of test organism was determined by exposing exponentially growing cells in GBM broth (O.D. 0.6-0.8 at 600 nm), suspended in 0.02 M phosphate buffer, to UV light from a 15 W germicidal lamp having an UV emission of 254 nm, for 0, 20, 40, 80, 120, 150 and 180 mins. 5 ml of the bacterial suspension was transferred to a petridish and gently stirred during irradiation. Petridishes containing unexposed cells served as control. Immediately after irradiation, cells were diluted in phosphate buffer (10^{-3},10^{-4},10^{-5}) and fractions of the survivors are calculated after plating 0.1 mL cell suspension of each dilution and subsequently observing the viable colonies. In both the qualitative and quantitative tests GBM agar plates were incubated in the dark for 24 hrs at 28±2°C. Survival curves are plotted as % UV survival Vs UV exposure (mins).

Mutagenesis and screening of non-luminescent mutants

The assays were performed according to previously described procedures (Czyz et al. 2000; 2002). The chemical mutagens nitrosoguanidine (NTG) Acridine orange, Ethidium bromide and 5-Bromo-uracil were used at final concentrations of 50 µg/ mL, 200 µg/ mL, 200 µg/ mL and 50 µg/ mL respectively. Exposure of cells to UV light for 100 mins was chosen to screen UV mutants. For initial treating of cells with mutagens, culture suspension (2 %) of absorbance 0.1 at 600 nm was incubated with respective mutagens in GBM broth at 28±2°C for 24 hrs. Required volume of cell suspension containing 3×10^6 Cfu/ml (estimated by measuring 600 nm) taken from mutagens treated cultures was plated on GBM agar plates and non luminescent bacterial colonies (putative mutants) were picked up for comparison with wild type (luminescent) with reference to metal and organo-metal tolerance limit (MIC), Exopolysaccharide (EPS) content and protein profile.

Effect of Chemical mutagens on growth and bioluminescence

Effect of chemical mutagens; Acridine Orange (50, 100, 150 and 200 µg/ml), Ethidium Bromide (50, 100, 150 and 200 µg/ml), and Nitrosoguanidine (MNNG) (10, 25, 50 and 75 µg/ml), on growth and bioluminescence was tested by plating the cells treated with these chemical mutagens on GBM agar plates after every subsequent serial sub culturing up to 8 sub cultures. Growth and bioluminescence was monitored visually after every subsequent serial sub culturing. Plates were incubated at 28± 2 °C for 24 hrs, and growth and bioluminescence was monitored visually .

Comparative study of mutant and wild type with reference to metal tolerance, exopolysaccharide production and protein profile

MIC determination of different metals and organo-metals for mutants

MIC of different metals and organo-metals for all mutants was determined according to Malik and Ahmad (1994) and Forbes et al. (1998). The experimental tubes were prepared by supplementing MSM with metal salts for cationic concentrations of 0, 0.001, 0.0025, 0.005, 0.007, 0.01 mM for Hg^{2+}; 0, 0.01, 0.025, 0.05 0.07, 0.1, 0.15 mM for Cd^{2+} 0, 1, 2, 5, 7.5, and 10 mM for, AsO_2; 0, 1, 2, 5, 7.5, and 10 mM AsO_4 ; 0, 0.5, 1, 2, 5, and 7.5 mM for $SnCl_2$ and organo-metals viz. TBTC; 0, 0.1 0.25, 0.5, 0.75 and 1 mM; DBTC., 0, 0.1 0.25, 0.5, 0.75 and 1 mM respectively. The metal compounds used were $HgCl_2$, $CdCl_2$, $NaAsO_2$, $NaHAsO_4$, $SnCl_2$, TBTC and DBTC. One milliliter of all the mutants cell suspension as inoculum (1×10^6 CFU/ml or O.D. of 0.25 at 600 nm) was added to each tube. The tubes were incubated for 24 hrs at 28±2 °C and visual turbidity was noted. The lowest concentration that inhibited growth compared with the control tube was defined as the MIC of respective metal and organo-metal. Comparative MIC for different metals in case of wild type and mutants were determined and presented as bar diagram.

Exopolymer production of NTG induced mutant

NTG induced mutant was characterized with reference to exopolymer (EPS) production. Luminous bacterial culture VB23 (wild type) and VB23 (mutant) were grown and maintained as batch cultures for exopolysaccharide production in optimized mineral salts

medium (MSM) with sodium chloride at a final concentration of 1.5 % (w/v), 0.2 % glucose as the carbon source, The pH of the medium was adjusted to 7.0 with 1 N NaOH. The medium (250 ml) dispensed in 500 ml Erlenmeyer flasks, sterilized and inoculated with 2 % (v/v) of an overnight culture grown in the same medium at room temperature (28±2 °C) on a rotary shaker at 160 rpm. The sub samples (5 ml) were drawn at regular intervals for both exopolysaccharide production. The yield of exopolymer (mg/ml) for mutant and wild type has been compared and graphs were plotted as mutant, wild type vs Exopolymer (mg/ml).

Protein profile of NTG induced mutant

NTG induced mutant was also characterized with reference to protein profile. The soluble protein concentration was determined by the Lowry method (Lowry et al., 1951) before performing gel electrophoresis. Proteins were resolved by Polyacrylamide gel electrophoresis (SDS-PAGE) (Laemelli, 1970), which was performed using 10 % homogenous gels (Protean II unit, Bio-Rad) overlaid with a 5 % stacking gel. Wells contained either 50 µg of proteins per sample or a constant amount of cellular material (O.D 0.6-0.7) as determined from A_{600} of original sample and later concentrated. Electrophoresis was carried out in 1X Tris-Glycine Electrophoresis buffer at 10 °C, with temperature control achieved by a cooling unit (Remi, Ltd). Samples were electrophoresed at 35 mA until the tracking dye entered the resolving gel, at which time the current was increased to 70 mA for 3 hours. Medium range molecular weight (MWM) (B. Genei) was used as standard markers. The Polyacrylamide gel was visualized by silver staining (Appendix G2), and photographed using gel documentation. NTG induced proteins are compared with the wild type.

Results and Discussion

Transformation of *E. coli* DH5α

To determine whether the metal and antibiotic resistance determinants were actually on plasmid DNA, transformation experiment was carried out using plasmid DNA isolated from four luminous bacterial strains VB6, VB23, BR9 and DN1W (Table 4.4). It is interesting to note that several conspicuous colonies appeared on separate LB agar plates containing ampicillin, (25 µg/ml), chloramphenicol (25 µg/ml) and rifamycin (5 µg/ml). Thus it clearly indicates that these transformants possess genes encoding antibiotic resistance only on plasmids. Using plasmid DNA of strains VB6, VB23, BR9 and DN1W for transformation of *E. coli* DH5α, it was noticed on LB agar plates containing ampicillin, (25 µg/ml), 120, 200, 40 and 100 discrete and conspicuous colonies (transformants) appeared in respective set of transformation experiment (Table 4.4). Similarly several transformants were observed on separate LB agar plates containing MIC concentrations of heavy metals, viz. Hg (II) 5 µM, Cd (II) 100 µM, As (III) 5 mM, As (V) 7 mM and Sn (II) 5 mM. Thus it is clearly evident that toxic metal (Hg, Cd, As, Sn) and antibiotic (amp, Chl, rif) resistance encoding genes are located on plasmids of respective bioluminescent bacterial strains. Genes encoding organo-metal resistance are not located on plasmids, since no transformants were observed. There are several reports and reviews of plasmid mediated antibiotic and heavy metal resistance. (Silver and Misra 1988; Cerventes et al. 1994; Silver and Ji 1994, Silver and Phung 1996).

Selection of *E. coli* cells were transformed with plasmid DNA of luminous bacteria VB6, VB23, BR9 and DN1W on LB agar containing MIC of test heavy metals and organo-metals viz. Hg (II) (5 µM), Cd (II) (100 µM), As (III) (5 mM), As (V) (7 mM) and Sn (5 mM); organo-metals viz. TBT (1 mM), DBT (0.75 mM). Several *E. coli* transformants showing resistance to the above heavy metals were successfully obtained, whereas no transformants were obtained on LB agar plates with organometals TBT and DBT. The genes encoding resistance to organometals may be present on chromosomal genome rather than on plasmid. From this experiment it is clearly understood that *E. coli* DH5α, which was sensitive to all the above mentioned heavy metals and antibiotics after transformation, became resistant to these due to plasmid acquisition from luminescent

bacterial strains. The transformation efficiency was very low for plasmids of BR9 and highest for VB23 (Table 4.2, 4.3 & 4.4). These results indicate that luminous *Vibrio harveyi* strains VB6, VB23, BR9 and DN1W carry some genes on plasmid conferring resistance to the test heavy metal. This is the first report describing a plasmid mediated heavy metal resistance of Hg, Cd, As, and Sn in *Vibrio harveyi* strains.

MIC of heavy metals and organometals for transformants

The MIC values of heavy metals and organo-metals for counter selection of transformants were examined using LB agar plates at concentration ranges of 0 to 0.01 mM Hg, 0 to 0.15 mM Cd, 0- 10 mM of AsO_2, AsO_4 and Sn, 0 to1 mM of TBT and DBT respectively. The heavy metals and organometals tested were $HgCl_2$, $CdCl_2$, $NaAsO_2$, $NaAsO_4$, $SnCl_2$, TBT and DBT. In agar plates, *the E.coli* DH5α transformants from the strains VB6, VB23, BR9 and DN1W were resistant to inorganic heavy metals. Interestingly, these transformed strains were relatively sensitive to the organo-metals such as TBT and DBT. *E. coli* Dh5α strain was susceptible to both inorganic and organic heavy metals except for 0.5 mM AsO_4 concentration. The concentration of the metal and organometals which permitted growth and beyond which abrupt inhibition of growth was considered as MIC of the metal against each transformant strain tested and selection marker concentrations were chosen on the basis of these antibiotic sensitivity patterns (Table 4.1). *E. coli* strain DH5α was used as control

Heavy metals tolerance of *E. coli* DH5α transformants possessing plasmids from different luminous bacterial strains (Table 4.1) revealed that plasmids are harboring genes conferring toxic metal resistance. Earlier results have also indicated the presence of antibiotic resistance encoding genes on the same plasmids. Therefore we can infer that antibiotic and heavy metal resistance shown by luminescent isolates is plasmid mediated. Similar findings correlating metal and antibiotic resistance mediated by plasmids have been reported earlier in various non-luminescent bacterial strains from the environment (Di Vicente et al. 1990; Chaudhury and Kumar 1996; Mc Arthur and Tuckfield 2000). Interestingly plasmids of luminescent strains were unable to confer TBT and DBT resistance in *E. coli* DH5α, which confirms that in natural isolates resistance to these organo-metals may be governed by chromosomal genes.

Similar observations were done on *Acidiphilium multivorum* AIU 301 possessing a (plasmid of size 56 kbp, pKW301), When transferred into *E. coli* JM109, an *E. coli* transformant carrying pKW301 exhibited resistance to sodium arsenite, sodium arsenate, and mercuric(II) chloride (Suzuki et al. 1997). In conclusion, the plasmids from four selected luminous bacteria (VB6, VB23, BR9 and DN1W) *carries* the genes responsible for resistance to inorganic heavy metals viz. Hg, As, Cd, and Sn and these genes are expressed in *E. coli* DH5ά by transformation. These results indicate that luminous bacterial plasmids carry some genes encoding proteins that make the host cells resistant to these heavy metal salts. This is the first report describing a plasmid encoding heavy metal resistance genes from luminous *Vibrio* spp *and Photobacterium* species.

Plasmid profile and restriction digestion of transformed *E.* coli DH5α

The plasmids were recovered from the transformant of *E. coli* DH5ά by alkaline lysis method, Plasmids extracted from luminous *Vibrio harveyi* strains VB6, VB23, BR9 and DN1W were separated on a 0.8 % agarose gel electrophoresis, the gel was stained in ethidium bromide solution for 20 min. The image of the gel was finally photographed by using gel documentation system. A similar pattern was obtained for the plasmid preparation from the *E. coli* DH5α transformant (Fig. 4.3), indicating that the small plasmid harbored in the transformant originated from luminous *Vibrio harveyi* strains VB6, VB23, BR9 and DN1W. This plasmid DNA band corresponding to that of original strain is the indication of successful transformation

Restriction digestion and mapping of the plasmid

To determine the size of *Vibrio harveyi* plasmid were digested with various restriction endonucleases and the resultant DNA fragments were analyzed by electrophoresis on 0.7% agarose gels with Tris-borate-EDTA buffer (Fig. 3). Restriction endonucleases *Not* I, *Eco* RI, *Sau*3a II, *Not* I, *Hind* III and *Bam* HI were purchased from Promega. 1 kbp DNA ladders were used as molecular size standards. The closed circular form of the plasmid DNA's appeared as a single band between 11 and 12 kbp (Fig. 3, lane 4). Three DNA

bands of 5, 4 and 2 kbp were detected when the plasmid was digested with *Bam* HI (lane 8), and no fragments were observed when digested with *Eco* RI, *Sau* 3a II, *Not* I, *Hind* III (lanes 1, 2, 3 and 6). These results indicated that the size of the intact plasmid VB23 was around 11 kbp. This size is extremely same compared with that of ordinary plasmids employed for the transformation of *E. coli.*

Curing of plasmid DNA

Inorder to confirm the loss of the plasmid mediated heavy metal resistance, an attempt was made to eliminate the plasmid of *Vibrio harveyi* VB23 cells with 3 different curing agents, Viz. acridnine orange, ethidium bromide and SDS. Earlier workers have reported that acridine orange inhibits the replication of bacterial plasmids by causing mutation in absence of light at the site of semi conservative DNA replication (Caro et al. 1984; Trevors, 1986). Acridine compounds and ethidium bromide are known to exert several biological effects: curing of plasmids, induction of frameshift mutations, inhibition of DNA repair, and inhibition of cell growth (Hollander et al. 1996).

Percent survival of the test organism exposed to different curing agents was determined prior to the curing experiment. The LD_{50} values for curing agents were 150 µg/ml for acridine orange, > 200 µg/ml for ethidium bromide and 0.5 % SDS respectively (Fig. 4.4). The cured derivatives of strain VB23 was obtained by incubation with chemical mutagens viz acridine orange, ethidium bromide and anionic detergent SDS. It was expected that the metal and antibiotic resistance of *Vibrio harveyi* VB23 might be plasmid mediated. Indeed, it was observed that total curing of plasmids from *Vibrio harveyi* VB23 caused concomitant loss of metal resistance. Interestingly, plasmid curing was achieved only by growing the luminous strain VB23 at SDS, 0.5 % and acridine orange, 150 µg/ml, whereas the curing experiment with ethidium bromide was unsuccessful. . It may be attributed to ethidium bromide resistance mediated by multidrug efflux pump operational in these organims. Similar mechanism of efflux and ethidium resistance was earlier reported in [illegible] bacteria *Pseudomonas* spp and *Vibrio paraheamolyticus* (Ma et al. 1994; Aries et al. 1999; Li et al. 1998).

Kulkarni and Kanekar (1998) and Deshpande et al. (2001) have recently showed the ability of *Pseudomonas aeruginosa* MCMB-427 to degrade dimethoate and degradation of e-caprolactam by *Pseudomonas putida* MCM B-408) is plasmid mediated and transferable to other strains as demonstrated by efficient curing agents viz. acridine orange, ethidium bromide, mitomycin C and SDS. Interestingly enough, we found some antibiotic and heavy metal resistant encoding gene determinants on plasmids after screening of transformants in various metals and antibiotics and subsequently confirmed the loss of plasmids after curing with SDS 0.5 %. Similar observations was done by Dhakephalkar et al. (1996) to study the chromate resistance and reduction in *Pseudomonas mendocina* MCM B-180, in which the resistance is mediated by plasmid and further confirmed by curing with mitomycin C, whereas as there are no reports of metal resistance and plasmid curing in the luminous bacteria.

PCR amplification of *merA* and *cadA* genes from plasmid DNA and genomic DNA

The primers, *mer* A1.F and *mer* A5-.R, effectively amplify the 288 bps segment of merA gene, when plasmid DNA as template of strain VB23 and VB6 were used for PCR (Fig 4.6). The amplified product is the expected size 288 bps gene fragment, while this *mer A* gene could amplify only a part of the whole gene. In case of luminous strains VB9 and VB01, No positive amplification of *mer A* gene segment was seen. This clearly mentions that only plasmids of strain VB23 and VB6 have obtained *mer A* gene, whereas the gene is absent in the plasmids of VB9 and VB01. It is interesting to note that mer A gene encoded polypeptide of 54 kDa, which is cearly visible in SDS–PAGE profile of strainVB23 grown in presence of 5 and 7 µM $HgCl_2$. Similar reports are available in number of mercury resistant gram-negative bacterial isolates including enteric bacteria from marine environment (Barkay et al. 1985; Barkay and Olson 1986; Barkay, 1987 Osborn et al. 1997; Ravel et al. 1998; Trajanovska et al. 1997; Muller et al. 2001; Hobman et al. 2002).

Amplification of *czc A* genes in the experiment involving amplification with czcA1-czcA2 primer pair yielded the expected ~ 1880 bp products from plasmid DNA of VB50. Most isolates yielded no amplification products with the primer pairs *czcA*1-*czcA*2 *czc A* gene amplicon (1.8 kbps) from plasmid DNA of VB23, DN1W, BR9 and VB9 yielded a product

of expected with the primer pair *czcA*1-*czcA*2 (Fig 4.7). As such there are hardly any reports on mercury and cadmium resistant luminous bacterial isolates and presence of mer A gene encoding resistance determinants on their plasmids.

MIC determination indicated that the luminous bacterial isolates used in the present investigation have developed resistance to several metal ion and organometals ions. The observation of high levels of mercury resistance in VB6 and VB23 is notable, as several reports indicated the existence of mercury-resistant Gram-negative isolates from contaminated sites and these often have mercury-resistance encoding plasmids or transposons (Diels and Mergeay 1990; Rochelle et al. 1991).

The prokaryotic mercury resistance mechanism plays a unique role in this paradigm by reducing Hg (II) to volatile elemental mercury, Hg (0) (Barkay et al. 2003). Microbial communities acclimate to life in the presence of mercury through (i) enrichment of resistant organisms (Barkay, 1987; Muller, 2001; Rasmussen and Sorensen, 2001), (ii) induction and synthesis of mer gene products (Nazaret et al., 1994; Schaefer et al., 2004), and possibly, (iii) horizontal gene transfer of mer determinants (Dronen et al 1998; Smit et al. 1998). In environments inhabited by acclimated communities, the enrichment and activities of resistant microbes may play a significant role in mercury biogeochemistry by reducing Hg (II) to elemental mercury Hg (0). This is supported by correlations between MerA activity and transcript abundance (Schaefer et al. 2004). Little is currently known about Hg (II) resistant microbes and MerA activities in marine environments and there are no reports on luminous bacteria. MerA and its activities were documented among strict anaerobes (Rudrick et al. 1985) and the formation of Hg (0) in anoxic sediment incubations has been demonstrated (Weber et al. 1998).

The genes encoding mercury resistance encoding genes are found to be located frequently on plasmids and transposons (Misra 1992; Silver and Walderhaug (1992). Bacteria resistant to cadmium, chromium, arsenic and nickel have been isolated from several contaminated sites and natural deposits. The observations that metal resistance determinants are located most frequently on plasmids and transposons have led to suggestions that these determinants are probably spread by horizontal transfer (Bogdanova et al.1988). Many microorganisms that resist high concentrations of heavy

metal salts are known to harbor plasmids that govern their resistances to various toxic heavy metals.

Response to toxic metals could be plasmid or chromosomally mediated, although most resistance systems appear to be encoded by plasmids. Resistance systems have been shown for Ag^{+}, AsO^{2-}, AsO_4^{3}, Cd^{2+}, Co^{2+}, CrO_4^{2-}, Cu^{2+}, Hg^{2+}, Ni^{2+}, Pb^{2+}, TeO_3^{2-}, Tl^{+}, and Zn^{2+} (Silver and Phung 1996). These systems are primarily energy dependent efflux systems although a few involve enzymatic transformations. Energy dependent efflux systems appear to function either as ATPases (i.e., Cd^{2+} and Zn^{2+} efflux systems in Gram positive bacteria) or as chemiosmotic ion/proton exchangers (i.e., Cd^{2+}, Zn^{2+}, Co^{2+} and Ni^{2+} in Gram negative bacteria) (Nies and Silver 1995). Resistance to arsenic can either be conveyed by an ATPase or by a chemiosmotic transporter. In some bacteria, resistance to arsenite is conferred by enzymatic oxidation to the less toxic arsenate (Cervantes et al. 1994).

Growth and bioluminescence in presence of chemical mutagens

In order to check the effect of chemical mutagens on growth and bioluminescence, serial subcultures were done for 8 generations. When subcultured in presence of chemical mutagens, growth was observed in all the concentrations of Acridine orange and Ethidium bromide, whereas with NTG 50 µg/ml growth was observed up to 3rd sub culture, whereas with NTG 75 µg/ml no visible growth was observed. Luminescence was observed In presence of NTG 10 and 25 µg/ml, visible growth was seen till 6 consecutive sub cultures, but luminescence was observed only till one generation. Luminescence was observed up to 2nd subculture and tends to be inhibited at higher concentrations of AcOr, and EthBr. The inhibition of luminescence in presence of Hg, Cd, TBT EthBr NTG and AcOr could be attributed due to the blocking of AHL signal molecules and ' β ' unit of the luciferase encoding *lux B* gene of the luminous bacteria (Baldwin and Zeigler 1992). Effect of toxic chemicals on luminescent bacterial system was previously reported by Bulich and Isenberg (1981), cyclodextrins (Bar and Ulitzur, 1994), mutagenic chemicals (Bulich et al. 1986; Czyz et al. 2000, 2002, 2003).(Table 4.5, Fig 4.11)

NTG mutagenesis of Vibrio harveyi VB23

In order to determine the LD 50 values of NTG, the cells were treated with NTG for regular time intervals (15, 30, 45, 60, 90 and 120 mins respectively). At 15 mins, a killing rate of 28 % in NTG 25 µg/ml, 68 % in 50 µg/ml, and 76 % in 100 µg/ml killing was observed. Whereas at 60 mins a killing rate of 74 % in NTG 25 µg/ml, 90 % in NTG 50 µg/ml and 98 % in NTG 100 µg/ml respectively. Likewise, it was observed that a killing rate of 92 % in NTG 25 µg/ml, 27 % in NTG 50 µg/ml and 100 % in NTG 100 µg/ml at 120 mins (Fig 4.8).

