Development of Biosensor for Heavy Metal Detection

A Thesis
Submitted in fulfillment of the requirement
for the award of the degree of

Doctor of Philosophy

In

Biotechnology & Environmental Sciences

By

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Contents Page No

Certificate

Certified that the thesis "**Development of Biosensor for Heavy Metal Detection**" which is submitted by Ms. Minakshi Datta, in fulfillment of the requirement for the award of the Degree of Doctor of Philosophy in the Department of Biotechnology & Environmental Sciences, Thapar University, Patiala, is a record of candidate's own independent and original research work carried out by herself under my supervision and guidance. The material embodied in this thesis has not been submitted in part or full to any other University or Institute for the award of any degree.

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Candidate's Declaration

I hereby declare that the work presented in the thesis entitled "Development of Biosensor for Heavy Metal Detection" submitted by me for the award of the degree of *Doctor of Philosophy* in the Department of Biotechnology and Environmental Sciences, Thapar University, Patiala, is true and original record of my own independent and original research work carried out under the supervision of Dr. Dinesh Goyal, Professor, Department of Biotechnology & Environmental Sciences, Thapar University. The matter embodied in this thesis has not been submitted in part or full to any other university or institute for the award of any degree in India or Abroad.

Minakshi

Minakshi Datta

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I would like to take this opportunity to thank many people who have helped and encouraged me through out this study. Without their help, support and encouragement, I would never have been able to accomplish this task. It is a pleasant aspect that I have now the opportunity to express my gratitude to all of them.

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Above all, I'm grateful to **Almighty God** for blessing me to complete this work successfully.

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Synopsis

Introduction

Heavy metal ions constitute a serious environmental problem due to their persistent and non-biodegradable nature. They are toxic to biological systems even at low concentration and there is an obvious need to determine them at trace level (Bontidean *et al.*, 1998). Bioaccumulation of the heavy metals has been reported to be higher in the upper trophic levels at concentrations surpassing those found in water supplies (Krawczyk *et al*., 2000)). The conventional methods used for the determination of the heavy metals based on spectrophotometry, chromatography, mass spectrometry and various hyphenated techniques; require sophisticated and expensive equipments, highly trained staff and is usually time-consuming (Dzyadevych *et al*., 2005; Sherma and Zweig 1983). The total amount of heavy metals detected by such means may not always be related to toxicity of such samples because the original biological availability of the metal ions is not taken into account. Thus need arises for the fast and inexpensive methods for the detection of bioavailable heavy metals. Biosensors are useful analytical devices in this respect. A biosensor is an analytical device, which converts a biological response into an electrical signal. . The two main components of a biosensor are bioreceptor and transducer.

Bioreceptor: It is a biomolecule or biocomponent like enzymes, DNA, metalloproteins or microbes etc. that recognizes the target molecule. Enzymes represent the largest class of bioreceptors, which are mainly proteins that catalyze different chemical reactions in cells. Enzyme reacts to a substrate molecule and produces a reaction that can be measured and is repeatable (i.e., the enzyme is stable). Enzymes/whole cells can be immobilized on to the transducer and their enzymatic activities can be studied electrochemically. These whole cell enzymatic biosensors have the advantage of being more stable as the enzymes are in their natural environment.

Transducer: It is a device for converting the recognition event or the interaction/reaction between analyte and bioreceptor into a measurable signal. Transducers can be subdivided into the following four main types.

- Electrochemical Transducers (Potentiometric, Conductometric, Voltammetric, FET-based sensors)
- Optical Transducers
- Piezo-Electric Devices
- Thermal Sensors