In order to isolate the NTG induced heavy metal and organometals resistant mutants strong chemical mutagen NTG was used. It is an alkylating agent, potent mutagen and carcinogen, which act through covalent modification of cellular DNA resulting in various types of primary lesions (Waters et al. 1990) insertional mutations (Miyauchi et al. 2002). Nitrosoguanidine (NTG) is one among the diverse group of highly electrophilic substances that act by transferring alkyl groups (methyl, ethyl etc.) to nucleophilic sites on purine and pyrimidine rings, especially vulnerable to N-7 and O6 positions of guanine and the O4 position of thymine. Alkylation of these sites can have potentially mutagenic effects (Miller, 1992). NTG is known to induce mutations at the replication fork leading to clustering of induced mutations (Hollander et al. 1996).

UV mutagenesis

When cells exposed to gradually increasing time intervals, up to 120 mins, the cells tends to be showing 50 % survival after 100 mins of UV light exposure, whereas after 180 mins, 6 % survival was observed. However, significantly less survival of cells was observed when UV-irradiated for more than 120 mins. Most of the non-luminescent mutants occurred among UV sensitive bacteria. One possible interpretation would be that *V. harveyi* cells, which are unable to emit light after 120 mins of UV irradiation, might be defective in their DNA repair caused by UV exposure. To test this hypothesis, we investigated the survival of UV-irradiated *V. harveyi* VB23 cells, which were subsequently grown in the dark or in the presence of external light. This type of experiment has previously been used to investigate the efficiency of photoreactivation, a process of DNA

repair by photolyase. One possible interpretation of the unexpectedly large proportion of non-luminescent mutants among UV sensitive bacteria was that *V. harveyi* cells unable to emit light might be defective in the repair of DNA lesions caused by UV light (Czyz et al. 2000, 2002). Most probably, stimulation of luminescence was caused by inactivation of the *LexA* repressor and subsequent induction of the SOS response.

Mutagenic response of the test organism VB3 to different mutagenic agents viz. acridine orange, ethidium bromide, NTG, 5-Bromouracil and UV light was significantly different as 170, non- luminescent mutants were obtained after treating the cells with acridine orange 200 µg/ml, similarly ethidium bromide 200 µg/ml and NTG 50 µg/ml, UV light exposure (100 mins induced 80, 65, and 65 mutants (Fig 4.10). The above results indicated that, the chemical mutagen NTG is the most potent mutagen as compared to the Ethidium bromide and acridine orange.

Interestingly, a decreased number of mutants were found at a relatively high concentration of the mutagen (NTG, EthBr, AcOr and 5-Bromouracil). This phenomenon may be caused by either toxicity of some mutagens at higher concentrations or by induction of so many mutations that a large proportion of resistant mutants contain also additional mutations, which can be deleterious or lethal. We can speculate that the higher number of mutants appearing after incubation of bacterial cultures with a high mutagen concentration in the minimal media arises from slower cell metabolism. Mutagenic effects of chemicals on cells depend not only on their potential to interact with a genetic material, but also on permeability of the cellular envelope to these compounds.

Base analogues and alkylating agents usually cause two way transitions (AT to GC or GC to AT), but alkylating agents can also cause transversions, while acridines (intercalating agents) cause frameshift mutations (Miller, 1992). Flouroquinolone resistance attributable to the efflux has been reported in a number of gram-negative organisms. An interesting feature of strains expressing efflux-mediated quinolone resistance is their cross-resistance to a number of structurally unrelated antimicrobial agents (Ma et al. 1994; Li et al. 1998; Aries et al. 1999). This might be due to the broad substrate specificity of the flouroquinolone efflux systems,

Comparative study of mutant and wild type with reference to metal tolerance, exopolysaccharide production and protein profile

The MIC values of heavy metals and organometals for counter selection of mutants were examined using MSM agar plates at a concentration ranges of 0 to 0.01 mM Hg, 0 to 0.20 mM Cd, 0- 12 mM AsO_2, AsO_4 and Sn, 0 to 1.25 mM TBT and DBT respectively. The heavy metals and organometals tested were $HgCl_2$, $CdCl_2$, $NaAsO_2$, $NaAsO_4$, $SnCl_2$, TBT and DBT in agar plates. The mutants from the strains VB6, VB23, BR9 and DN1W were resistant to inorganic heavy metals (Figs 4.13 and 4.14).

It is interesting to note that exopolysaccharides (EPS) production of NTG induced mutant is significantly repressed (50%) as compared to wild type strain, during early log phase of growth (10 hrs). but after 24 hrs of incubation, a moderate increase in the EPS was noticed. This may be attributed to repressed synthesis of polypeptides for exopolysaccharides, in case of NTG mutant, since proteins are the second major constituents of the EPS (exopolysaccharide) after carbohydrates (Fig 4.15). Induction of two novel proteins of 48 kDa and 55 kDa size occurred at NTG (25 µg/ml), whereas when the cells treated with NTG (10 µg/ml only low molecular weight proteins are expressed. The synthesis of high molecular weight proteins (polypeptides are significantly repressed in NTG induced mutants as compared with the wild type. High molecular weight proteins of NTG (50 µg/ml) induced mutants of strain *Vibrio harveyi* VB23 seems to be involved in metal resistance specifically Cd, Arsenate and arsenite, because, NTG induced mutant is highly sensitive to Cd, Arsenate and arsenite (Fig 4.16). As of now there are hardly few reports on mutagenecity assays on bioluminescent bacteria.

S.No.	Heavy metals and Organo-metals	MIC values in (mM) of *E. coli* DH5α transformed with plasmids of			
		VB6	VB23	BR9	DN1W
	Inorganic heavy metals				
1	$HgCl_2$- Hg^{2+}	0.005	0.005	0.0025	0.0025
2	$CdCl_2$-Cd^{2+}	0.1	0.1	0.50	050
3	$NaAsO_2$-As^{3+}	7.5	7.5	7.5	7.5
4	$NaHAsO_4$-As^{5+}	10	10	10	10
5	$SnCl_2$- Sn^{2+}	5	5	5	5
	Organic heavy metals	--	--	--	--
6	TBTC-Sn				
7	DBTC-Sn	--	--	--	--

Table 4.1. MIC of transformants for heavy metals and organometals (mM)

S.No	Plasmids containing donor strains		No: of Transformants
1	$2.65X10^6$	VB6	120
2	$2.36X10^6$	VB23	200
3	$2.72X10^6$	BR9	40
4.	$2.25X10^5$	DN1W	100

Table 4.2. Transformation ability of *E. coli* DH5α for different plasmids on LB + Ampicillin (25 µg/ml)

Table 4.3. Confirmation of presence of genetic determinants for resistance to heavy metals and organo-metals

S.No	Heavy metal-Organo-metals	Plasmids from strains			
		VB6	VB23	BR9	DN1W
1	$HgCl_2$- Hg^{2+} (5 µMl)	+	+	+	+
2	$CdCl_2$-Cd^{2+} (20 µM)	+	+	+	+
3	$NaAsO_2$-As^{3+} (2.5 mM)	+	+	+	+
4	$NaHAsO_4$-As^{5+} (5 mM)	+	+	+	+
5	$SnCl_2$- Sn^2 (5 mM)	+	+	+	+
6	TBTC -- (0.5 mM)	--	--	--	--
7	DBTC -- (0.25 mM)	--	--	--	--

Table 4.4. Confirmation of presence of genetic determinants for resistance to antibiotics

S.No	Antibiotics	Plasmids from strains			
		VB6	VB23	BR9	DN1W
1	Kanamycin (10 µg/ml)	--	--	--	--
2	Ampicillin (25 µg/ml)	+	+	+	+
3	Streptomycin (10 µg/ml)	--	--	--	--
4	Chloramphenicol(25 µg/ml)	+	+	+	+
5	Novobiocin (10 µg/ml)	--	--	--	--
6	Tetracycline (10 µg/ml)	--	--	--	--
7	Rifamycin (5µg/ml)	+	+	+	+

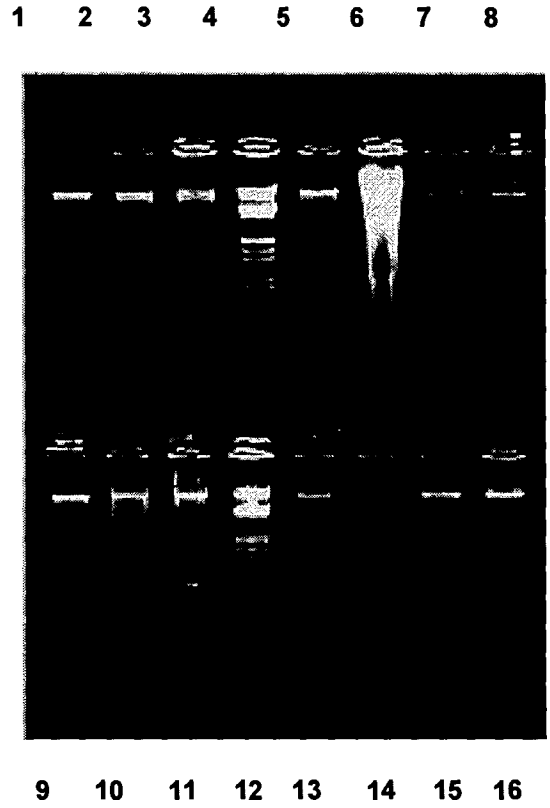


Fig. 4.1. Agarose gel electrophoresis of plasmid DNAs from various luminous bacterial isolates

Lane 4 and 12, 1 kbp DNA molecular weight marker,

Lanes (1-3, 5-8, 9-11, 15-16) plasmid DNA of 15 luminous bacterial isolates,

Lane 14 , absence of plasmid

Fig 4.1. Screening of plasmids from luminous bacterial strains

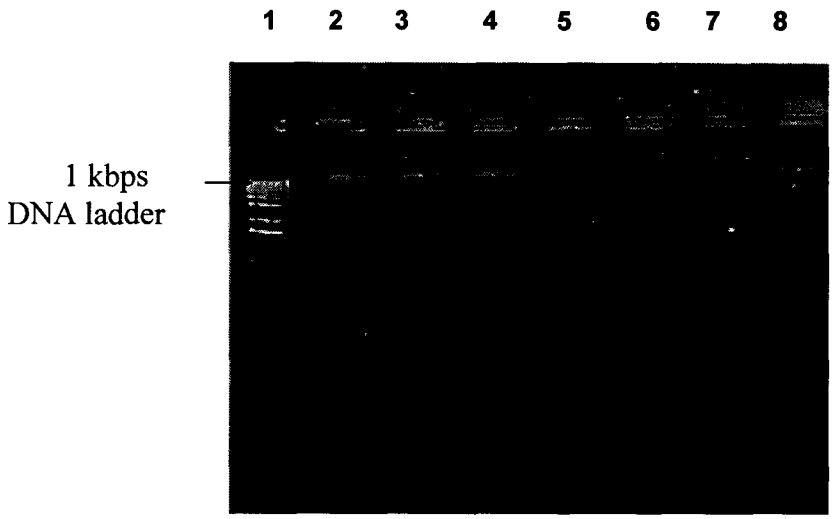


Fig. 4.2. Screening of Transformants carrying plasmids of strains (VB6, VB23, BR9 and DN1W in Lanes 2, 3 4 and 5, Lane 6 , wild type VB23 plasmid, Lane 1, 1 Kbp DNA ladder

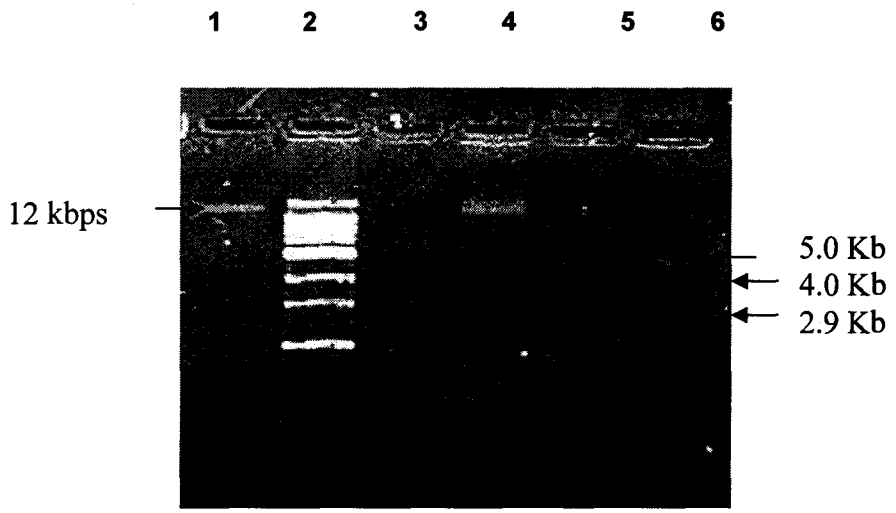
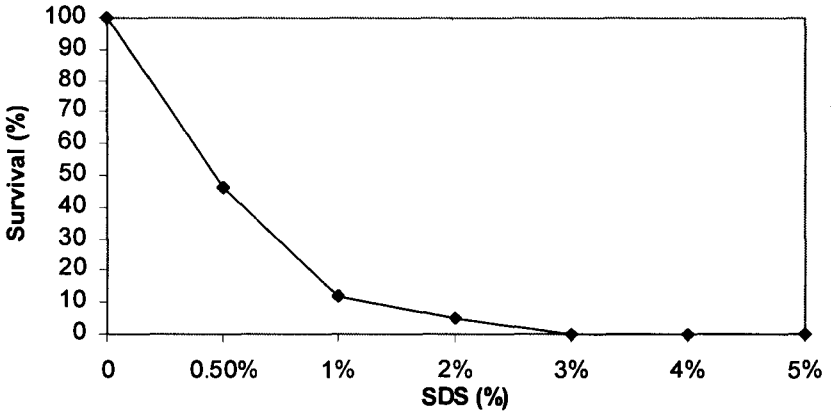
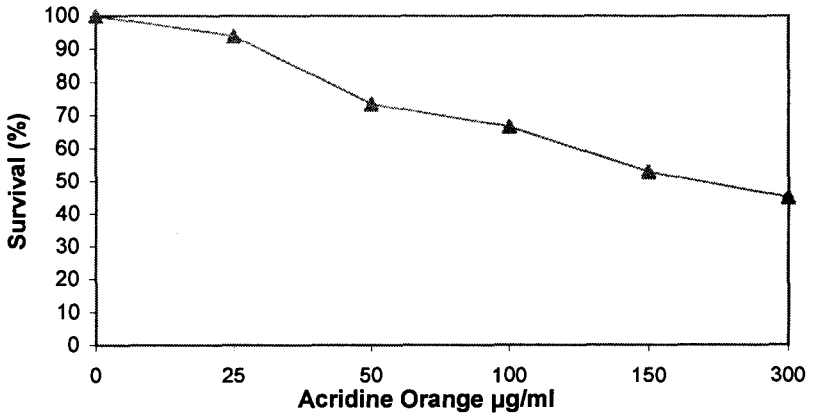


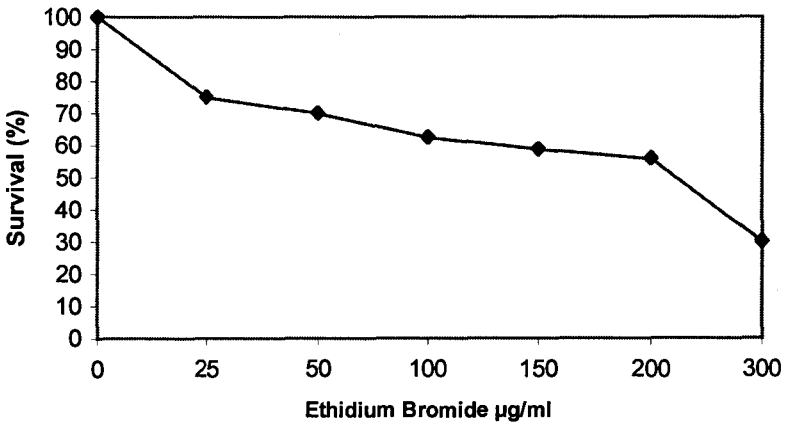
Fig 4.3. Lane 1, Uncut plasmid of strain DN1W, lane 2, 1 Kbps DNA ladder, Lane 3, *Eco* RI digest of plasmid DNA of strain DN1W, Lane 4, *Sau3A* 1 digest of plasmid DNA of strain DN1W, Lane 5, *Hind* III digest of plasmid DNA of strain DN1W, Lane 6, *Bam HI* digest of plasmid DNA of strain DN1W



A

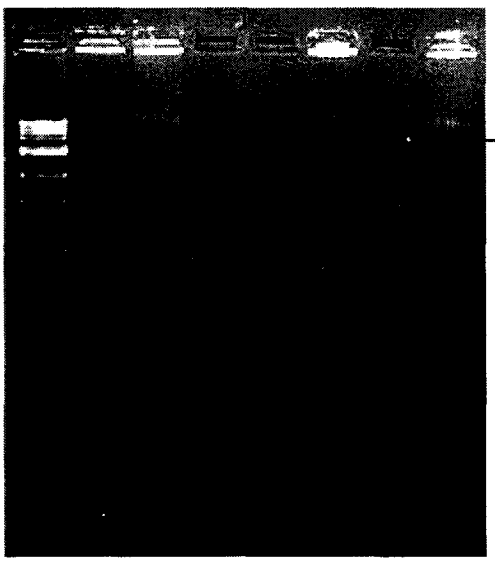


B



C

(Fig. 4.4 A, B, and C) Percent survival curves for plasmid Curing agents. SDS, AcOr and EthBR



1 2 3 4 5 6 7 8

Fig. 4.5 Agarose gel electrophoresis of plasmid DNA of *Vibrio harveyi* VB23 treated and untreated Curing agents

Curing of plasmid strain VB23 using SDS, AcrOr, EthBr

Lane 1	1 Kbp DNA ladder,
Lane 2-3	plasmid profile of culture ethidium bromide treated curing
Lane 4-5	plasmid profile of culture cured with Sodium dodecyl sulphate (0.5 % w/v)
Lanes 5-6	plasmid profile of culture treated with Acridine Orange
Lane 7	plasmid profile of culture untreated with curing agents (control)

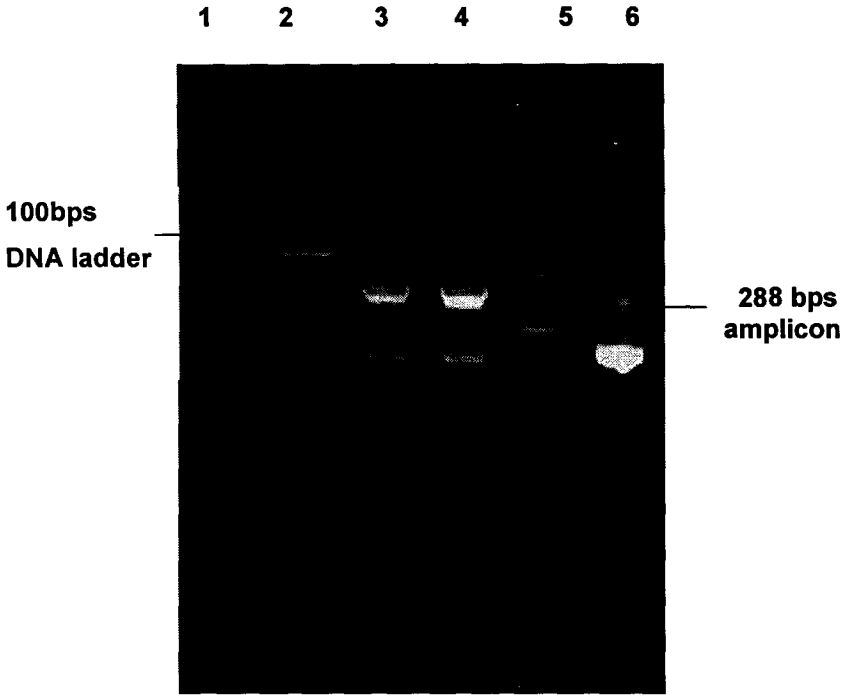


Fig. 4. 6. Agarose gel electrophoresis of PCR products of plasmid DNAs with primer pair

(*mer* A 5 R' 5' ACC ATC GTC AGG TAG GGG AAA AA 3')
(*mer* A1 F' 5' ACC ATC GGC GGC ACC TGC GT 3'

Lane 1	Negative control (10X Diluted genomic DNA of *E. coli* as template DNA
Lane 2,	100 bps DNA ladder
Lane 3-4	PCR product of 288 bps *merA* amplicon *(mer A)* using plasmid DNA from strains VB23 and VB6 respectively
Lane 5-6	No amplification of *merA* amplicon using plasmid DNA template from strain VB9 and VB01 respectively

Fig 4.6. Detection of mercury (*merA)* genes from luminous bacterial isolates by PCR amplification.

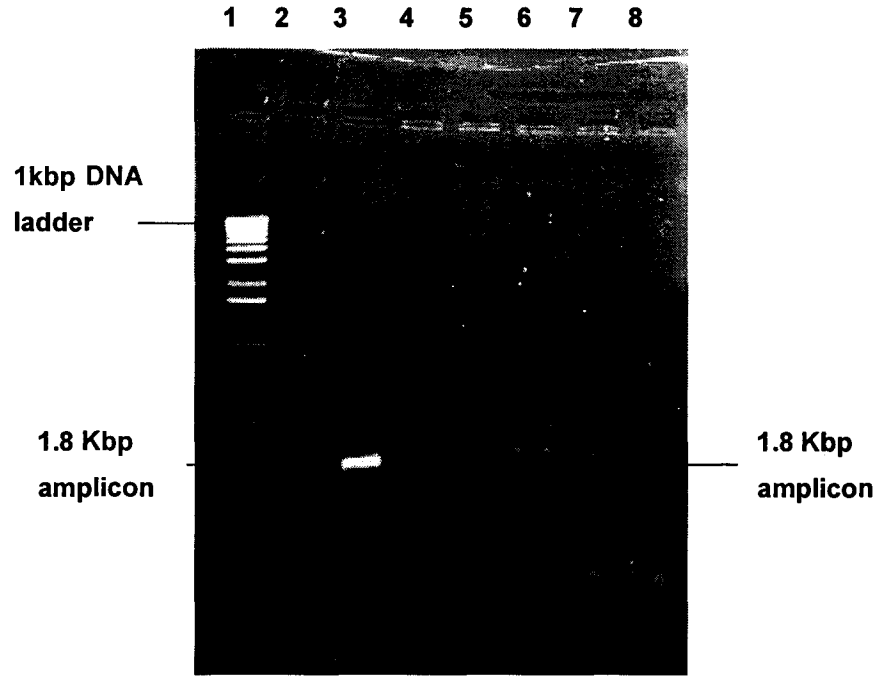


Fig. 4.7. Agarose gel electrophoresis of PCR products of plasmid DNAs with primer pair

Forward primer (*czc*A1 5' GTT TGA ACG TAT CAT TAG TTT C 3'
Reverse primer (*czc*A2 5' GTA GCC ATC CGA AAT ATT CG 3'

Lane 1,	1 kbps DNA ladder
Lane 2,	Negative control (10X Diluted genomic DNA of *E. coli* as template DNA
Lane 3	*czc A* gene amplicon (1.8 kbps approximately from plasmid DNA of VB6 Template
Lanes 4 & 5	PCR products not clear/no amplification
Lanes 6, 7 & 8	*czc A* gene amplicon (1.8 kbps) from plasmid DNA of VB23, DN1W, BR9 and VB9

Fig. 4.7. Detection of Cadmium (*czcA)* gene from luminous bacterial isolates by PCR amplification.

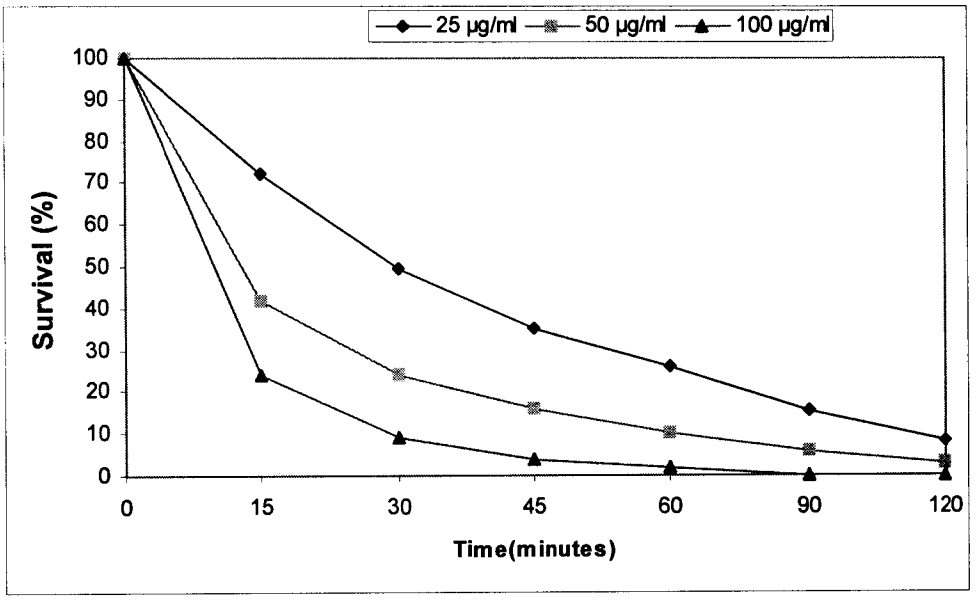


Fig 4.8 Survival of *Vibrio harveyi* strain VB23 in presence of NTG at regular time interval

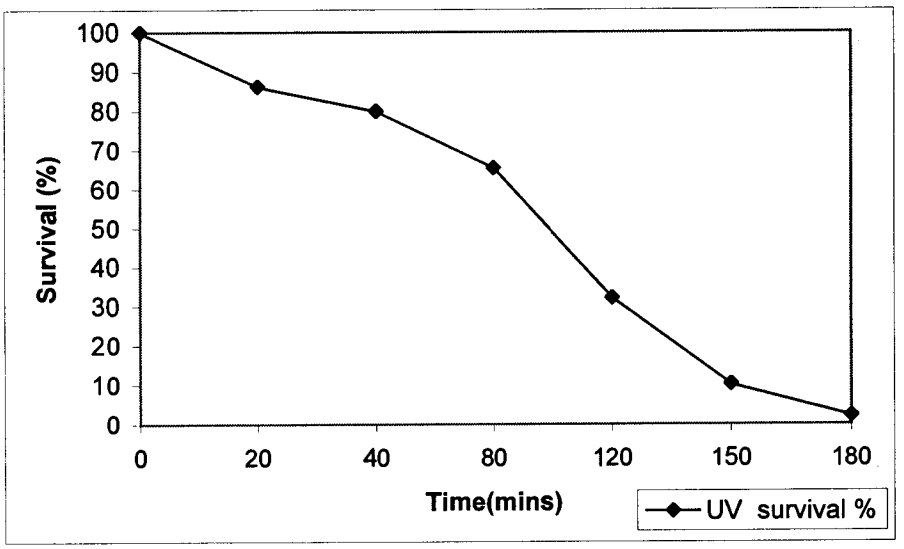


Fig 4.9. Survival of *Vibrio harveyi* strain VB23 exposed to UV light at regular time interval

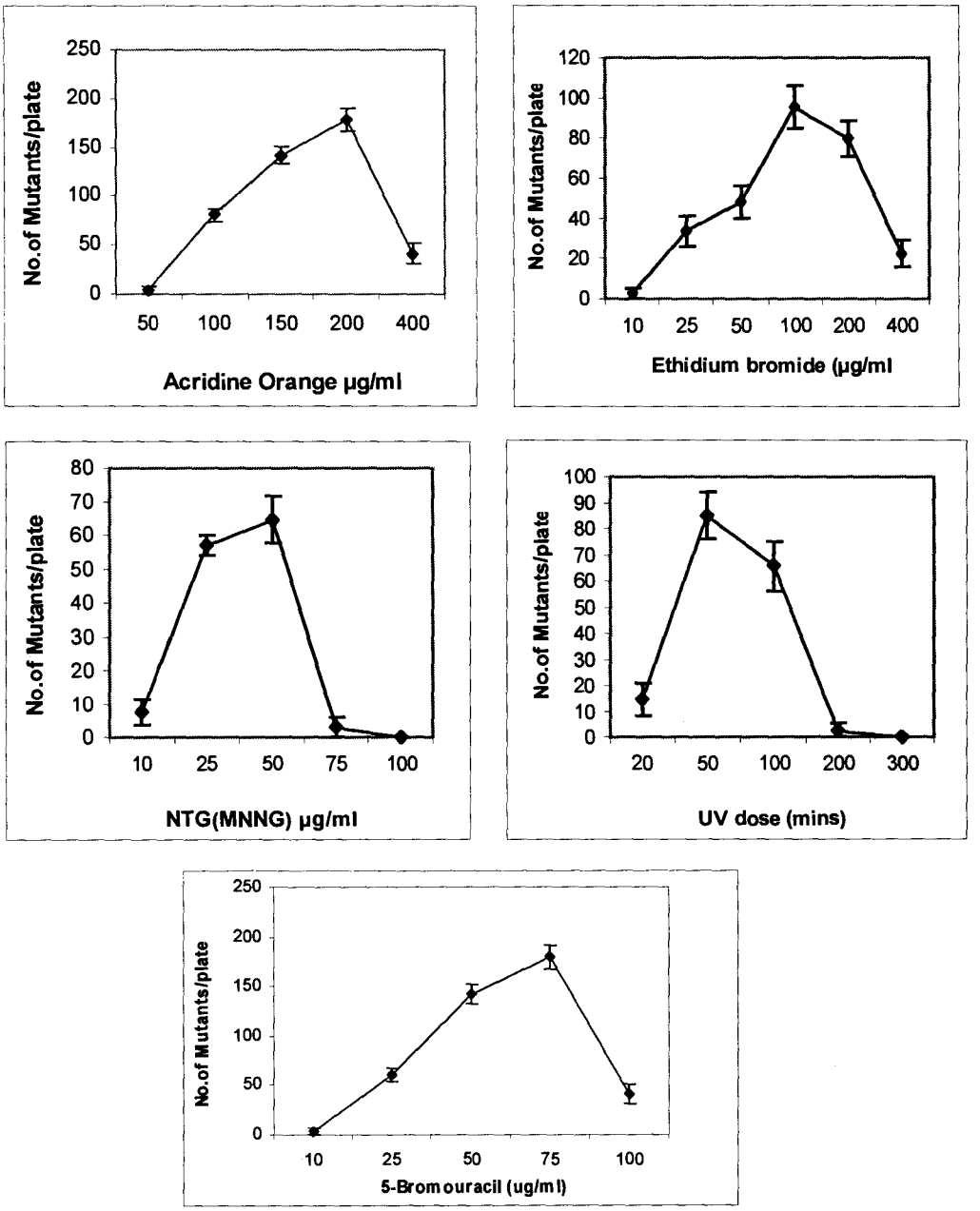
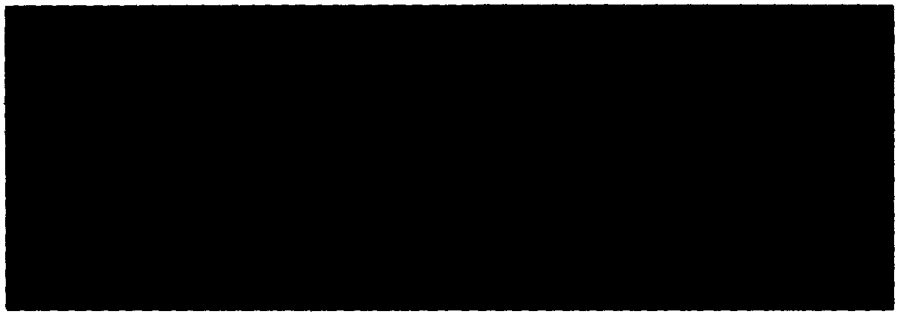


Fig 4.10. Mutagenesis and screening of mutants

Table. 4.5. Effect of chemical mutagens on growth and bioluminescence of strain *Vibrio harveyi* VB23

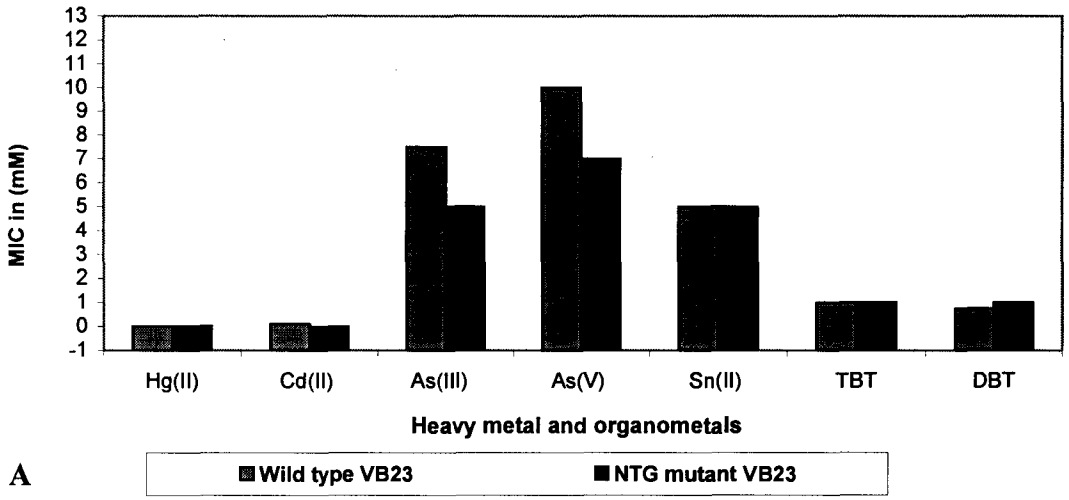
Chemical mutagens	Serial subculturing on GBMA								Growth-G Luminescence-L
	1	2	3	4	5	6	7	8	
Acridine Orange	+	+	+	+	+	+	+	+	G
AcOr 50 µg/ml	L	L	--	--	--	--	--	--	L
	+	+	+	+	+	+	+	+	G
AcOr 100 µg/ml	L	L	--	--	--	--	--	--	L
	+	+	+	+	+	+	+	+	G
AcOr 150 µg/ml	L	--	--	--	--	--	--		L
	+	+	+	+	+	+	+	+	G
AcOr 200 µg/ml	L	--	--	--	--	--	--	--	L
Nitrosoguanidine(NTG)									
NTG 10µ g/ml	+	+	+	+	+	+	+	+	G
	L	--	--	--	--	--	--	--	L
NTG 25 µg/ml	+	+	+	+	+	+	--	--	G
	L	--	--	--	--	--	--	--	L
NTG 50 µg/ml	+	+	+	--	--	--	--	--	G
	L	--	--	--	--	--	--	--	L
NTG 75 µg/ml	+	--	--	--	--	--	--	--	G
	L	--	--	--	--	--	--	--	L
Ethidium Bromide									
EthBr 50 µg/ml	+	+	+	+	+	+	+	+	G
	L	L	--	--	--	--	--	--	L
EthBr 100 µg/ml	+	+	+	+	+	+	+	+	G
	L	L	--	--	--	--	--	--	L
EthBr 200 µg/ml	+	+	+	+	+	+	+	+	G
	L	L	--	--	--	--	--	--	L
EthBr 300 µg/ml	+	+	+	+	+	+	--	--	G
	--	--	--	--	--	--	--	--	L



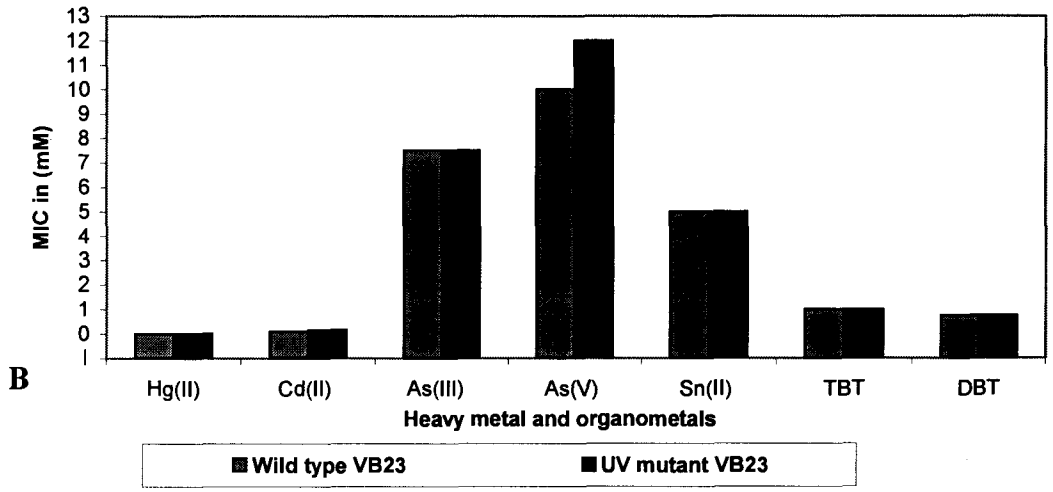
A

B

Fig 4.11 (A &B) .**Luminous bacterial strain grown in increasing concentrations of chemical mutagens Eth-Br (50, 100 and 200 µg/ml) and Acr Or (50, 100 and 200 µg/ml)**



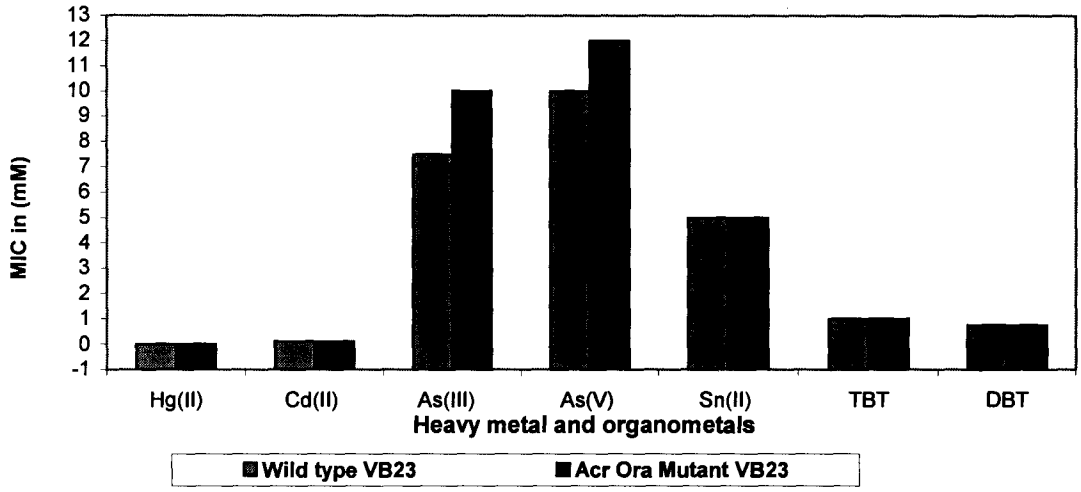
A

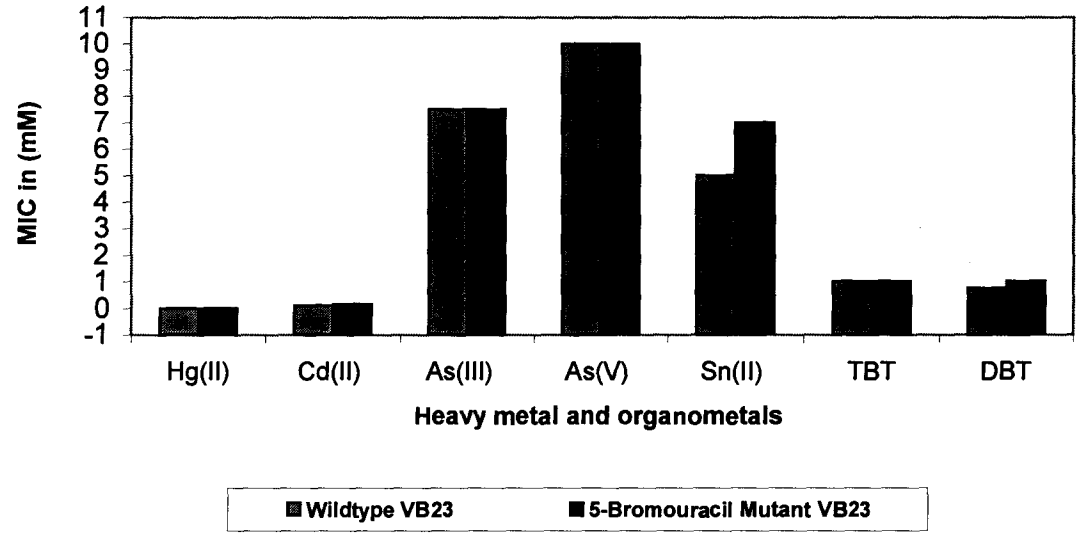


B

Fig 4.12 (A &B). Minimal inhibitory concentrations (MIC) of the NTG and UV mutants

A





B

Fig 4.13 (A& B). Minimal inhibitory concentrations (MIC) of the AcOr and 5-Bromouracil mutants

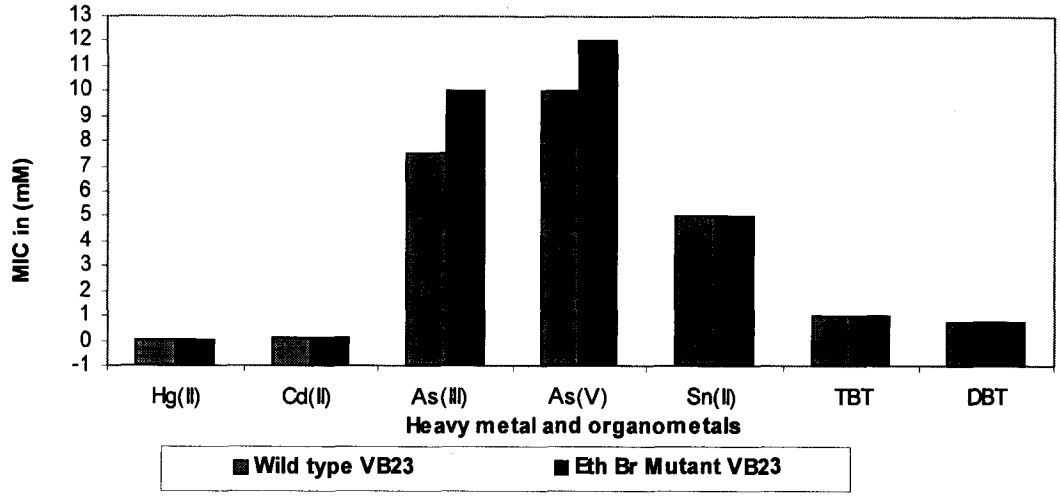


Fig 4.14. Minimal inhibitory concentrations (MIC) of the EthBr mutant

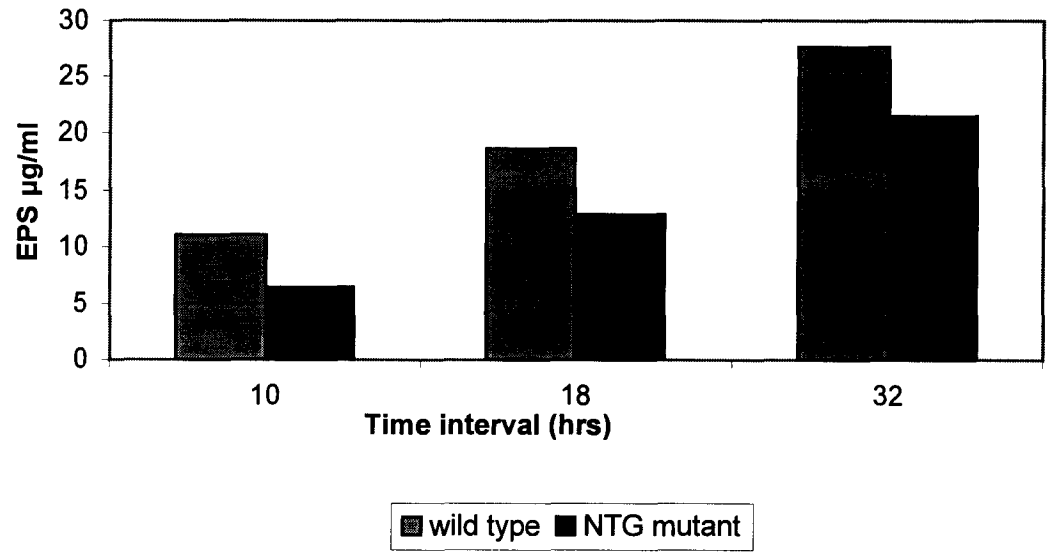


Fig. 4.15 Comparison of EPS production of NTG induced mutant and wild type strain of VB23