Several biosensor configurations have been described in the past for heavy metal detection. Wide spectrum of biological recognition elements and transducer systems has been used for the fabrication of biosensors (Castillo *et al*., 2004; Amine *et al*., 2006; Bentley *et al*., 2001). Enzymes are the most widely used biological sensing element in the fabrication of biosensors (Turner *et al.*, 1992; Mulchandani *et al*., 1998; Tran, 1993; Mikkelson and Corton, 2004). Although purified enzymes have very high specificity for their substrates or inhibitors, their application in biosensors construction may be limited by the tedious, time-consuming and costly enzyme purification, requirement of multiple enzymes to generate the measurable product or need of cofactor/coenzyme. Microorganisms provide an ideal alternative to these bottle-necks (Arikawa *et al*., 1998). The many enzymes and co-factors that co-exist in the cells give the cells the ability to consume and hence detect large number of chemicals; however, this can compromise the selectivity. They can be easily manipulated and adapted to consume and degrade new substrate under certain cultivating condition. Additionally, the progress in molecular biology/recombinant DNA technologies has opened endless possibilities of tailoring the microorganisms to improve the activity of an existing enzyme or express foreign enzyme/protein in host cell. All of these make microbes excellent biosensing elements. Of the different matrices used for the fabrication of biosensors, conducting polypyrrole (PPy) has attracted attention of various researchers due to its operational compatibility at physiological pH and the ease of conductivity modulation (with the counter ions). The electrical conductivity of polypyrrole can be modulated in the range of 10^{-3} to 10^{3} Ω/cm (Kros *et al.*, 2005; Diaz *et al*., 2005; Kwon *et al*., 1991).Various forms of polypyrroles can be easily prepared by electrochemical techniques and oxidation of pyrrole in presence of desired dopant ions results in a doped film deposited at the surface of the electrode (Zotti *et al*., 1992; Teasdale *et al.*, 1993; Sadik, 1995; Belanger *et al*., 1989). Conducting polymer matrices have been reported to have improved environmental stability, biocompatibility, increased polymerization growth with higher compactness and conductivity when used with large polymeric anions such as *para*-toluene sulfonate (pTS),

polystyrene sulfonate (PSS), polyvinyl sulfonate (PVS) that helps in maintaining the charge neutrality during reduction process (Chaubey *et al*., 2000; Gaikwad *et al*., 2006; Otero *et al*., 1996; Hallik *et al*., 2001). It has been suggested that size of dopant ions induces changes in molecular confirmation resulting in increased electrical conductivity (Kumar *et al*., 2001). Polypyrrole-polyvinyl sulfonate composite membrane has been shown to play important role as a 'charge controllable membrane' in which the fixed charges can be controlled electrochemically by an internal electrode (Shimidzu *et al*., 1988).

The second category of the biorecepotors used for the fabrication of the heavy metal sensors are metalloenzymes/metalloproteins and are potentially most promising because of their specificity for metal binding (McCall *et al*., 2000). Different metalloproteins/peptides have been used for developing heavy metal sensors (Cherian *et al*., 2003; Chow *et al*., 2005). The high selectivity of these metal binding molecules even in complex natural solutions like sea water or blood when combined with a suitable transducer has a great promise as an indicator system that may in the future replace the current techniques of measuring very low concentrations of metal ions (Thompson *et al*., 1996; Kielland, 1937). In the present study bacteria were isolated from soil on the basis of urease and alkaline phosphatase activities. Enzyme activities of the bacterial isolates were monitored in free and immobilized state and effect of three metal ions Cu, Zn and Cd was observed to determine the threshold concentrations of the metal ions. Two transducer systems potentiometric and amperometric were used to assemble the whole cell and electrode system to monitor the enzyme reactions and inhibition by metal ions electrochemically in order to develop a biosensor for heavy metal detection.

A comprehensive study was under taken with the following objectives.

Objectives

1. Screening, isolation and identification of microbes sensitive to Cu, Zn and Cd ions.
2. Metal-microbe interaction studies to determine threshold concentration of metal ions.
3. Immobilization and process optimization for whole cell-electrode assembly and its application for detection of heavy metals.

1. Screening, isolation and identification of microbes sensitive to Cu, Zn and Cd ions.

For isolation of microbes with high Urease and Phosphatase activity soil samples were collected from sites within Thapar University Campus, Patiala. Urease and alkaline phosphatase were chosen as the parameters to monitor the sensitivity of microbes towards Cu, Zn and Cd.

Morphological, biochemical and molecular characterization based on 16S rDNA sequence analysis of the isolates was carried out and the isolates P8 and P10 were identified as *Bacillus* sp. MD028 (FJ005050) and *Bacillus subtilis* strain MD008 (EU780733) respectively. Optimization of pH and temperature for the whole cell urease and phosphatase activity and effect of substrate concentration were studied.