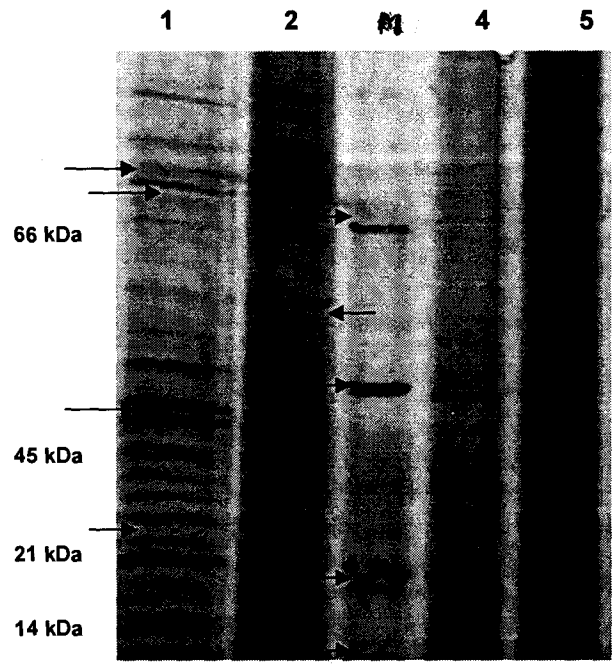


Fig. 4.16. SDS-PAGE anlaysis of protein profile of of NTG mutant and wild type strain Lane 1, protein profile of mutant (NTG 25 µg/ml), Lane 2, protein profile of mutant NTG 10 µg/ml, Lane M, medium range protein mol weight maker, Lane 5 – Control, Lane 4, protein profile of mutant NTG (50 µg/ml)

Fig. 4.16 Comparison of protein profile of NTG mutant and wild type strain VB23

References

Aires, J. R., Kohler, T., Nikaido, H. and Plesiat, P. 1999. Involvement of an active efflux system in the natural resistance of *Pseudomonas aeruginosa* to aminoglycosides. Antimicrob. Agents Chemother. 43: 2624–2628.

Baldwin, T. O. and Ziegler, M.M. 1992. The biochemistry and molecular biology of bacterial bioluminescence. Chem. Biochem. Flavoenzymes. 3: 467-524.

Barkay, T, Fouts, D. L and Olson, B.H. 1985. Preparation of a DNA gene probe for detection of mercury resistance genes in Gram negative bacterial communities. Appl. Environ. Microbiol. 49: 686–692

Barkay, T. and Olson, B.H. 1986. Phenotypic and genotypic adaptation of aerobic heterotrophic sediment bacterial communities to mercury stress. Appl Environ Microbiol 52:403– 406

Barkay, T. 1987. Adaptation of aquatic microbial communities to Hg^{2+} stress. Appl. Environ Microbiol 53: 2725-2732.

Belkin, S. 1998. Stress-responsive luminous bacteria for toxicity and genotoxicity monitoring. P.71–183.In: P.G. Wells, K. Lee and C. Blaise (ed.) Microscale Aquatic Toxicology – Advances, Techniques and Practice. CRC Press, Boca Raton, FL.

Bogdanova, E. S, Mindlin, S. Z, Kalyaeva, E. S. and Nikiforov, V.G. 1988. The diversity of mercury reductases among mercury resistant bacteria. FEBS Lett. 234: 280–282

Bulich, A. A. and Isenberg, D.L. 1981. Use of the luminescent bacterial system for the rapid assessment of aquatic toxicity. ISA Trans. 20:29–33.

Bulich, A. A. 1986. Bioluminescence assays. p.57-74, *In* : Bitton, G., and Dutka, G.J. (*eds*), Toxicity testing using microorganisms, Vol I. CRC Press, Boca Raton, Florida.

Caro, L., Churchward, G. and Chandler, M. 1984. Study of plasmid replication *in vivo*. In Methods in Microbiology, Vol. 17, eds Bennett P.M. & Grinsted J. pp. 97-122. London: Academic Press.

Cervantes, C., Ji, G., Ramirez, J. L. and Silver, S. 1994. Resistance to arsenic compounds in microorganisms. FEMS Microbiol Rev 15:355–367.

Chaudhary, P. and Kumar, R. 1996. Association of metal tolerance with multiple antibiotic resistance of enteropathogenic organisms isolated from coastal region of Deltaic Sunderbans. Ind. J. Medical Research 104:148-151.

Czyz, A., Jasiecki, J., Bogdan, A., Szpilewska, H., Wêgrzyn, G. 2000. Genetically modified *Vibrio harveyi* strains as potential bioindicators of mutagenic pollution of marine environments. Appl Environ Microbiol 66: 599–605.

Czyz, A., Szpilewska, H., Dutkiewicz, R., Kowalska, W., Biniewska-Godlewska, A. and Wegrzyn G, 2002. Comparison of the Ames test and a newly developed assay for detection of mutagenic pollution of marine environments. Mutat Res 519: 67–74.

Czyz, A., Kowalska, W. and Wegrzyn, G. 2003. *Vibrio harveyi* mutagenicity assay as a preliminary test for detection of mutagenic pollution of marine water. Bull Environ Contamin Toxicol. 70: 1065–1070.

Dhakephalkar, P.K., Bhide, J. V. and Paknikar, K.M. 1996. Plasmid mediated chromate resistance and reduction in *Pseudomonas mendocina* MCM B-180. Biotechnology Letters 18 (10), 1119-1122.

De Vicente, A., Aviles, M., Codina, J.C., Borrego, J.J and Romero, P. 1990. Resistance to antibiotic and heavy metals of *Pseudomonas aeruginosa* isolated from natural waters. J. Appl Bacteriol. 68: 625–632.

Deshpande, N. M., Dhakephalkar, P.K and. Kanekar, P.P. Plasmid-mediated dimethoate degradation in Pseudomonas aeruginosa MCMB-427. Lett. Appl. Microbiol 2001, 33: 275-279.

Diels, L. and Mergeay, M. 1990. DNA probe-mediated detection of resistant bacteria from soils highly polluted by heavy metals. Appl. Environ. Microbiol. 56: 1485–1491.

Dronen, A.K., Torsvik, V., Goksoyr, J., and Top, E.M. 1998. Effect of mercury addition on plasmid incidence and gene mobilizing capacity in bulk soil. FEMS Microbiol Ecol 30 27: 381-394. 31.

Hobman, J. L., Essa, A. M. M. and Brown, N. L. 2002. Mercury resistance (*mer*) operons in enterobacteria. Biochem Soc Trans 30: 719-722.

Hollander, M. C., Alamo, I. and Fornace, A. J. Jr. 1996. A novel DNA damage-indelible transcript, *gadd7*, inhibits cell growth, but lacks a protein product. *Nucleic Acids Res.*, 24, 1589–1593.

Kulkarni, R. S. and. Kanekar, P.P. Effects of some curing agents on phenotypic stability in Pseudomonas putida degrading e-caprolactam. World J Microbiol. Biotechnol 14: 255-257.

Klein, G., Walczak, R., Krasnowska, E., Baszczak, A. and Lipińska, B. 1995. Characterization of heat-shock response of the marine bacterium *Vibrio harveyi*. Mol Microbiol 16: 801–811.

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

Li, X. Z., Zhang, L. and Poole, K. 1998. Role of the multidrug efflux systems of *Pseudomonas aeruginosa* in organic solvent tolerance. J. Bacteriol. 180: 2987–2991.

Liebert, C. A., Wireman, J., Smith, T., and Summers, A.O. 1997. Phylogeny of mercury resistance (*mer*) operons of Gram-negative bacteria isolated from the fecal flora of 38 primates. Appl Environ Microbiol 63: 1066-1076. 39

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

Ma, D., Cook, D. N., Hearst, J. E. and Nikaido, H. 1994. Efflux pumps and drug resistance in Gram-negative bacteria. Trends Microbiol. 2:489–493. Regulation of the *Pseudomonas aeruginosa* outer membrane porin

Mc Arthur, J. V. and Tuckfield, R. C. 2000. Spatial patterns in antibiotic resistance among stream bacteria: effects of industrial pollution. Appl.Environ.Microbiol. 66(9): 3722-3726.

Miller, J. H. 1992. A Short Course in Bacterial Genetics. A laboratory Manual and Handbook for *Escherichia coli* and Related Bacteria. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

Misra, T. K. 1992. Bacteria resistance to inorganic mercury salts and organomercurials. Plasmid 27:4–16.

Miyauchi, K., Lee, H.S., Fukuda, M, Masamichi, T., Nagata, Y. and Ku, B. 2002. Cloning and characterization of lin R, involve din regulation of downstream pathway for gamma heachloro-cyclohexane degradation in *Spingomonas paucimobilis* UT26. Appl. Environ.Microbiol. 68: 1803-1807.

Müller, A. K. 2001. The effect of long-term mercury pollution on the soil microbial community. FEMS Microbiol Ecol 36: 11-19.

Muller, K. A., Rasmussen, L. D. and Sorensen S. J. 2001 Adaptation of the bacteria community to mercury contamination. FEMS Microbiol Lett 204:49–53

Nazaret, S., Jeffrey, W. H., Saouter, E., von Haven, R., and Barkay, T. 1994. *merA* gene expression in aquatic environments measured by mRNA production and Hg (II) volatilization. Appl Environ Microbiol 60: 4059-4065.

Nies, D. H., Nies, A., Chu, L. and Silver, S. 1989. Expression and nucleotide sequence of a plasmid-determined divalent cation efflux system from *Alcaligenes eutrophus*. Proc. Natl. Acad. Sci. USA. 86: 7351–7355

Nies, D. H.1992. Resistance to cadmium, cobalt, zinc and nickel in microbes. Plasmid 27: 17–28

Nucifora, G., Chu, L, Misra, T. K. and Silver, S. 1989. Cadmium resistance from *Staphylococcus aureus* plasmid pI258 *cadA* gene results from a cadmium-efflux ATPase. Proc. Natl. Acad. Sci. U.S.A. 86: 3544–3548.

Osborn, A. M., Bruce, K. D., Strike, P. and Ritchie, D. A. 1997. Distribution, diversity and evolution of the bacterial mercury resistance (mer) operon. FEMS Microbiol Rev 19:239–262.

Rasmussen, L. D., and Sorensen, S. J. 2001. Effects of mercury contamination on the culturable heterotrophic, functional and genetic diversity of the bacterial community in soil. FEMS Microbiol Ecol 1231: 1-9.

Ravel, J., Amoroso, J.M., Colwell, R.R., and Hill, R.T. 1998. Mercury-resistant actinomycetes form the Chesapeake Bay. FEMS Microbiol Lett 162:177–184.

Rochelle, P. A., Wetherbee, M. K and Olson, B.H. 1991. Distribution of DNA sequences encoding narrow- and broad-spectrum mercury resistance. Appl. Environ. Microbiol. 57: 1581–1589.

Rudrick, J. T., Bawdon, R. E., and Guss, S. P. 1985. Determination of mercury and organomercurial resistance in obligate anaerobic bacteria. Can J Microbiol 31: 276-281.

Sambrook, J, Fritsch, E.F. and Maniatis, T. 1989. Molecular cloning: a laboratory manual. (Ed. II). Cold Spring Harbor Laboratory, Cold Spring Harbor, New York.

Schaefer, J. K., Letowski, J., and Barkay, T. 2002. mer-mediated resistance and 41 volatilization of Hg (II) under anaerobic conditions. Geomicrobiol J 19: 87-102

Sheng, Y., V. Mancino, and Birren, B. 1995. Transformation of *Escherichia coli* with large DNA molecules by electroporation. Nucleic Acids Res. 23:1990–1996.

Silver, S, Misra, T.K.1988. Plasmid-mediated heavy metal resistances. Annu Rev Microbiol 42:717–743

Silver, S. and Walderhaug, M. 1992 Gene regulation of plasmid and chromosome-determined inorganic ion transport in bacteria. Microbiol. Rev. 56: 195–228

Silver, S. and Ji, G. G. 1994. Newer systems for bacterial resistances to toxic heavy metals. Environ. Health Perspect. 102: 107-113.

Silver S. and Phung L.T. 1996. Bacterial heavy metal resistance: new surprises. Annu Rev Microbiol 50:753–789

Suzuki, K., Wakao, N., Sakurai, Y., Kimura, T., Sakka, K. and Ohmiya, K. 1997. Transformation of *Escherichia coli* with a large plasmid of *Acidiphilium multivorum* AIU 301 encoding arsenic resistance. Appl. Environ.Microbiol.63: (5) 2089–2091.

Trajanovska, S., Britz, M. L. and Bhave, M. 1997. Detection of heavy metal ion resistance genes in Gram-positive and Gram-negative bacteria isolated from a lead-contaminated site. Biodegradation 8: 113–124.

Trevors, J. T.1986. Plasmid curing in bacteria. FEMS Microbiology Reviews 32: 149-157. 85.

Ulitzur, S., Weiser, I. and Yannau, S. 1980. A new, sensitive and simple bioluminescence test for mutagenic compounds. Mutation Res., 74, 113–124.

Waters, M.D., Brady, A.L., Stack, F. and Brockman, H.E. 1990. Antimutageecity profile for some model compounds.Mut.Res./ Review on Gen.Toxicol. 23: 57-85.

Weber, J.H., Evans, R., Jones, S.H., and Hines, M.E. 1998. Conversion of mercury (II) into mercury (0), monomethylmercury cation, and dimethylmercury in salt marsh sediment slurries. Chemosphere 36: 1669-1687.

SUMMARY

- Surface water samples were collected from various marine and estuarine sites of west coast of Goa, India and physico-chemical characteristics; Alkalinity (2.08-2.28 meq. L^{-1}) Dissolved oxygen (2.40-3.58 $ml.L^{-1}$; Phosphates (0.45-1.24 $mol.dm^{-3}$; nitrates (2.64 -4.04 $\mu mol.dm^{-3}$); nitrites (0.18-0.58 $\mu mol.dm^{-3}$) of water samples were determined.

- Total bacterial viable and luminous counts revealed the abundance of bioluminescent bacteria in the estuarine waters as compared to marine waters. The optimum temperature, pH and salinity for growth of the five selected luminous bacterial isolates were found to be 28°C, pH 8-8.5, and salinity 3-4 % respectively.

- Selected bioluminescent bacterial strains were identified using biochemical tests (according to Bergey's Manual of Systematic Bacteriology, and Alsina and Blanch's set of keys for biochemical identification, FAME analysis as well as molecular techniques (16 s rDNA - PCR amplification). Size of a segment of 16 s rDNA amplicon for *Vibrio harveyi* isolates was 413 bps using species specific primers VH1 and VH2. *Vibrio harveyi, Photobacterium leiognathi, Vibrio fischeri and Vibrio splendidus* were identified by PCR amplification of complete 16s rDNA (amplicon size: 1300 bp) using universal 16 s rDNA specific primers and subsequent restriction fragment length polymorphism (RFLP) analysis using restriction endonuclease *Hha* I.

- Strain *Vibrio harveyi* VB23 was selected for further characterization of growth in different media (varying from minimal to rich) and physiological impact of various concentrations of selected (metals and organometals), viz. Hg^{2+}, Cd^{2+}, As^{3+}, As^{5+}, Sn^{2+}, Cr^{6+}, TBT and DBT on growth was assessed to find out their lethal and sub-lethal levels for inducing stress responses.

- Selected metal and organo-metal pollutants were found to seriously affect the cell viability of bioluminescent bacteria. MIC levels for different metals and organo-

metals to the strains VB6 and VB23 were determined as follows; 7 µM for $HgCl_2$, 150 µM for $CdCl_2$, 100 µM for CrO_4, 5 mM for $SnCl_2$, 5 mM for $NaAsO_2$, 7.5 mM for $NaHAsO_4$ 0.75 mM for DBT, and 1 mM for TBT respectively. This clearly indicates that these two luminescent bacterial strains are highly resistant to test metals and organo-metals.

- Chelating agent (Na_2EDTA) and Glutathione significantly reduced the toxicity of heavy metals cadmium, mercury and TBT to luminescent bacterial strain VB23, as compared to other thiol compounds. viz. DTT, β-mercaptoethanol and Cysteine. It indicates that Na_2EDTA and Glutathione are the most effective chelating and ameliorative agents to reduce the toxicity of metals Cd (II), Hg (II) and organo-metal TBT

- Stimulation of light emission (RLI) was observed in presence of Hg, Cd, Na_2EDTA, Chloramphenicol, Rifamycin, Acridine orange and short term UV exposure. Whereas reduction in the light emission was observed in presence of arsenite, arsenate, inorganic tin, tributyltin, dibutyltin, kanamycin and cyclohexamide. Synergistic stimulation of light emission was observed in the following combinations; Hg (5 µM) +Na_2EDTA (5 µM), Chloramphenicol (25 µM) +Hg (5 µM), Chloramphenicol (25 µM) + UV irradiation (40 mins exposure).

- SDS-PAGE analysis of *Vibrio harveyi* VB23 clearly revealed that heavy metal and organo-metal stress proteins were induced within 12-16 hrs of growth. A novel 20 kDa protein was uniquely found in cadmium (25 µM, 50 µM) arsenite stresses (5 mM). Another novel protein of 54 kDa was induced by Hg (II) 2, 5 and 7 µM which seem to be mercuric reductase enzyme. 15 kDa, 20 kDa and 23 kDa and 54 kDa were induced at 2 and 5 µM only. Because cells recovered viability on prolonged exposure to Hg^{2+}, a detoxification mechanism may have been present. Stress induced proteins in Cd (II) 25 µM, a 54 kDa protein was induced, whereas Cd (II) 50 µM, induced 20 kDa, 52 kDa, 70 kDa and 78 kDa proteins; Induction of 36 kDa and 38 kDa proteins was observed in presence of As (III) 5 mM. In presence of As (V) 5 mM the levels of certain protein synthesis were apparently declined.

Induction of two novel proteins of 32 kDa and 40 kDa size occurred at DBT 0.5 mM and 0.75 mM, whereas 50 kDa protein was induced in TBT 0.5 mM. A constitutive protein of 58 kDa protein was more expressed at DBT 0.5 mM, 0.75 mM and 0.5 mM TBT. These metal induced stress proteins may serve as an important tool to sequester specific toxic metals. Thus these bacterial isolates can also be used for metal bioremediation of specific metal pollutants.

- The rate of EPS production in batch cultures was highest during the late log phase of growth as compared to stationary growth phase. Chemical and physical analyses (GC, FTIR, and ESI-MS) of exopolysaccharide (EPS) revealed that it is primarily composed of neutral sugars, uronic acids, proteins and sulphates having prominent characteristic groups such as hydroxyl, carboxylic and amides corresponding to a typical heteropolysaccharide and also possesses good emulsification activity. Heavy metal Cd (II) induces high level of EPS production in the strain VB23.

- The luminous bacterial pigment VB23 was induced at different metal and organo-metal responses. Toxic metals and organo-metals tested caused enhanced synthesis of pigments in *Vibrio harveyi* VB23, which may be involved in biochemical resistance against toxic metals and organo-metals possibly through intracellular sequestration.

- Correlation of heavy metal resistance and antibiotic resistance was initially confirmed by determining MIC levels of antibiotics and heavy metals and further confirmed by screening of plasmids in luminous bacterial strains, screening of transformants (clones) antibiotics and heavy metals and by subsequent cloning experiments.

- Transformation experiment confirms that metal as well as antibiotic resistance in these natural bioluminescent strains is plasmid mediated, whereas organo-metal resistant genes encoding may be located on genomic DNA. Plasmid mediated metal resistance was substantiated by curing experiment. SDS proved best curing agent than acridine orange and ethidium bromide. PCR amplification of metal

resistance genes (*mer A*, 288 bps amplicon) and *(cad A,* 1800 bps amplicon)using plasmid DNA as template confirmed that Cd (II) and Hg (II) resistance is plasmid mediated .It was further confirmed that metal resistance is plasmid mediated.

- Several non luminescent bacterial mutants were screened by treating the naturally luminescent strain VB23, with mutagenic chemicals such as NTG, AcOr, EthBr, 5-bromouracil and UV radiation.

- NTG induced mutants were more sensitive to toxic test metals and organo-metals than the wild type, whereas other mutagens induced highly tolerant mutants.

- NTG induced mutants produce less exopolysaccharide (EPS) than the wild type.

- Novel NTG induced proteins were present in the NTG treated mutant, whereas wild type lacks the same set of proteins.

- Genes encoding metal - induced stress proteins and other resistance mechanisms may be used to develop microbial sensors for environmental biomonitoring of specific toxic metal pollutants using *lux* reporter gene technology

FUTURE PROSPECTS OF THE RESEARCH

- Screening of some more genes encoding metal and organo-metal resistance in all the strains of luminescent bacteria

- Identification and characterization of metal and organo-metal induced stress proteins in luminous bacterial strains by western blotting, 2D-gel electrophoresis, N-Terminal peptide sequencing to find out the predominant by using 2D, gel electrophoresis, N-terminal peptide sequencing and find out the predominant amino acid residues of screening genes responsible for induction of metal responsive stress proteins by reverse genetics.

- Further biochemical characterization of stress proteins by SDS-PAGE and western blotting.

- Luminous *Vibrio harveyi* causes a major threat in biofouling and aquaculture industries. Recently it has been reported that quorum sensing signal molecules (AHL) has an important role in biofilm formation and pathogenesis in *Vibrio* spp, therefore much focused research is required to find out the other roles of signaling molecules, Acyl-homo serine lactones (AHL) in bacterial biofouling (Biofilm formation) and pathogenesis besides bioluminescence.

- Extraction and isolation of bioactive compounds from marine luminous bacteria, its application in industrial biotechnology and dig out the genes responsible for the exopolysaccharide production mediated by heavy metal sequestration.

- Genes encoding metal - induced stress proteins and other resistance mechanisms may be used to develop microbial sensors for environmental biomonitoring of specific toxic metal pollutants using *lux* reporter technology

Appendix

(A.1). Glycerol based Marine Agar (GBM)

Peptone	5 g
Yeast Extract	1 g
Glycerol	3 ml
Agar Agar	15 g
NaCl	15 g
$MgSO_4.7H_2O$	1.5 g
Potassium chloride	0.38 g
Double distilled water	500 ml
Aged sea water	500 ml
pH	7.5

(A.2).Thiosulfate Citrate Bile Sucrose Agar (TCBS) –Selective agar for *Vibrio sp*. (Kobayashi et al, 1963)

Peptone from casein	5.0 g
Peptone from meat	5.0 g
Yeast extract	5.0 g
Sodium citrate	10.0 g
Sodium thiosulfate	10.0 g
Ox bile	5.0 g
Sodiumcholate	3.0 g
Sucrose	20.0 g
Sodium chloride	10.0 g
Iron (III) citrate	1.0 g
Thymol blue	0.04 g
Bromothymol blue	0.04 g
Agar	14.0 g
pH:	8.6 ± 0.2

Preparation: 88 g media was suspended in 1 liter and boiled to dissolve the agar. Media was not autoclaved. Do not autoclave. The media is clear and green-blue in colour.

(A.3). *Vibrio harveyi* agar (VHA) (Harris et.al., 1996)

D-cellobiose	2 .0 g
L-ornithine	2.0 g
NaCl	30.0 g
Tris [hydroxymethyl] aminomethane	1.21 g
Agar	20.0 g
K_2HPO_4	0.075 g
Thymol blue	0.04 g
Bromothymol blue	0.04 g
Peptone	0.1 g
Yeast extract	0.1 g
Distilled water	1000 ml.

The solution is boiled for 30 min, Cooled to 56 °C and adjusted to pH 9.0 by the addition of 1 M NaOH and dispensed into sterile petri dishes. When solidified, VHA is an azure blue color

(A.4). **Mineral Salts Media (Single strength)** (Mahtani and Mavinkurve, 1979)

Ferrous sulphate (green crystals)	0.06 g
Dipotassium hydrogen ortho phosphate 12 %	100 ml
Potassium dihydrogen ortho phosphate 18.2 %	20 ml
Ammonium nitrate 10 %	20 ml
Magnesium sulphate 1 %	0.2 ml
Manganese sulphate 0.6 %	0.2 ml
Sodium molybdate 0.6 %	0.2 ml
Calcium chloride.(dihydrate) 1 %	15 ml
Double distilled water	1000 ml

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

(A.5). Luria Bertani (L.B.Broth) (Gerhardt et al.1994).

Tryptone	10.0 g
Yeast extract	5.0 g
Sodium chloride	10.0 g
Double distilled water	1000 ml

Adjusted to pH 7.0 with 0.1 N NaOH

For L.B agar, 1.5 g of agar is added to 100 ml L.B broth. Dissolved by boiling and sterilized accordingly.

B. Reagents for Estimation of Salinity, Dissolved oxygen, Nitrite, Nitrate and Phosphate contents

(B.1). Reagents for Salinity estimation: (Grasshoff, 1983)

1. $AgNO_3$ solution: 27.25 g of crystallized Silver nitrate was dissolved in 1L of double distilled water. This solution was mixed and stored in amber colored bottle.

2. Potassium chromate indicator solution: 10 g of Potassium chromate was dissolved in 100 ml of double distilled water.

(B.2). Reagents for Dissolved Oxygen (Koreleff , 1983)

1. Winkler's A reagent: 40 g of manganous chloride is dissolved in 60ml of distilled water and the volume made to 100 ml.

2. Winkler's B reagent: 60 g of potassium iodide and 30 g of potassium hydroxide are dissolved separately in minimum amount of distilled water and combined. The solution is made up to 100 ml with distilled water.

3. H_2SO_4 solution: 50 ml of concentrated Sulphuric acid is added carefully to 50 ml of chilled distilled water at 4°C.

4. Sodium thiosulphate method: 0.25 g of $Na_2S_2O_3$ is dissolved in 80ml of distilled water and volume is made to 100 ml.

5. Starch solution: 1 g of starch is dissolved in 50ml of distilled water by boiling and the starch solution is made to 100 ml with distilled water after cooling.

6. Potassium iodate solution: 0.3567 g of potassium iodate is dissolved carefully and made up to 1000 ml with distilled water.

(B.3). Reagents for Nitrite estimation (Koreleff , 1983)

1. Sulphanilamine: Sulphanilamine (1 g) was dissolved in 10 ml concentrated HCl and the solution was made to 100 ml with double distilled water.

2. N-(1-napthyl) ethylene di-amine di-hydrochloride: 0.1 g was dissolved In 100 ml of double distilled water

3. Standard $NaNO_2$ solution: 0.1725 g was dissolved in 250 ml of double distilled water which equals to 1 ml contains 10 µg atom of NO_2-N.

4. Working solution A: 2.5 ml of $NaNO_2$ diluted to 250 ml with double distilled water to make 0.1 µg atom of NO_2-N per ml.

5. Working solution B: 50 ml working solution A was diluted to 500 ml with distilled water to make 0.01-µg atom of NO_2-N per ml..

(B.4).Reagents for Nitrate estimation (Koreleff , 1983)

1. Concentrated Ammonium Chloride (NH_4Cl): 62.5 g of NH_4Cl was dissolved in 200 ml distilled water and volume was made to 250ml in volumetric flask.

2. Diluted Ammonium Chloride (NH_4Cl): 5 ml of above concentrated NH_4Cl was diluted to 200 ml with distilled water.

3. Amalgamated Cadmium granules: 100 g was treated with 500 ml of 2 % $CuSO_4$.Then the amalgamated Cadmium granules are washed several times with distilled water and is stored in dilute NH_4Cl.

4. Sulphanilamide: 1 g was dissolved in 10 ml of concentrated HCl and the volume was made to 100 ml with distilled water.

5. N-(1-napthyl)- ethylene diamine dihydrochloride : 0.1 g was dissolved in 100 ml of distilled water to prepare the stock solution of the reagent.

6. KNO_3 solution: 0.1 g of KNO_3 was dissolved in 100 ml of distilled water.

7. Working solution of KNO_3: 2.5 ml 0.1% $KNO_3$of was diluted to 250 ml with distilled water.

(B.5). Reagents for Phosphate estimation:

1. 9 N H_2SO_4: 25ml of concentrated H_2SO_4 was added to 75 ml double distilled water.

2. Ammonium molbydate solution: 9.5 g of Ammonium molbydate was dissolved in 100 ml of double distilled water.

3. Ascorbic acid: 7 g of Ascorbic acid was dissolved in 100 ml double distilled water.

4. Potassium antimonyl tartarate: 3.25 g of Potassium antimonyl tartarate was dissolved in 100 ml double distilled water.

5. Mixed Reagent: 22.5 ml of Ammonium molbydate solution, 100 ml of H_2SO_4 and 2.5 ml of Potassium antimonyl tartarate solution was mixed together.

6. Phosphate solution: .0.1361 g Potassium dihydrogen ortho phosphate (KH_2PO_4) was dissolved in 100 ml of double distilled water.

C. Biochemicals, stains, reagents, buffers, media composition, microbiological, biochemical assays for bacterial identification, basic techniques in microbiology, phase contrast microscopy (Gerhardt, et al. 1994).

(C.1). Scanning Electron Microscopy (S.E.M Sample preparation)

Concentration Acetone %	Volume Acetone	Volume Distilled water	Total Volume
10	5	45	50
20	10	40	50
50	25	25	50
80	40	10	50
90	45	5	50
95	47.5	2.5	50
100	50	0	50

(D). Preparation of stock solutions (Organometals)

(D.1). Tributyltin chloride (TBTC) ($C_{12}H_{27}ClSn$) (M.W. 325.49)

Stock solution of TBTC

Absolute ethanol (Filter sterilized) (72.2 ml) + TBTC (27.8 ml) (3.7 M) was mixed to make up the final volume of 100 ml. The solution was preserved in amber colored bottle at 4 °C dark. Fresh stock solutions were prepared when required.

(D.2). Dibutyltin chloride (DBTC) ($C_8H_{18}Cl_{12}Sn$) (F.W. 303.83)

Stock solution of DBTC

Absolute ethanol (Filter sterilized) (50 ml) + DBTC (15.1989 g) (1 M) was mixed to make up the final volume of 50 ml. The solution was preserved in amber colored bottle at 4 °C in dark. Fresh stock solutions were prepared when required.

(D.3). Preparation of Heavy metals stock solutions

The stock solutions for Heavy metal ions $HgCl_2$ (10 mM), $CdCl_2$ (10 mM), $NaAsO_2$ (50 mM), $NaAsO_4$ (100 mM) and $SnCl_2.2H_2O$ (10 mM). All stock metal solutions, with the exception of Sn^{2+}, were made in double-distilled water and filter sterilized (0.22 µm, Millipore) into sterile glass vials. Sn^{2+} was dissolved in 50 % ethanol and stored in a sterile polypropylene tube

(D.4). Thiols and Chelating agents:

2-mercpatoethanol - (MW- 78.13, Specific gravity –111.5)
1mM stock solution - 2-mercpatoethanol
714 µl in 10 ml Double distilled water

L-Cysteine (MW - 157.62)
1mM stock solution- L-Cysteine
0.0157 g in 10 ml Double distilled water

Glutathione (MW - 307.32)
1mM stock solution- Glutathione
0.03073 g in 10 ml Double distilled water

Dithiotreitol (MW - 154.25)
1 mM stock solution- Dithiotritol
0.01542 g in 10 ml Double distilled water

Disodium EDTA - (MW-372.34)
1mM stock solution -Disodium EDTA
0.0372 g in 10 ml Double distilled water

(D.5). Preparation of antibiotics stock solutions

Antibiotics*	Stock solutions	Mode of resistance
Ampicillin	100 mg/ml in D.water	β-lactamase hydrolyzes ampicillin before it enters the cell
Carbenicillin	50 mg/ml in D.water	In place of ampicillin
Chloramphenicol	50 mg/ml in methanol	Chloramphenicol acetyltransferase inactivates chloramphenicol.
Erythromycin	10 mg/ml in D.water	Macrolides inhibit protein synthesis by binding to 50 S subunit of 70 S ribosomes, mutation in modification of a protein in the 50S ribosomal unit.
Kanamycin	50 mg/ml in D.water	Aminoglycoside phosphotransferase (neomycin Phosphotransferase) inactivates kanamycin
Nalidixic acid	10 mg/ml in 1 N NaOH	Mutation in the host DNA gyrase prevent nalidixic acid from binding.
Neomycin	10 mg/ml in D.water	Neomycin (aminoglycoside) phosphotransferase inactivates neomycin.
Rifampicin	10 mg/ml in methanol	Mutation in the β subunit of RNA polymerase prevents rifampin from complexing.
Streptomycin	50 mg/ml in D.water	Aminoglycoside phosphotransferase inactivates Streptomycin; mutation in *rpsL* (*strA*) resulting in modified S12 protein, prevents streptomycin from binding.
Novobiocin	100 mg/ml in D.water	no data
Tetracycline	50 mg/ml in 70% ethanol	Active efflux of drug from cell.
Cyclohexamide	50 mg/ml in water	Inhibits protein synthesis by binding to 50 S subunit of ribosomes, mutation in modification of a protein

Stock solutions of antibiotics dissolved in Milli-Q water and sterilized by filtration through 0.22 µm filter (Millipore, GS). Antibiotics dissolved in ethanol were not sterilized. All solutions were stored in amber colored glass vials at 4 °C, except tetracycline, which was stored at – 20 °C and working solutions were freshly made prior to prescribed.

(E.) Stock solution of chemicals for Bioluminescence measurement

Heavy metals and organometals - same as for Impact studies
Antibiotics - same as for MIC determination studies

Chemical mutagens
NTG (1-Methyl-3-Nitro-Nitrosoguanidine) - 1 mg/ml
Acridine Orange - 10 mg/ml
Ethidium Bromide - 10 mg/ml
Sodium aizde - 10 mg/ml
5-Bromouracil - 5 mg/ml

Substrate (luciferase)
n-Decanaldehyde (MW-156.27)
To make 10mM, 0.0015 g was dissolved in 1ml of distilled water.

UV irradiation - 15 watts mercury vapour germicidal lamp

(F). Reagents for chemical estimation of Exopolysaccharides

(F.1). Reagents for Uronic acid estimation (Carbazole method) (Dische, 1968)

1. **Sulphuric acid reagent:**
   0.025 M Na –tetraborate in Concentrated H_2SO_4 (0.5485 g in 100 ml).

2. **Carbazole reagent**:
   0.125 % carbazole in 20 ml of ethanol.

3. **Standard Galactouronic acid:** - 0.1 mg of galactouronic acid dissolved in 1ml of double distilled water.

Procedure:

5 ml of H_2SO_4 reagent in tube was cooled to 4 °C using ice bath. To this a known amount of test sample was added and the tube was closed with a stopper and shaken gently and later on vigorously on a vortex mixer with constant cooling .the tube was heated for 10 mins in a boiling water bath and cooled at room temperature 0.2 ml of carbazole reagent was then added and after mixing, the samples were heated in a boiling water bath for 15 mins, cooled to room temperature and the absorbance was measured against reagent blank at 530 nm. Concentration of uronic acid was determined using Galactouronicacid (0-100 µg/ml) as standard.

(F.2). Reagents for sugar estimation

Phenol sulphuric acid method for Total Carbohydrates (Dubois et al. 1956).

1. **5 % phenol:** 5 g of redistilled phenol dissolved in double distilled water and diluted to 100ml

2. **Sulphuric acid:** 96 %, analytical grade

3. **Standard Glucose solution:** 0. 1 mg of glucose dissolved in 1ml of Double distilled water

Procedure:

To the known amount of glucose sample, 1 ml of Concentrated H_2SO_4 was added followed by the addition of 1ml of 2.5 % aqueous phenol and 4 ml of Conc. H_2SO_4.The

tubes were rapidly kept in ice bath and held for 10mins at room temperature. The yellow arrange color obtained was measured at 490 nm against distilled water blank. Standard curve was plotted using glucose (0-100 µg/ml). Factor F was calculated and the concentration of the total sugars in the samples was determined.

(F.3). Cysteine Hydrogen chloride method for Methyl pentoses (Dische et al.1948)

Reagents for Methyl pentoses

1. **H_2SO_4: H_2O mixture:** Added 1:6 (v/v) of H_2O and H_2SO_4.
2. **3 % Cysteine hydrogen chloride:** 0.3 g in 10 ml of Double distilled water
3. **Standard Arabinose solution:** 0.1 mg of arabinose dissolved in 1ml of Double distilled water.

Procedure:

To the known amount of sample, 4.5 ml of chilled 1:6 (v/v) of H_2O and H_2SO_4 was added and the mixure was then held at room temperature for few minutes and then in a boiling water for 3-10 mins and cooled under running tap water 0.1 ml of the 3 % Cysteine hydrogen chloride was added and the absorbance of greenish yellow color was read at 396 nm and 430 nm; Rhamnose (0-100 µg/ml) was used as a standard and the readings of 430 nm were subtracted from 396 nm in order to calculate the F factor.

(F.4). Barium chloride - Gelatin method for sulphate estimation (Terho and Hartiala, 1971).

1. **$BaCl_2$-Gelatin reagent:** Dissolved 2 g of gelatin in 400 ml of hot Double distilled water (60-70 °C) and allowed to stand at 4 °C overnight. Further dissolved in 2 g of $BaCl_2$ in the semi gelatinous fluid and the resultant cloudy solution allowed standing for 2-3 mins before use.

2. **3% w/v Tricholoroacetic acid:** Dissolved 3 g of TCA in 100 ml of Double distilled water.

3. **Standard K_2SO_4 solution:** Dissolved 1.1814 g of K_2SO_4 in 10 ml of 1N corresponds to 100 µg/ml of sulphate.

Procedure:

To the known amount of sample, 1ml of 1 N HCl was added in a glass ampoule. The ampoule was flushed with N_2 gas and sealed .the sample was hydrolyzed in an oven for 105 °C for 16 hrs. The content of the tube was cooled and mixed before opening the tube and the content added to another tube containing 3.8 ml of 3 % TCA 0.1 ml of $BaCl_2$-Gelatin reagent was then added. Mixed thoroughly and kept at room temperature for 20 mins. Absorbance of the white sulphate precipitate was measured at 360 nm against a blank containing 1ml of 1 N HCl. Factor F was calculated using K_2SO_4 (0-100 µg/ml) as the standard and the concentration of sulphates in the sample was determined.

(F.5). Ammonium molybdate method for Inorganic Phosphates (Gerhardt et al.1994)

1. **Copper-acetate buffer pH 4.0:** Dissolved 0.25 g of $CuSO_4$ and 4.6 g of Sodium acetate in 100 ml of 2 N acetic acid.

2. **Reducing agent:** Dissolved 2 g of p-methylaminophenol sulphate in 10 g/ml solution of sodium sulphite and stored in dark bottle till required.

3. **5 % Ammonium molybdate:** Dissolved 5 g of ammonium molybdate in 100 ml of Double distilled water.

4. **Standard KH_2PO_4:** Dissolved 34 mg of KH_2PO_4 in 100 ml of water and volume made up to 250 ml of Double distilled water. This solution has 1 µmol of phosphorous/ ml.

Procedure:

To the known amount of sample, 3ml of copper acetate buffer was added, 0.5 ml of ammonium molybdate solution.0.5 ml of reducing agent and incubated at room temperature for 10 mins. Absorbance was read at 680 nm against stock solution of PO_4 containing K_2HPO_4.

(F.6). Reagents for Protein Estimation (Lowry et al. 1951)

a. **Reagent A: Sodium Carbonate reagent**

Na_2CO_3	2 g
NaOH (0.1N)	100 ml

b. **Reagent B: Copper sulphate solution**

Sodium potassium tartarate	1 g
Copper sulphate	0.5 g
Double distilled water	100 ml

c. **Reagent C: Alkaline Copper sulphate solution**

Reagent A:	50 ml
Reagent B:	1 ml

Reagent C was prepared fresh at the time of estimation

d. **Reagent D – Folin Ciocalteau reagent**

FC reagent commercial grade	10 ml
Double distilled water	20 ml

Freshly prepared at the time of estimation.

Procedure

To the known amount of sample, 5ml of the Copper sulfate solution was added and kept at room temperature in the dark for 10 mins. 0.25 ml of Folin-ciocalteau phenol reagent was added and kept in the dark for 20 mins. Absorbance was measured at 660 nm against reagent blank and the concentrations of the unknown samples were determined from standard graph and factor F was calculated using Bovine serum albumin as the standard (0-100 µg/ml).

(G). Reagents, Buffers and Staining solutions for Protein and DNA

(G.1). Reagents for protein separation by SDS-PAGE (Laemelli. 1970)

Monomer solution (30 % T, 27 % C)

Acrylamide	29.2 g
Bis-acrylamide	0.8 g
Double distilled water	100 ml

Stored at 4 °C in dark place

TEMED (use as purchased)

10% Ammonium per sulfate (prepare freshly)

1 mg ammonium persulfate
Dissolve in 1.0 mL Double distilled water

Buffers

Resolving Gel buffer (1.5 M Tris, pH 8.8)

Tris base	45.4 g
Double distilled water	150ml

pH adjusted to 8.8 with 6 N HCl
Fill to 250 mL with distilled water

Stacking gel buffer (1.0 M, pH 6.8)

Tris Base	3 g
Double distilled water	50 ml

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

10 % SDS

Sodium dodecyl sulphate	1 g
Double distilled water	10 ml

10 X Electrophoresis Running Buffer

(250 mM Tris, 1.92 M glycine, 1 % SDS)
30.3 g Tris base
144.0 g Glycine
10.0 g SDS
Fill to 1 liter with distilled water
To make 1 liter of I X running buffer, add 900 mL of distilled water to 100 mL of I0 X running buffer.

Guide for the Preparation of Gels of Different Acrylamide Concentrations									
	7.0% Acrylamide concentration			10% Acrylamide concentration			12% Acrylamide concentration		
	1 gel	2 gels	4 gels	1 gel	2 gels	4 gels	1 gel	2 gels	4 gels
Distilled water	2.5 mL	5.0 mL	10.0 mL	2.0 mL	4.0 mL	8.0 mL	2.5 mL	3.4 mL	6.7 mL
1.5 M Tris-HCl, pH 8.8	1.3 mL	2.5 mL	5.0 mL	1.3 mL	2.5 mL	5.0 mL	1.3 mL	2.5 mL	5.0 mL
SDS (10%)	50 mL	100 mL	200 mL	50 mL	100 mL	200 mL	50 mL	100 mL	200 mL
Acrylamide (30%)	1.2 mL	2.3 mL	4.7 mL	1.7mL	3.3 mL	6.7 mL	2.0 mL	4.0 mL	8.0 mL
Ammonium persulfate* (10%) fresh	25 mL	50 mL	100 mL	25 mL	50 mL	100 mL	25 mL	50 mL	100 mL
TEMED*	3 mL	5 mL	10 mL	3 mL	5 mL	10 mL	3 mL	5 mL	10 mL
Total volume	5 mL	10 mL	20 mL	5 mL	10 mL	20 mL	5 mL	10 mL	20 mL

Table for SDS-PAGE preparation

Sample Buffer with SDS

(60 mM Tris, 2 % SDS, 5 % β-mercaptoethanol, 10 % glycerol, 0.025 % Bromophenol blue)
25.0 mL distilled water
6.58 mL 0.5 M Tris, pH 8.8
5.26 mL glycerol
10.5 mL of 10 % SDS
2.63 mL of 0.5 % Bromophenol blue

Fill to 50 mL with water in a volumetric flask
To prepare fresh working sample buffer, add 50 µl of β-mercaptoethanol to 950 µl of the stock sample buffer before adding to the sample

(G.2). Protein Staining and Developing Solutions

Staining Dye

(0.2 % Coomassie Brilliant Blue-R250, 50 % methanol, 10 % acetic acid)

Coomassie Blue-R-250	0.4 g
Methanol	100 mL
Glacial acetic acid	20 mL

Made to 200 mL with distilled water

Destaining Solution

(10 % acetic acid, 20 % methanol)

Glacial Acetic acid	100 mL
Methanol	200 mL

Made to 1 liter with distilled water

Reagents for Silver staining (All solutions should prepare fresh)

Sol- I Fixative solution

Methanol	50 ml
Glacial acetic acid	2 ml
Formaldehyde	50 µl

Volume made to 100 ml with milli-Q water

Sol-II Wash solution

Methanol	50 ml

Volume made to 100 ml with milli-Q water.

Sol-III Sodium thiosulphate

Sodium thiosulphate	20 mg (0.02 g)
Milli-Q water	100 ml

Sol-IV silver stain (wrap with aluminum foil)

$AgNO_3$	0.2 g
Formaldehyde	75 µl
milli-Q water	100ml

Sol-V Developer solution (prepare fresh)

Na_2CO_3	6.0 g
Sodium thiosulphate	0.4 mg (1crystal only)
Formaldehyde	50 µl
milli-Q water	100 ml

(G.3). Reagents for Genomic DNA isolation (Jones and Bartlett 1990)

TE buffer: (10 mM Tris HCl, 1 mM EDTA, pH 8.0
10 % w/v Sodium dodecyl sulphate
20 mg/ml Proteinase K

Phenol chloroform (1:1)
Isopropanol
70 % ethanol
3 M Sodium acetate (pH -5.2)

(G.4). Reagents for plasmid DNA isolation (Sambrook et al. 1989)

Alkaline Lysis Method

i) Solution I (pH 8.0)

Glucose	0.9 g
Tris- chloride	0.394 g
EDTA	0.292 g
Double distilled water	100 ml

ii) Solution II

SDS	1.0 g
0.2 N NaOH	100 ml

iii) Solution III (pH 5.2)

5M Potassium acetate	60.0 ml
Glacial acetic acid	11.5 ml
Double distilled water	28.5 ml

TE buffer:

Tris HCl	0.156 g
EDTA	0.029 g
Double distilled water	100 ml
pH	8.0

Tris acetate EDTA (TAE) buffer (pH 8.0)

50X: Tris Base	: 2.42 g
0.5 M EDTA	: 1 ml

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

(G.5). Agarose gel Electrophoresis (Ausubel et al. 1992)

Agarose	0.8 g
1X TAE buffer	100 ml

Ethidium bromide solution

Ethidium bromide	10 mg
Distilled water	1 ml

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

Loading dye

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

Tracking dye was stored at 4 °C

(G.6). Plasmid Curing agents

Sodium Dodecyl sulphate	1 g in 10 ml)
Acridine Orange	10 mg/ml
Ethidium Bromide	10 mg/ml

(G.7). Mutagenic chemicals and radiation

NTG (1-Methyl-3-Nitro-Nitrosoguanidine)	1 mg/ml
Acridine Orange	.10 mg/ml
Ethidium Bromide	10 mg/ml
UV light	254 nm

(G.8). Reagents for Mutagenesis study

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

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

Fresh stock solution was prepared and kept in amber colored bottle at 4 °C in dark.

Citrate buffer (pH.5.5)

Citric acid	10.5 g
NaOH	4.4 g
Double distilled water	500 ml

Phosphate Buffer (pH 7.0)

Potassium dihydrogen phosphate	6.8 g
NaOH	1.16 g.
Double distilled water	100 ml

pH was adjusted to 7.0 with 2 N NaOH and autoclaved

References

Ausubel, F.M., Brent, R., Kingston, R.E., Moore, D.D., Seidman, J.G., Smith, J.A. and Struhl, K. 1992. Short protocols in molecular biology, second edition. Green Publishing Associates and John Wiley & Sons, New York.

Dische, Z. and Shettles, L.B. 1948. A specific colour reaction of methyl pentoses and a spectrophotometric micro method for their determination. J. Biol Chem. 175: 595-603.

Dische, Z. 1962. Color reactions of hexuronic acids. Methods in Carbohydrate Chemistry 1: Pp497.

Dubois, M., Gilles, K. A., Hamilton, J. K., Rebers, P.A. and Smith, F. 1956. Colorimetric method for determination of sugars and related substances. Analytical Chemistry. 28: 350–356.

Gerhardt, P., Murray, R.G.E., Wood, W.A. and Krieg, N.R. (eds) 1994. Methods for General and Molecular Bacteriology. American Society for Microbiology, Washington, DC, Pp791.

Grasshoff, K. 1983. Determination of salinity. *In* Methods of seawater analysis. Ed. 2nd. By Grasshoff, K., Ehrhardt. M., Kremling, K. Verlag chemie publication. Pp31.

Kobayashi, T., Enomoto, S., Sakazaki, R. and Kuwahara, S. 1963. A new selective isolation medium for pathogenic vibrios: TCBS-Agar. - Jap. J. Bact. 18: 391-397.

Koroleff, F.1983. Determination of nutrients. In: Methods of seawater analysis. 2nd Ed.. By Grasshoff, K., Ehrhardt. M., Kremling, K. Verlag chemie publication. Pp125.

Harris, L., Owens, L. and Smith, S. 1996. Selective and differential medium for *Vibrio harveyi.* Appl Environ Microbiol, 62: 3548–3550.

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

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

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

Sambrook, J., Fritsch, E.F. and Maniatis, T. 1989. Molecular Cloning: A Laboratory manual. Ed.2nd. Cold Spring Harbor Laboratory, New York, U.S.A.

Terho, T.T. and Hartiala, K. 1971. Method for determination of the sulfate content of glycosaminoglycans. Analytical Biochemistry. 41: 471-476.

List of Publications:

1. **Veera Bramhachari P.** and S. K. Dubey (2006). Isolation and characterization of exopolysaccharide produced by a marine bioluminescent bacteria, *Vibrio harveyi* VB23. Lett. Appl. Microbiol. (Under revision).

2. **Veera Bramhachari P.** and S. K. Dubey (2005). Rapid and specific detection of luminous and non-luminous *Vibrio harveyi* isolates by PCR amplification. Curr. Sci. (Accepted).

3. **Veera Bramhachari P** and S. K. Dubey (2005). Biochemistry, Molecular Biology of Bacterial bioluminescence and Applications of *lux* genes. **In:** Cellular and Biochemical Sciences (Ed. G. Tripathi), I. K. International Publishers, New Delhi (In press).

4. Sangham, S., Jayasree, D., Janardhan Reddy, K., **Chari, P.V.B**, Sreenivasulu, N. and Kavi Kishor P.B. (2005). Salt tolerance in plants-Transgenic approaches. J. Plant. Biotechnol. 7: 1-15.

5. Dubey, S. K. and **P. V. Bramhachari** (2004). Cyanobacteria in heavy metal polluted environment: Environmental Biotechnology Perspective. **In:** Biotechnological applications of cyanobacteria. (Ed. Kiran Singh), pp.225-244.

6. Dubey, S. K. and **P. V. Bramhachari** (2005). Cyanobacterial Metallothioneins: Structure, Function and Future prospects. Microbes & Environment (Communicated).

Letters in Applied Microbiology ISSN 0266-8254

ORIGINAL ARTICLE

Isolation and characterization of exopolysaccharide produced by *Vibrio harveyi* strain VB23

P.V. Bramhachari and S.K. Dubey

Laboratory of Bacterial Genetics & Environmental Biotechnology, Department of Microbiology, Goa University, Taleigao Plateau, Goa, India

Keywords
emulsification activity, exopolysaccharides, heteropolysaccharide, precipitation, *Vibrio harveyi*.

Correspondence
Santosh Kumar Dubey, Department of Microbiology, Laboratory of Bacterial Genetics & Environmental Biotechnology, Goa University, Goa 403206, India. E-mail: dubey_santosh1@rediffmail.com

2006/0048: received 16 January 2006, revised 14 April 2006 and accepted 18 April 2006

doi:10.1111/j.1472-765X.2006.01967.x

Abstract

Aims: The aim of the study was to isolate and characterize exopolysaccharide (EPS) produced by *Vibrio harveyi* strain VB23.

Methods and Results: Growth and EPS production by *V. harveyi* strain VB23, was studied in mineral salts medium supplemented with NaCl (1·5%) and glucose (0·2%). The rate of EPS production in batch cultures was highest during the late log phase of growth when compared with stationary growth phase. The exopolymer was recovered from the culture supernatant by using a cold ethanol precipitation–dialysis procedure. Chemical analyses of EPS revealed that it is primarily composed of neutral sugars, uronic acids, proteins and sulfates. The purified EPS revealed prominent functional reactive groups, such as hydroxyl, carboxylic and amides, which correspond to a typical heteropolymeric polysaccharide and the EPS, also possessed good emulsification activity. The gas chromatographic analysis of an alditol acetate-derivatized sample of EPS revealed that it is composed primarily of galactose and glucose. Minor components found were rhamnose, fucose, ribose, arabinose, xylose and mannose.

Conclusions: The EPS produced by *V. harveyi* strain VB23 is a heteropolysaccharide possessing good emulsification activity. EPS was readily isolated from culture supernatants, which suggests that the EPS was a slime-like EPS.

Significance and Impact of the Study: This is the first report of EPS characterization in luminous *V. harveyi* bacteria, which describes the isolation and characterization of an EPS expressed by *V. harveyi*. The results of the study contributes significantly towards an understanding of the chemical composition and applications of the EPS in environmental biotechnology and bioremediation.

Introduction

Exopolysaccharides (EPS) are organic macromolecules that are formed by polymerization of similar or identical building blocks, which may be arranged as repeated units within the polymer. Bacterial exopolymers are important in the interaction between bacteria and their environment and are chemically diverse. The major organic fractions of the EPS are carbohydrates, proteins and humic substances (Nielsen and Jahn 1999). A wide range of chemical structures of homopolymeric or heteropolymeric type, made up of sugar and nonsugar components is possible and the range of monosaccharide combinations, together with noncarbohydrate constituents and varied linkage types, makes the exopolymer an excellent emulsifying agent and attributes diversity in bacteria (Keene and Lindberg 1983). The bacterial exopolymers are usually acidic heteropolysaccharides possessing the functional groups (e.g. hydroxyl, carboxyl and phosphoric acid) associated with EPS, which exhibits high affinity towards certain metal ions (Mittleman and Geesey 1985). Many interesting physical and chemical properties of microbial exopolysacharides, have found a wide range of applications in the field, e.g. stabilizing, suspending, thickening, gelling, coagulating, film-forming and water-retention capability, e.g. in detergents, textiles, adhesives, paper, paint, food

	L A M	1 9 6 7	B	Dispatch: 15.5.06	Journal: LAM	CE: Siva
	Journal Name	Manuscript No.		Author Received:	No. of pages: 7	PE: Bhuvana

and beverage industries (Sutherland 1996), oil recovery, mining industry and petroleum industries (Sutherland 1998). A wide range of bacteria from various environmental habitats are known to produce complex and diverse EPS occurring as capsular polysaccharides intensively associated with the cell surface or as slime polysaccharides, loosely associated with the cell (Whitfield 1988).

Bacterial growth is often accompanied by production of EPS, which have relevant ecological and physiological functions. The nutrient status and growth phase of surface-associated bacteria may influence the quality and composition of the EPS produced (Decho 1990). In recent years there has been a growing interest in the isolation and characterization of microbial EPS owing to their importance in adhesion, nutrient sequestration, chelation of heavy metals, detoxification of toxic compounds and protection against osmotic shock (Decho 1990; Hoagland *et al.* 1993). Despite their importance, very few studies have been carried out on chemical characterization of EPS by marine fouling bacteria (Rodrigues and Bhosle 1991; Majumdar *et al.* 1999; Muralidharan and Jayachandran 2003). Keeping in view of the commercial importance of the EPSs, the present investigation deals with the isolation, chemical and physical characterization of extracellular polysaccharides from the bacteria *Vibrio harveyi* strain VB23.

Materials and methods

Culturing of exopolymer-producing bacteria

Exopolymer-producing halophilic luminescent bacteria were isolated from water samples collected from different sampling sites of river Mandovi and Zuari estuarine network connecting the coastal regions of Goa. The luminescent bacterial isolates were obtained by serial dilution plating on TCBS Agar and Glycerol-based marine agar plates incubated at 28 ± 2°C for 24 h. A total of about 48 colonies were isolated, and the exopolymer-producing bacteria were screened for their ability to produce exopolymer based on colony morphology (mucoid phenotypes). Among the screened isolates, one halophilic luminescent *V. harveyi* strain VB23 having the ability to form a viscous exopolymer after the ice-cold ethanol precipitation was tentatively identified as *V. harveyi* based on the morphological and physiological characteristics using biochemical tests according to Bergey's Manual of Systematic Bacteriology and Alsina and Blanch's (1994) set of keys for biochemical identification of marine *Vibrio* sp. (Baumann and Schubert 1984; Alsina and Blanch 1994). The isolate VB23 was further selected for isolation, purification and characterization of an exopolymer from *V. harveyi* strain VB23. The culture VB23 was grown and maintained in optimized mineral salts medium containing 0·2% of glucose, 12·6% of K_2HPO_4, 18·2% of KH_2PO_4, 10% of NH_4NO_3, 1% of $MgSO_4{\cdot}7H_2O$, 0·6% of $MnSO_4$, 1% of sodium molybdate, 1% of $CaCl_2{\cdot}2H_2O$, 0·06% of $FeSO_4{\cdot}2H_2O$ and 1·5% of NaCl in 1 l of distilled water.

Growth curve and exopolymer production of strain VB23

Luminescent bacterial cultures for EPS production was grown and maintained as batch cultures in 200 ml of MSM medium supplemented with NaCl to a final concentration of 1·5% (w/v), 0·2% glucose in 500 ml Erlenmeyer flasks on a rotary shaker at 28 ± 2°C for 2 days. The pH of the medium was adjusted to 7·0 with 1 mol l^{-1} NaOH. The medium (250 ml) was dispensed in 500 ml Erlenmeyer flasks and inoculated with 2% (v/v) of an overnight grown culture in the same medium at room temperature (28 ± 2°C) on a rotary shaker at 160 rev min^{-1}. The subsamples of 5 ml aliquots were drawn at regular intervals for turbidity measurements of bacterial growth ($A_{600\ nm}$) and EPS production.

Extraction and purification of VB23 exopolysaccharide

The culture volume of 200 ml was centrifuged at 15 000 *g* for 20 min at 4°C. The cell pellets were freeze-dried and weighed. The supernatants were pressure-filtered through cellulose nitrate filters with the following pore sizes: 0·8, 0·45 and 0·25 μm (Millipore filters). EPS were precipitated from the final filtrate after the addition of three volumes of cold ethanol and the solution was chilled at 4°C overnight. The resulting precipitate was recovered by vacuum filtration through a scintered glass apparatus. An additional 100 ml cold ethanol was added to the filtrate and the solution was placed at −20°C overnight. The precipitate was recovered as above. The precipitates were washed with 70–100% ethanol–water mixtures. After washing with ethanol, the EPS were combined and dried in a desiccator and stored at room temperature till needed. To remove excess salts, the EPS was redissolved in distilled water and dialysed at 4°C for 24 h (molecular weight cut-off of 13 kDa; Sigma-Aldrich Chemie GmbH, Germany) against distilled water. Excess water was removed under vacuum before lyophilization. Exopolysaccharide extracted was lyophilized using a Labonco lyophilizer (Kansas City, MO, USA) at 3000 psi. The lyophilized EPS was stored at room temperature until chemical and physical analyses could be performed.

Quantitative analysis of VB23 exopolysaccharide

Lyophilized EPS was hydrolysed with 2 mol l^{-1} HCl for 2 h at 100°C in ampoules flushed with N_2 before seal-

ing. After hydrolysis, the solution was evaporated to dryness under reduced pressure at 40°C (Read and Costerton 1987). Wet weight and dry weight of the EPS were determined (data not shown). The lyophilized sample was used for further chemical and physical characterization.

Emulsifying activity of VB23 exopolysaccharide

The emulsifying activity of EPS was assayed by modifying the method described by Rosenberg *et al.* (1979). Lyophilized EPS (0·5 mg) was dissolved in 0·5 ml deionized water by heating at 100°C for about 15–20 min and allowed to cool to room temperature (28 ± 2°C). The volume was then made up to 2 ml using phosphate-buffered saline (PBS). The sample was vortexed for 1 min after the addition of 0·5 ml hexadecane. The absorbance at 540 nm was read immediately before and after vortexing (A_0). The fall in absorbance was recorded after incubation at room temperature for 30 and 60 min (A_t). A control was run simultaneously with 2 ml PBS and 0·5 ml hexadecane. The emulsification activity was expressed as the percentage retention of emulsion during incubation for time *t*: $A_t/A_0 \times 100$.

Chemical analysis of VB23 exopolysaccharide

Lyophilized exopolymer was dissolved in ultrapure milli-Q water 0·1 g l^{-1} for chemical analyses. Exopolymers were assayed for total carbohydrate content using the phenol sulfuric acid assay with glucose as standard (Dubois *et al.* 1956). Uronic acids were assayed using the method described by Dische (1962) with glucuronic
8 acid as standard. Methyl pentoses (Dische and Shettles 1948). Sulfated sugars were determined by measuring sulfates according to the method of Terho and Hartiala (1971) after hydrolysis of the polymer (K_2SO_4 was used as standard). The protein content of the EPS was determined according to Lowry *et al.* (1951) (BSA was taken as standard).

Fourier-transformed infrared spectroscopy

The major structural groups of the purified EPS were detected using Fourier-transformed infrared (FTIR) spectroscopy (Abu *et al.* 1991). Pellets for infrared analysis were obtained by grinding a mixture of 2 mg EPS with 200 mg dry KBr, followed by pressing the mixture into a 16-mm-diameter mould. The FTIR spectra were recorded on a Bruker Vector 22 instrument (Bruker SA, Wissembourg, France) in the region of 4000–
9 400 cm^{-1} and the spectra was traced with a Hewlett Packard plotter.

Analytical gas chromatography

Alditol acetate derivatives of the monosaccharide sugars released by hydrolysis (Bhosle *et al.* 1995) from lyophilized EPS were analysed by a capillary gas chromatogra-
10 phy (Perkin-Elmer model 8310) equipped with a fused silica capillary column coated with CP Sil-88 (25 m, i.d. 0·32 mm, ds 0·12; Chrompack, Middleburg, the Netherlands). A flame ionization detector (FID) was used to separate the alditol acetate mixture, and oxygen-free dry helium was used as carrier gas at a flow rate of 25–30 ml min^{-1}. The resulting methyl glycosides were converted to their trimethylsilyl derivatives. The gas chromatogram oven temperature was initially programmed at 70°C and was then rapidly raised to 150°C after the sample was injected. The final analysis temperature was set at 230°C for 40 min using inositol (1 mg ml^{-1}) as the internal standard.

Results and discussion

Bacterial isolates were identified by standard morphological, physiological and biochemical tests. Bacterial identification was made by following the schemes of Baumann and Schubert (1984) and Alsina and Blanch (1994). The bacterial strain VB23 was Gram-negative, facultative anaerobe, motile rods, oxidase-, catalase- and gelatinase-positive and produced green mucoid colonies on TCBS agar. In addition, the isolate failed to utilize inositol, sorbitol, saccharose and melibiose, the isolate was positive to lysine decarboxylase, ornithine decarboxylase-positive and sensitive to the vibriostatic agent, 0/129. The isolate was tentatively identified as *V. harveyi*. Most of the EPS-producing marine bacteria, isolated from various sites are Gram-negative rods belonging to the genus *Vibrio*, *Flavobacterium*, *Pseudomonas*, *Alteromonas* sp., *Alteromonas atlantica* and *Alteromonas colwelliana* are known to produce acidic polysaccharides (Geesey *et al.* 1992).

The rate of EPS production in batch culture was highest during the late log phase of growth to stationary growth. The exopolymer yields of the batch culture were 12 μg ml^{-1} at 6 h to 27·8 μg ml^{-1} after 28 h of incubation and did not vary much, followed by the decrease of EPS production after 32 h (Fig. 1). When the cultures were centrifuged at 15 000 *g*, for 30 min, the supernatants from two isolates were viscous and formed stringy precipitates with cold ethanol (95%) and to enhance the precipitation, the samples were stored at 4°C for 24 h. The addition of two volumes of ice-cold ethanol showed a better precipitation and recovery of the biopolymer. The
11 precipitates recovered by centrifugation at 12 000 rpm for 20 min were vacuum dried to obtain a crude biopolymer or were directly dissolved in distilled water. This VB23

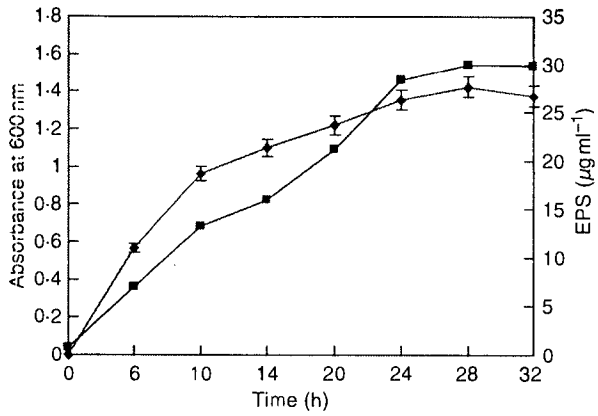


Figure 1 Growth and exopolysaccharide (EPS) production of *Vibrio harveyi* strain VB23. Culture conditions: MSM broth + 1·5% NaCl + 0·2% glucose, temperature 28°C, pH 7·0 and agitator speed 160 rev min^{-1}. Absorbance at 600 nm – (■) filled square; EPS $\mu g\ ml^{-1}$ – (◆) filled diamond.

EPS exhibited good solubility in distilled water. Most EPSs are produced both in the exponential and stationary phases (Uhlinger and White 1983), an exception has been noted by Williams and Wimpenny (1977) for another nonmarine pseudomonad, which produces EPS only in the stationary phase. Various methods including high-speed cold centrifugation (Decho 1990), mild alkali, ethylenediaminetetraacetic acid (EDTA), NaCl (Bhosle *et al.* 1995) have been reported for extraction of EPS from microbial cultures. However we found fairly effective extraction of exopolysacchardies by cold ethanol precipitation and incubated at 4°C for 24 h.

Chemical analysis

Chemical analyses of the EPS expressed by strain VB23 showed gross differences in composition of the EPS. The analysis showed that the exopolymer produced by *V. harveyi* was acidic in nature and the contents of the neutral sugars (total carbohydrates), proteins, uronic acids and methyl pentoses were 211·71, 186, 114·62 and 32 $\mu g\ mg^{-1}$ respectively (Table 1). The sulfate contents were below detection limits. Among the two strains selected during initial screening, *V. harveyi* VB23 showed the highest exopolymer production with reference to yield ($\mu g\ ml^{-1}$) and chemical contents, i.e. total carbohydrates, uronic acids, proteins and methyl pentoses. Fazio *et al.* (1982) have previously shown that exopolymer from the marine bacterium contains high quantities of galacturonic acid.

Table 2 Hexadecane, 0·5 ml, was added to 0·5 ml EPS (1 mg ml^{-1}) diluted to 2 ml with PBS, vortexed for 1 min and the absorbance monitored at 540 nm (a control was run with 2 ml PBS without EPS)

Exopolymer [EPS (VB23)]	Incubation time (min)	Sample OD at A_{540nm}	Emulsifying activity (%)
Nondialysed	0	0·22	100
	30	0·08	36·36
	60	0·02	9·0
Dialysed, lyophilized	0	0·47	100
	30	0·19	40·42
	60	0·16	34·04

EPS, exopolysaccharide; PBS, phosphate-buffered saline.

Emulsification activity

The emulsification activity of the exopolymer is determined by its strength in retaining the emulsion breaks rapidly within an initial incubation of 30 min. The absorbance reading after 30 and 60 min gives a fairly good indication of the stability of the emulsion. The dialysed fraction of the exopolymer produced by *V. harveyi* retained 40·42% and 34·04% of the emulsification activity after 30 min and 60 min respectively. But the nondialysed fraction of the exopolymer produced 36·36% and 9·0% after 30 and 60 min (Table 2). The stability of the emulsion by exopolymer from *V. harveyi* VB23 is comparable with other EPS-producing bacteria as per the bacterial isolates reported earlier (Rosenberg *et al.* 1979).

Table 1 Chemical characterization of exopolymer components (%) isolated from *Vibrio harveyi* strain VB23 in comparison with other bacterial isolates

Strain	Total carbohydrates	Proteins	Uronic acid	Sulfates	Methyl pentoses	References
Vibrio harveyi (VB23)	21·171	18·6	11·462	ND	3·2	Present study
Vibrio sp.	51·9	1·793	14·389	ND	ND	Majumdar *et al.* (1999)
Alteromonas infernus	57·0	4·0	42·0	8·8	ND	Guezennec *et al.* (1998)
Alteromonas macleodi	42·0	4·0	38·0	5·0	ND	Rougeaux *et al.* (1998)
Marinobacter	16·89	43·89	17·58	2·69	ND	Bhaskar (2003)

ND, not detected.

Fourier-transformed infrared spectroscopy

The FTIR spectrum of the purified EPS of *V. harveyi* strain VB23 revealed characteristic functional groups, such as broad stretching hydroxyl group at 3412 cm^{-1} and a weak C-H stretching peak of methyl group at 2925 cm^{-1} (Fig. 2). Further, an asymmetrical stretching peak was noticed at 1636 cm^{-1}, which corresponds to carboxyl groups and a peak at 1546 cm^{-1} could be assigned to amide II (protein; Helm and Naumann 1995). A broad stretching of C-O-C, C-O at 1000–1200 cm^{-1} corresponds to the presence of carbohydrates (Bremer and Geesey 1991). Specifically, the peaks at 1000–1125 cm^{-1} range ascertain the presence of uronic acid, o-acetyl ester linkage bonds. The FTIR spectra of the polymer evidenced the presence of carboxyl groups, which may serve as binding sites for divalent cations. A comparison of functional groups presents that EPS having a higher number of variable functional groups was more complex than the other EPSs reported previously. The presence of acidic sugars in the EPS may be important, considering the heavy metal-binding properties of this polymer. The EPS excreted by *V. harveyi* is highly surface active, which is probably due to an uronic acid-containing polymer. Grobe *et al.* (1995) have analysed the chemical composition of EPS excreted by *Pseudomonas aeruginosa*.

Composition of exopolysaccharides

The carbon source used for growth determines both the quality and quantity of polysaccharide formation (Cerning *et al.* 1994; Meningitsu *et al.* 1994; Nourani *et al.* 1998). Sugar nucleotides play an important role in the EPS synthesis as activated precursors (Fett *et al.* 1995). The sugar composition of the EPS, analysed using gas chromatography (Fig. 3), shows the percentage relative contribution of hexoses (galactose, 10·08%; glucose, 3·6%), deoxyhexoses (rhamnose, 0·7%; fucose, 0·15%) and pentoses (ribose, 0·2%; arabinose, 0·3%; xylose, 0·45%; mannose, 1·56%). In *V. harveyi* strain VB23 galactose and glucose were the most abundant neutral sugars in differential amounts. EPS produced by *Vibrio diabolicus*, in batch cultures in the presence of glucose, produced an innovative EPS possessing high contents of both uronic acids and hexosamines (Raguenes *et al.* 1997), whereas *Vibrio parahaemolyticus* was shown to produce abundant levels of polysaccharide, consisting of four major sugars, viz. glucose, galactose, fucose and *N*-acetylglucosamine (Enos-Berlage and McCarter 2000). Similar biochemical compositions were observed in previous studies of EPS from *Alteromonas* species isolated from hydrothermal vent communities (Rougeaux *et al.* 1996; Raguenes *et al.* 1997, 2003) and adhesive EPS from *Pseudomonas* (Read and Costerton 1987), whereas the EPSs produced by *V. cholerae* TSI-4/R was found to have a composition of *N*-acetyl-D-glucosamine, D-mannose, 6-deoxy-D-galactose and D-galactose at a molar ratio of (7·4 : 10·2 : 2·4 : 3·0; Wai *et al.* 1998). Apparently the monosaccharide composition appears to be variably composed from that of EPS produced by a number of marine bacteria-like *Flavobacterium* sp. and *Pseudoalteromonas* sp. (Mancuso Nichols *et al.* 2004). The presence of different sugar moieties suggests that the exopolymer is a heteropolysachharide. The occurrence of nonsugars (uronic acids, methyl pentoses and sulfate and proteins) indicates the acidic nature of the exopolymer. The heteropolysachharide-containing multiple sugars have been reported in different Gram-negative bacteria, such as *Pseudomonas flourescens* strain III 3, which was found to contain glucose, glucosamine, rhamnose, fucose, arabinose and acetate, the plant pathogen *Pseudomonas andropogonis*, which produces an acidic exopolymer-containing glucose, glucuronic acid, man-

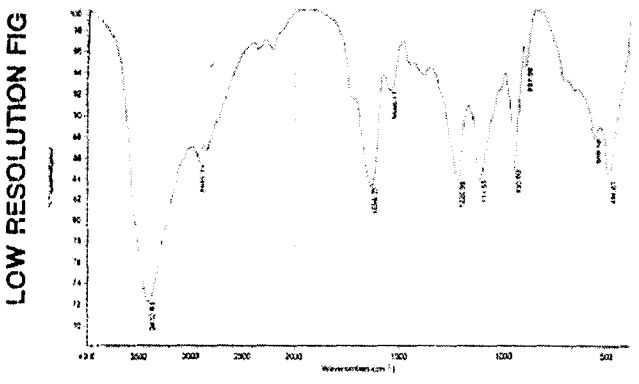


Figure 2 Fourier-transformed infrared (FTIR) spectrum of the exopolysaccharide produced by *Vibrio harveyi* VB23.

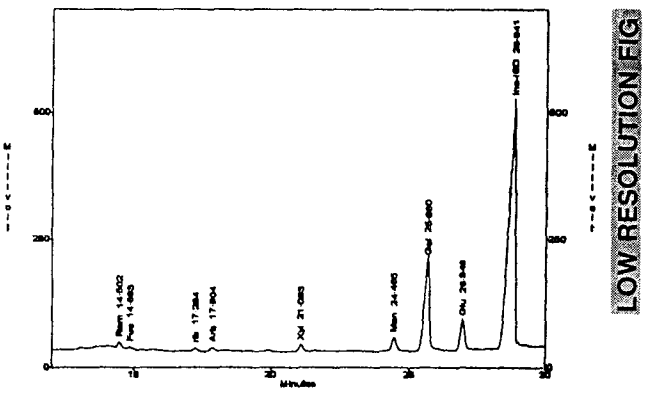


Figure 3 Gas chromatogram of alditol acetate derivative of hydrolysed exopolysaccharide from *Vibrio harveyi* VB23.

nose, rhamnose and galactose (Fishman *et al.* 1997), the exopolymer of *Pseudomonas mendocina* P_2d was found to contain rhamnose, fucose, glucose, ribose, arabinose and mannose.

Upon biochemical examination of the EPS produced by *V. harveyi* strain VB23, several prominent functional groups typical for heteropolysaccharides, such as hydroxyl, carboxylic and amides were found. The EPS also possessed good emulsification activity. High level of EPS production in *V. harveyi* strain VB23 may possibly be involved in biofilm formation which is regulated by quorum-sensing signal molecule. Similar report is available in *P. aeruginosa* where quorum-sensing signals are involved in development of biofilms (Parsek and Greenberg 1999). Implications for the role of these luminescent bacterial polysaccharides in the marine environment requires further characterization using nuclear magnetic resonance (NMR) and mass spectroscopy to elucidate the complete molecular structure of EPS produced by *V. harveyi* strain VB23. In addition to this we intend to study the emulsification activity of EPS using petroleum hydrocarbons, such as crude oil, lubricants, diesel and petrol, beside n-hexadecane. Biotechnological uses for microbially produced EPS include environmental bioremediation of toxic heavy metals, polyaromatic hydrocarbons, biological recovery of crude oils and several other industrial applications, as reviewed by Gutnick and Bach (2000) and Sutherland (1998, 2001). Though biotechnological potential of the biopolymer produced by *V. harveyi* strain VB23 from marine environment of India remains largely untapped. The present study will improve our knowledge on the ecological significance and provide insight into the biotechnological potential of the biopolymer.

Acknowledgements

The authors thank Dr N.B. Bhosle and Dr C.G. Naik, from National Institute of Oceanography, Goa, for making available the gas chromatography, FTIR and their scientific support. This study was supported by the financial assistance from Goa University as Research studentship to P.V. Bramhachari.

References

Abu, G.O., Weiner, R.M., Rice, J. and Colwell, R.R. (1991) Properties of an extracellular adhesive polymer from the marine bacterium, *Shewanella colwelliana*. *Biofouling* 3, 69–84.

Alsina, M. and Blanch, A.R. (1994) A set of keys for the biochemical identification of environmental *Vibrio* species. *J Appl Bacteriol* **76**, 79–85.

Baumann, P.S. and Schubert, R.H.W. (1984) The family II Vibrionaceae Veron. In *Bergey's Manual of Systematic Bacteriology*, Vol. 1 ed. Krieg, N.R. pp. 515–538. Baltimore, MD, USA: Williams and Williams.

Bhaskar, P.V. (2003) Studies on some aspects of marine microbial exopolysaccharides. PhD Thesis (T-281), Goa University, Goa, India.

Bhosle, N.B., Sawant, S.S., Garg, A. and Wagh, A.B. (1995) Isolation and partial chemical analysis of exopolysaccharides from the marine fouling diatom *Navicula subinflata*. *Bot Mar* **38**, 103–110.

Bremer, P.J. and Geesey, G.G. (1991) An evaluation of biofilms development utilizing non-destructive attenuated total reflectance Fourier transform infrared spectroscopy. *Biofouling* **3**, 89–100.

Cerning, J., Renard, C.M.G.C. and Thibault, J.F. (1994) Carbon source requirements for EPS production by *Lactobacillus casei* CG11 and partial structure analysis of the polymer. *Appl Environ Microbiol* **60**, 3914–3919.

Decho, A.W. (1990) Microbial exopolymer secretion in ocean environment, their roles in food webs and marine processes. *Oceanol Mar Biol Ann Rev* **28**, 73–153.

Dische, Z. (1962) Color reactions of hexuronic acids. *Methods Carbohydr Chem* **1**, 497–501.

Dische, Z. and Shettles, L.B. (1948) A specific colour reaction of methyl pentoses and a spectrophotometric micro method for their determination. *J Biol Chem* **175**, 595–603.

Dubois, M., Gilles, K.A., Hamilton, J.K., Rebers, P.A. and Smith, F. (1956) Colorimetric method for determination of sugars and related substances. *Anal Chem* **28**, 350–356.

Enos-Berlage, J.L. and McCarter, L.L. (2000) Relation of capsular polysaccharide production and colonial cell organization to colony morphology in *Vibrio parahaemolyticus*. *J Bacteriol* **182**, 5513–5520.

Fazio, S.A., Uhlinger, D.J., Parker, J.H. and White, D.C. (1982) Estimations of uronic acids as quantitative measures of extracellular and cell wall polysaccharide polymers from environmental samples. *Appl Environ Microbiol* **43**, 1151–1159.

Fett, W.F., Wells, J.M., Cescutti, P. and Wijey, C. (1995) Identification of exopolysaccharide produced by fluorescent Pseudomonads associated with commercial mushroom (*Agaricus bisporus*). *Appl Environ Microbiol* **61**, 513–517.

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

Geesey, G.G., Bremer, P.J., Smith, J.J., Muegger, M. and Jang, L.K. (1992) Two-phase model for describing the interactions between copper ions and exopolymers from *Alteromonas atlantica*. *Can J Microbiol* **38**, 785–793.

Grobe, S., Wingender, J. and Trufper, H.G. (1995) Characterization of mucoid *Pseudomonas aeruginosa* strains isolated from technical water systems. *J Appl Bacteriol* **79**, 94–102.

Guezennec, J., Ortega-Morales, O., Raguenes, G. and Geesey, G. (1998) Bacterial colonization of artificial substrate in the vicinity of deep-sea hydrothermal vents. *FEMS Microbiol Ecol* **26**, 89–99.

Gutnick, D.L. and Bach, H. (2000) Engineering bacterial biopolymers for the biosorption of heavy metals; new products and novel formulations. *Appl Microbiol Biotechnol* **54**, 451–460.

Helm, D. and Naumann, D. (1995) Identification of some bacterial cell components by FTIR spectroscopy. *FEMS Microbiol Lett* **126**, 75–80.

Hoagland, K.D., Rosowski, J.R., Gretz, M.R. and Roemer, S.C. (1993) Diatom extracellular polymeric substances: function, fine structure, chemistry and physiology. *J Phycol* **29**, 537–566.

Keene, L. and Lindberg, B. (1983) Bacterial polysaccharides. In *The Polysaccharides*, Vol. 2 ed. Aspinall, G.O. pp. 287–363. New York, USA: Academic Press.

Lowry, O.H., Rosenbrough, N.J., Farr, A.L. and Randall, R.J. (1951) Protein measurement with the Folin phenol reagent. *J Biol Chem* **193**, 265–275.

Majumdar, I., D'Souza, F. and Bhosle, N.B. (1999) Microbial exopolysaccharides: effect on corrosion and partial chemical characterization. *J Indian Inst Sci* **79**, 539–550.

Mancuso Nichols, C.A., Garon, S., Bowman, J.P., Raguenes, G. and Guezennec, J. (2004) Production of exopolysaccharides by Antarctic marine bacterial isolates. *J Appl Microbiol* **96**, 1057–1066.

Meningitsu, Y., Edwards, C. and Saunders, J.R. (1994) Continuous culture studies on the synthesis of capsular polysaccharides of *Klebsiella pneumoniae* K1. *J Appl Bacteriol* **76**, 424–430.

Mittleman, M.W. and Geesey, G.G. (1985) Copper-binding characteristics of exopolymers from a fresh water-sediment bacterium. *Appl Environ Microbiol* **49**, 846–851.

Muralidharan, J. and Jayachandran, S. (2003) Physicochemical analyses of the exopolysaccharides produced by a marine biofouling bacterium, *Vibrio alginolyticus*. *Process Biochem* **38**, 841–847.

Nielsen, P.H. and Jahn, A. (1999) Extraction of EPS. In *Microbial Extracellular Polymeric Substances: Characterization, Structure and Function* ed. Wingender, J., Neu, T.R. and Flemming, H.C. pp. 50–72. Berlin, Germany: Springer-Verlag.

Nourani, G.L., Blondeau, K. and Simonet, J.M. (1998) Influence extraction and purification of EPS from *Klebsiella pneumoniae*. *J Microbiol Methods* **9**, 211–220.

Parsek, M.R. and Greenberg, E.P. (1999) Quorum sensing signals in development of *Pseudomonas aeruginosa* biofilms. *Methods Enzymol* **310**, 43–55.

Raguenes, G., Christen, R., Guezennec, J., Pignet, P. and Barbier, G. (1997) *Vibrio diabolicus* sp. nov., a new polysaccharide-secreting organism isolated from a deep-sea hydrothermal vent polychaete annelid, *Alvinella pompejana*. *Int J Syst Bacteriol* **47**, 989–995.

Raguenes, G., Cambon-Bonavita, M.A., Lohier, J.F., Boisset, C. and Guezennec, J. (2003) A novel, highly viscous polysaccharide excreted by an *Alteromonas* isolated from a deep-sea hydrothermal vent shrimp. *Curr Microbiol* **46**, 448–452.

Read, R.R. and Costerton, J.W. (1987) Purification and characterization of adhesive exopolysaccharides from *Pseudomonas putida* and *Pseudomonas fluorescens*. *Can J Microbiol* **33**, 1080–1090.

Rodrigues, C. and Bhosle, N.B. (1991) Exopolysaccharide production by *Vibrio fischeri*, a fouling marine bacterium. *Biofouling* 4, 301–308.

Rosenberg, E., Zuckerberg, A., Rubinovitz, C. and Gutnick, D.L. (1979) Emulsifier of *Arthrobacter RAG-1*: isolation and emulsifying properties. *Appl Environ Microbiol* **37**, 402–408.

Rougeaux, H., Pichon, R., Kervarec, N., Raguenes, G.H.C. and Guezennec, J.G. (1996) Novel bacterial exopolysaccharides from deep-sea hydrothermal vents. *Carbohydr Polym* **31**, 237–242.

Rougeaux, H., Talaga, P., Carlson, R.W. and Guezennec, J. (1998) Structural studies of an exopolysaccharide produced by *Alteromonas macleodii* subsp *fijiensis* originating from a deep-sea hydrothermal vent. *Carbohydr Res* **312**, 53–59.

Sutherland, I.W. (1996) Extracellular polysaccharides. In *Biotechnology*, Vol. 6 ed. Rehm, H.J. and Reed, G. pp. 615–657. Weinheim: VCH.

Sutherland, I.W. (1998) Novel and established applications of microbial polysaccharides. *Trends Biotechnol* **16**, 41–46.

Sutherland, I.W. (2001) Biofilm exopolysaccharides: a strong and sticky framework. *Microbiology* **147**, 3–9.

Terho, T.T. and Hartiala, K. (1971) Method for determination of the sulfate content of glycosaminoglycans. *Anal Biochem* **41**, 471–476.

Uhlinger, D.J. and White, D.C. (1983) Relationship between physiological status and formation of extracellular polysaccharide glycocalyx in *Pseudomonas atlantica*. *Appl Environ Microbiol* **45**, 64–70.

Wai, S.N., Mizunoe, Y., Takade, A., Kawabata, S.I. and Yoshida, S.I. (1998) *Vibrio cholera* O1 strain TSI-4 produces the exopolysaccharide materials that determine colony morphology, stress resistance and biofilms formation. *Appl Environ Microbiol* **64**, 3648–3655.

Whitfield, C. (1988) Bacterial extracellular polysaccharides. *Can J Microbiol* 34, 415–420.

Williams, A.G. and Wimpenny, J.W.T. (1977) Exopolysaccharide production by *Pseudomonas* NICB 11264 grown in batch culture. *J Gen Microbiol* **102**, 255–263.

30. Wolfe, K., Wu, X. and Liu, R. H., Antioxidant activity of apple peels. *J. Agric. Food Chem.*, 2003, **51**, 609–614.
31. Burritt, E. A., Malechek, J. C. and Provenza, F. D., Changes in concentration of tannins, total phenolics, crude proteins and *in vitro* digestibility of browse due to mastification and insalivation by cattle. *J. Range Manage.*, 1987, **40**, 409–411.
32. Jia, Z., Tang, M. and Wu, J., The determination of flavonoid content in mulberry and their scavenging effect on superoxide radicals. *Food Chem.*, 1999, **64**, 555–599.
33. Jayajothi, E., Elavarasu, T., Hamsaveni, M. and Sridhar, S. K., Antioxidant activity and total phenolic content of *triphala* churna. *Nat. Prod. Sci.*, 2004, **10**, 16–19.
34. Weiss, J. F. and Landauer, M. R., Protection against ionising radiation by antioxidant nutrients and phytochemicals. *Toxicology*, 2003, **189**, 1–20.
35. Arora, R. *et al.*, Radioprotection by plant products: present status and future prospects (review). *Phytother. Res.*, 2005, **19**, 1–22.

ACKNOWLEDGEMENTS. We thank Dr T. Mukherjee, Associate Director, Chemistry Group, BARC for encouragement and support.

Received 18 May 2005; revised accepted 28 December 2005

Rapid and specific detection of luminous and non-luminous *Vibrio harveyi* isolates by PCR amplification

P. V. Bramha Chari and Santosh Kumar Dubey*

Laboratory of Bacterial Genetics and Environmental Biotechnology, Department of Microbiology, Goa University, Goa 403 206, India

***Vibrio harveyi* is the major causal organism of luminous vibriosis, which causes potential devastation to diverse ranges of marine invertebrates over a wide geographical area. These microorganisms, however, are extremely difficult to identify because they are phenotypically diverse. Biochemical identification techniques involve many tests which may be time-consuming and expensive. The development and sustainability of shrimp aquaculture industry requires a simple, fast and reliable technique for species-specific identification of *V. harveyi* in order to control it effectively. The present communication describes a simple, cost-effective and rapid PCR-based and species-specific detection technique to facilitate early detection and identification of luminous and non-luminous *V. harveyi* isolates. Many of these isolates are also resistant to multiple antibiotics such as ampicillin, chloramphenicol, nalidixic acid, rifampin, polymyxin-B, trimethoprim and penicillin.**

Keywords: Luminous *V. harveyi*, PCR amplification, shrimps, *Vibrio harveyi*.

VIBRIO harveyi, a marine bacterium, is not only ubiquitous in the marine environment but is also considered one of the main bacterial species which is part of normal microflora of healthy shrimps[1]. However, over the past few decades, bacterial strains of this species have been recognized as significant pathogenic agents and a cause of high rates of shrimp mortality in the shrimp culture industry worldwide[2,3]. In various parts across the globe, *V. harveyi* drastically affects the production of *Litopenaeus vannamei*[4], the most extensively cultured penaeid in all the zones. Vibriosis, especially luminous disease has caused serious loss in prawn hatcheries. *V. harveyi* was reported as the causative bacterium of vibriosis in pearl oyster (*Pinctada maxima*), black tiger prawn (*Penaeus monodon*) and kuruma prawn (*Penaeus japonicus*)[5,6]. Larval prawns are particularly susceptible to *V. harveyi*, succumbing to what has been termed as luminescent bacterial disease[6]. This disease has been identified as a major problem in the Philippines, Japan, Southeast Asia and European countries, causing severe losses of juvenile prawns in several hatcheries[7].

Among the common technologies used for diagnosis and detection of *V. harveyi* in shrimp farms are biochemical tests[8,9]. Although these phenotype-based identifications of marine bacteria are useful, they are time-consuming and can generate false-positive results[1]. The development and sustainability of shrimp aquaculture industry urgently requires a simple reliable and fast method for species-specific identification of *V. harveyi* for its adequate control. Likewise several highly powerful molecular techniques, e.g. ELISA, amplified fragment length polymorphism (AFLP) and repetitive extragenic palindromic elements polymerase chain reaction (REP–PCR), FAFLP, and IGSPCR have become readily available for the identification of bacteria, including *Vibrios*[10–14]. Keeping in view these important facts, we have developed a simple, cost-effective reliable and fast PCR-based technique to identify *V. harveyi* isolates. This genomic approach used for the identification and typing of *Vibrio* strains is useful for taxonomic studies, including identification up to the subspecies level. The genus *Vibrio*[15,16] contains a large number of closely related bacterial species with 16 S rRNAs differing in nucleotide sequence from less than 1 up to 6%. Our results have clearly demonstrated the rapid detection of marine luminous and non-luminous *V. harveyi* isolates for molecular epidemiology purpose. This approach may also be applied to the detection of other marine *Vibrio* species involved in aquaculture diseases. These *V. harveyi* isolates were also screened for antibiotic sensitivities[17]. Interestingly, several isolates were found to be resistant to ampicillin, chloramphenicol, nalidixic acid, rifampin, polymyxin-B, trimethoprim, and penicillin; few isolates were sensitive to tetracycline, streptomycin and novobiocin.

Environmental isolates of luminous and non-luminous *V. harveyi* screened were obtained from sea water samples collected from various sampling sites along the west coast of Goa, i.e. Goa Shipyard Limited, Western India Shipping Limited and beaches such as Majorda, Benaulim, Miramar, Donapaula, Anjuna, Tirakol, Colva and Kakra

*For correspondence. (e-mail: dubey_santosh1@rediffmail.com)

(at 15°N lat. and 73°E long.; Figure 1). To enumerate the *Vibrio* sp., 10 ml of sea water was filtered through Whatman no. 1 filter paper and bacteria were subsequently collected on 0.4 µm pore size (Millipore) membrane filters. Bacteria in the membrane were resuspended by vortexing in 2 ml of alkaline peptone water. Thereafter, 100 µl of the bacterial suspension was streaked on thiosulphate citrate bile sucrose agar (selective agar for *Vibrio* sp.) plates containing 2% NaCl. These plates were incubated overnight for 16–18 h at 30°C and the emerging colonies were counted (Figure 2). All the bacterial strains (luminous and non-luminous *V. harveyi* isolates) used in this study have been previously confirmed using the method of Alsina and Blanch's set of biochemical keys for identification of environmental *Vibrio* isolates[9] (data not shown).

V. harveyi strains were cultured in tryptic soy broth supplemented with 2% NaCl at 30°C with continuous shaking until the stationary phase of growth was reached. DNAs were extracted and purified. Bacterial cultures (50 ml) were harvested by centrifugation at 10,000 *g* for 10 min. The resultant pellets were lysed with a 1% sodium dodecyl sulphate–1 mg/ml, proteinase K solution, and the bacterial nucleic acids were extracted by a phenol–chloroform–isoamyl alcohol (25 : 24 : 1, vol/vol/vol) mixture as described by Sambrook *et al*[18]. Extracted DNAs were resuspended in 1X Tris-EDTA buffer and stored at –20°C until required. The DNA sample was subjected to PCR amplification.

PCR was carried out using species-specific primers, VH1 F′ (5′ ACC GAG TTA TCT GAA CCT TC 3′) and VH2 R′ (5′ GCA GCT ATT AAC TAT ACT ACT 3′)[19], which have the ability to cause specific amplification of a 413 bp fragment of the 16S rRNA sequences from a number of *V. harveyi* isolates. The reaction mixture contained 5 µl of 10X polymerase buffer, 3 mM magnesium chloride, 200 µM each dNTP, 1 U per 50 µl *Taq* polymerase, 10 pmol each primer and 50–250 ng template genomic DNA per 50 µl. The thermal cycler was programmed to perform 35 cycles consisting of initial denaturation at 94°C for 2 min, 35 cycles of 94°C for 1 min, 65°C for 1 min, 72°C for 1 min followed by a final extension step of 5 min at 72°C for 2 min. The same primers were used to set up a positive control with *Escherichia coli* DNA and a negative control with sterile double-distilled water. On gel electrophoresis of 10 µl of PCR product through 0.8% agarose gel and staining with ethidium bromide, amplification was visualized, in order to confirm the size of the PCR product. The 50 and 100 bp DNA ladders were used as standard markers, and the agarose gel picture with PCR product was recorded by gel documentation system (Figure 3).

Stock cultures of *V. harveyi* strains were grown on tryptic soy agar supplemented with 2% NaCl at 30°C for 24 h. The bacteria were suspended in sterile PBS buffer (0.9% NaCl, 0.02% KCl, 0.02% KH_2PO_4, 0.115% Na_2HPO_4, 10% glycerol, pH 7.2) and diluted to a turbidity equivalent to optical density of 0.1 at 600 nm. The bacterial suspension (0.1 ml) was spread onto Mueller–Hinton agar and then antibiotic discs were dispensed in the centre of the plate. The plates were incubated at 30°C for 18 h and the inhibition zones of the bacteria were determined (unpublished data). Minimal inhibitory concentrations for ten antibiotics were determined by the agar dilution method on Mueller–Hinton broth[20]. Each antibiotic was initially dissolved in appropriate solvent. Based on these experiments, percentage of resistant and sensitive *V. harveyi* isolates was determined (Table 1).

The species-specific PCR primers VH-1 and VH-2 corresponded to variable regions of *Escherichia coli* 16S sequence (GenBank accession J01859) at bases 59–87 and 453–473. It was consequently found that these primers corresponded to *Vibrios*, as described by Dorsch *et al.*[15]. The PCR amplified product of VH-1 and VH-2 is 413 bp from all the isolates of non-luminous and luminous *V.*

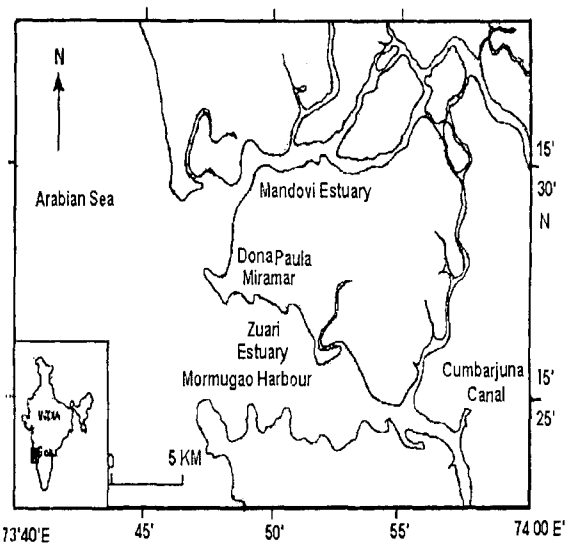


Figure 1. Map showing sampling sites for collection of luminous and non-luminous *Vibrio harveyi* isolates.

Table 1. Antibiotic sensitivity of *Vibrio harveyi* isolates (luminous and non-luminous) from different sites in the coastal regions of Goa

Antibiotic (µg/ml)	No. of isolates tested	Per cent resistant isolates
Ampicillin (75)	34	88.23
Chloramphenicol (50)	34	70.58
Tetracycline (25)	22	36.36
Streptomycin (25)	34	29.41
Nalidixic acid (50)	22	72.72
Rifampin (25)	22	81.82
Novobiocin (50)	22	54.52
Polymyxin-B (75)	22	63.63
Trimethoprim (75)	22	85.29
Penicillin (75)	22	77.27

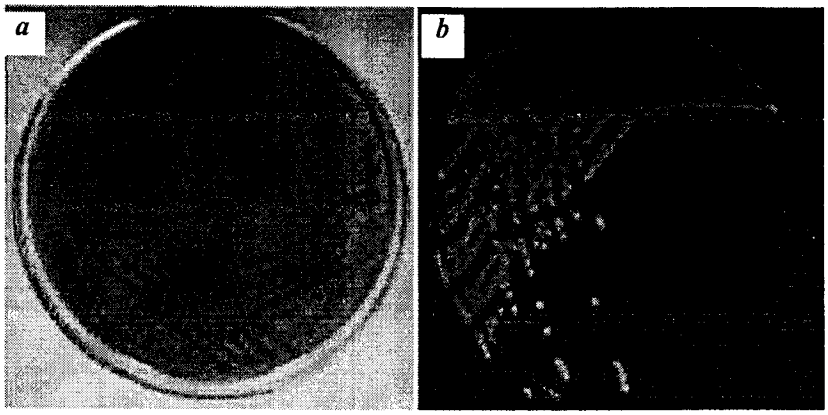


Figure 2. *V. harveyi* colony on TCBS agar (*a*) and glycerol-based marine agar (*b*) plates.

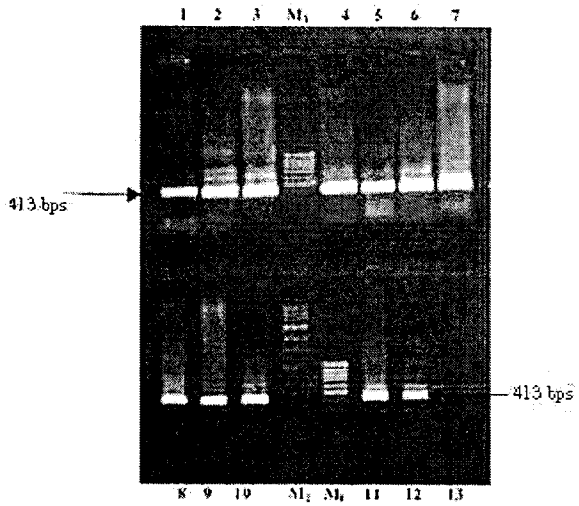


Figure 3. Agarose gel electrophoresis of 16S ribosomal DNA (PCR products) from 12 representative bacterial isolates (*V. harveyi*). Lane M_2, 50 bp and Lane M_1, 100 bp molecular weight markers; lanes 1–12, isolates (1–7, isolates of luminous *V. harveyi* and 8–12, isolates of non-luminous *V. harveyi*); lane 13, Negative control, *Escherichia coli*.

harveyi. This PCR method has been found to reduce the time and resource required for confirmation of identity of *V. harveyi*. The identification of *Vibrios* isolated from the aquaculture environment has been imprecise and labour-intensive, requiring many biochemical and physiological tests[21], or dichotomous keys that takes weeks to perform[9]. With our PCR-based technique, an isolate suspected to be luminous or non-luminous *V. harveyi* could be confirmed in less than 24 h. The positive-reacting cultures would require growth characteristics, VP test, and use of mannose, L-leucine, acetate or propionate as a sole carbon source, to confirm the identity of *V. harveyi* isolates. However, this PCR-based taxonomic identification of *V. harveyi* could easily initiate antibacterial therapy and thus prevent the rapid spread of specific *V. harveyi* in the estuarine and freshwater aquaculture.

The antibiotic sensitivity study revealed that *V. harveyi* developed resistance to many of the antibacterial agents tested. Most of the Gram-negative bacteria acquire resistance to antibacterial agents by changing the permeability of outer membrane porin channels, consequently leading to reduced drug influx into the bacterial cell and antibiotic resistance gene determinants present on the plasmids, transposons and integrons[22]. A large number of *V. harveyi* strains were found to be resistant to ampicillin (88.23%), chloramphenicol (70.58%), nalidixic acid (72.72%), rifampin (81.82%), polymyxin-B (63.63%), trimethoprim (85.27%), and penicillin (77.27%; Table 1). Occurrence of these antibiotic-resistant *V. harveyi* isolates is mainly due to the continuous usage of several broad spectrum antibiotics in the aquaculture sites. From the above results, it can be inferred that the virulence of the luminous and non-luminous *V. harveyi* strains increased slightly due to increased resistance to various antibacterial agents, including antibiotics.

This PCR-based detection technique facilitates the identification of bacterial isolates suspected to be *V. harveyi*, much more rapidly and economically than any other conventional techniques used for detection. This technique is also cost-effective, simple and fast, therefore *V. harveyi* can be controlled effectively and efficiently.

1. Vandenberghe, J. *et al.*, *Appl. Environ. Microbiol.*, 1999, **65**, 2592–2597.
2. Karunasagar, I., Pai, R., Malathi, G. R. and Karunasagar, I., *Aquaculture*, 1994, **128**, 203–209.
3. Alabi, A. O., Jones, D. A. and Latchford, J. W., *Aquaculture*, 1999, **178**, 1–11.
4. Aguirre-Guzmán, G., Vazquez-Juárez, R. and Ascencio, F., *J. Invertebr. Pathol.*, 2001, **78**, 215–219.
5. Pass, D. A., Dybadahl, R. and Mannion, M. M., *Aquaculture*, 1987, **65**, 149–169.
6. Lavilla-Pitogo, C. R., Baticados, M. C. L., Cruz-Larcierda, E. R. and de la Pena, L. D., *Aquaculture*, 1990, **91**, 1–13.

7. Lavilla-Pitogo, C. R., Leano, E. M. and Paner, M. G., *Aquaculture*, 1998, **164**, 337–349.
8. West, P. A. and Colwell R. R., In *Vibrios in the Environment* (ed. Colwell, R. R.), John Wiley, New York, 1984, pp. 285–363.
9. Alsina, M. and Blanch, A. R., *J. Appl. Bacteriol.*, 1994, **76**, 79–85.
10. Robertson, P. A. W., Xu, H. S. and Austin, B., *J. Microbiol. Methods*, 1998, **34**, 31–39.
11. Rademaker, J. L. *et al.*, *Int. J. Syst. Evol. Microbiol.*, 2000, **50**, 665–677.
12. Versalovic, J., Koeuth, T. and Lupski, J. R., *Nucleic Acids Res.*, 1991, **19**, 6823–6831.
13. Thompson, F. L., Hoste, B., Vandemeulebroecke, K. and Swings, J., *Syst. Appl. Microbiol.*, 2001, **24**, 520–538.
14. Gomez-Gil, B., Rodriguez, S. S., Gasca, A. G., Roque, A., Juarez, R. V., Thompson, F. L. and Swings. J., *Microbiology*, 2004, **150**, 1769–1777.
15. Dorsch, M., Lane, D. and Stackebrandt, E., *Int. J. Syst. Bacteriol.*, 1992, **42**, 58–63.
16. Ruimy, R., Breittmayer, V., Elbaze, P., Lafay, B., Bouseemart, O., Gauthier, M. and Christen, R., *Int. J. Syst. Bacteriol.*, 1994, **44**, 416–426.
17. Abraham, T. J., Manely, R., Palaniappan, R. and Dhevendran, K., *J. Aquat. Trop.*, 1997, **12**, 1–8.
18. Sambrook, J., Fritsch, E. F. and Maniatis, T., *Molecular Cloning: A Laboratory Manual*, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY, 1989, 2nd edn.
19. Oakay, H. J., Levy, N., Bourne, D. G., Cullen and Thomas, A., *J. Appl. Microbiol.*, 2003, **95**, 1293–1303.
20. Mohney, L. L., Bell, T. A. and Lightner, D. V., *J. Aquat. Anim. Health*, 1992, **4**, 257–261.
21. Vandenberghe, J., Thompson, F. L., Gomez-Gil, B. and Swings, J., *Aquaculture*, 2003, **219**, 9–20.
22. Dever, L. A. and Dermody, T. S., *Arch. Int. Med.*, 1991, **151**, 886–895.

ACKNOWLEDGEMENTS. P.V.B. thanks Goa University for financial assistance in the form of research studentship.

Received 3 November 2005; revised accepted 28 December 2005

Detection of genetic variability among chrysanthemum radiomutants using RAPD markers

Sudhir Kumar[1], K. V. Prasad[2,*] and M. L. Choudhary[3]

[1]Department of Biotechnology, Bundelkhand University, Jhansi 284 128, India
[2]Division of Floriculture and Landscaping, Indian Agricultural Research Institute, New Delhi 110 012, India
[3]Horticulture Commissioner, Krishi Bhawan, New Delhi 110 001, India

Eleven radiomutants from two chrysanthemum cultivars Ajay and Thai Chen Queen were characterized by RAPD to understand the extent of diversity and relatedness. Out of 40 random primers screened, 21 gave reproducible polymorphic bands. PCR product of radiomutant genome revealed a total of 156 bands, out of which 118 were found to be polymorphic. Cluster analysis of the radiomutants indicated that they fell into three major groups. Yellow and Bright Orange mutants derived from cv. Thai Chen Queen have been placed in a separate group, indicating their high genetic diversity from the rest of the mutants and parents. The study revealed that RAPD molecular markers can be used to assess polymorphism among the radiomutants and can be a useful tool to supplement the distinctness, uniformity and stability analysis for plant variety protection in future.

Keywords: Chrysanthemum, genetic variability, polymorphism, radiomutants, RAPD markers.

CHRYSANTHEMUM is the second largest cut-flower after rose among the ornamental plants traded in the global flower market. It is cultivated both as a cut-flower and as a potted plant (pot mums). The commonly grown chrysanthemums are hexaploid complex with average number of 54 chromosomes[1]. It is propagated vegetatively as it has a strong sporophytic self-incompatibility system as shown by all members of Asteraceae family[2].

Mutation breeding by radiation, an agricultural application of nuclear technology has been widely utilized to upgrade the well-adapted plant varieties by one or two major traits and also develop new varieties with improved agricultural characteristics. Although most cultivated chrysanthemum cultivars are polyploids with high genetic heterogeneity, mutants with altered flower colour, shape, floret size and shape are often recovered. Altered flower colours with chimeric tissue can be easily induced by radiation and can be isolated using *in vitro* tools. Identification and characterization of cultivars is extremely important in horticultural crops in order to protect the plant breeders' rights. Earlier, new varieties were identified based on horticultural and physiological parameters. New cultivars of chrysanthemum are developed from a single progenitor either spontaneously (sports) or by radiation-induced mutation[3]. Since the effect of mutation in ornamentals is clearly visible, selection for changed flower colour, shape, and size is possible in the M1 generation itself because most of the ornamental crops are vegetatively propagated. Novelty visible in any form is of high value in ornamental crops and hence mutation breeding played a key role in the improvement of ornamental crops in general and chrysanthemum in particular.

Williams *et al.*[4] developed DNA fingerprinting using RAPD markers during 1990. Since then, chrysanthemum cultivars and other closely related family members of Asteraceae have been characterized based on RAPD[1,5,6] and DAF analysis in other parts of the world. Genetic variation between two genetically diverse tissues and three chimeral cell types in leaf has also been demonstrated earlier using the RAPD technique[1].

*For correspondence. (e-mail: kvprasad66@rediffmail.com)