Isolation of alkaline phosphatase and urease producing Bacteria

a) The phosphate-solubilizing bacteria were screened and isolated on Pikovskya media (Pikovskya, 1948). Formation of clear halo zones around the colonies confirmed the solubilization of the insoluble phosphates by the phosphatases released by the bacterial colony. Bacteria with different colony morphology were streaked on separate agar slants and checked for alkaline phosphatase activity using *para*- nitrophenyl phosphate method (Barnes and Morris, 1956). Along with the selected bacterial isolates, *Pseudomonas striata* procured from IARI, New Delhi was also checked for alkaline phosphatase activity and was found to show maximum activity (3 U/mg (Dry cell weight) among all the isolates and was selected for further enzymatic assay.

b) Urease producing bacteria were isolated from the agricultural fields of Thapar University Campus, Patiala (India) using urea enriched Nutrient Agar (Stanier *et al.*, 1987). Qualitatively the isolates were screened for urease activity using Phenol red indicator media. Urease producing isolates were further quantitatively screened for the urease production using the colorimetric estimation by phenol-hypochlorite method (Natarajan, 1995). Among all the different urease producing strains P8 was found to be most efficient in urease production and showed high urease activity (12 U/mg (Dry cell weight).

Identification of bacterial isolates

Partial characterization of these isolates was carried by 16S rDNA procedure using standard protocols (Sambrook *et al*., 1989). The cloned genes were then amplified and sequenced using SP6 and T7 primers, at the DNA sequencing facility, Labindia, Gurgaon (India). Multiple sequence alignment was carried out using BLAST (Altschul *et al.*, 1997) followed by phylogenetic analysis using MEGA 4 (Dudley *et al.*, 2007). The strains were identified as *Bacillus sp.* MD028 (FJ005050) and *Bacillus subtilis* strain MD008 (EU780733).

2. Metal-microbe interaction studies to determine threshold concentration of metal ions.

Threshold concentrations (Linear range and Detection limits) of the three metal ions viz., Cu, Zn and Cd were determined for whole cell enzyme activities (alkaline phosphatase and urease) of *Pseudomonas striata* and *Bacillus sp.* MD028 (FJ005050) respectively. The metal-microbe interaction studies were conducted using lyophilized biomass of *P. striata* and *Bacillus* sp. MD028. The trend observed for whole cell alkaline phosphatase inhibition by heavy metal ions was Zn>> Cd>> Cu. Zinc caused maximum alkaline phosphatase (APL) inhibition of 93.9% at 1.7 mg l^{-1} and the decrease in the APL activity of *P. striata* was linear for zinc concentration in the range of 0.01-1 mg l^{-1} and the limit of detection for the determination of Zn^{2+} ions was found to be 10 µg l^{-1}. Cadmium caused a maximum inhibition of 86.3 % at a concentration of 4 mg l^{-1}. Two linear ranges for cadmium detection were observed from 0.1-2.5 mg l^{-1} and from 2.5-4 mg l^{-1}. The detection limit for cadmium was found to be 100 µg l^{-1}. Copper was least inhibitory of the three metal ions and caused a maximum inhibition of 65.6% at 34 mg l^{-1}. The decrease in the APA activity of *P. striata* was linear for copper concentration in the range of 1-30 mg l^{-1} and the limit of detection for the determination of Cu^{2+} ions was 1 mg l^{-1}.

Bacillus sp. MD028 urease inhibition by heavy metal ions exhibited a different trend of Cu>> Zn>>Cd. Cu caused maximum inhibition of 91.4% at 1.5 mg l^{-1} and inhibition was linear in the Cu concentration range of 0.01-1 mg l^{-1}. Linear detection ranges for Zn and Cd were 0.7-30 mg l^{-1} and 1-30 mg l^{-1} respectively.

3. Immobilization and process optimization for whole cell-electrode assembly and its application for detection of heavy metals.

The microbes were immobilized in two different matrices: polyvinyl chloride (PVC) membranes and electropolymerised films of polypyrrole-polyvinylsulphonate (Ppy-PVS). The work is a novel approach for bacterial cells immobilization in PVC and Ppy-PVS and their use in integration with suitable transducers systems to develop a biosensor. The immobilization matrices (polyvinylchloride and polypyrrole-polysulphonate) were integrated with suitable transducer systems (Digital potentiometer, (Equiptronics EQ602) and Autolab Potentiostat/Galvanostat (Eco Chemie, Netherlands) which recorded the chemical changes of enzyme reactions as electrical signals. Potentiometer was used to record the potential difference

generated across the PVC membrane containing bacterial cells in response to different metal ion concentrations (Potentiometric Biosensor Assembly), whereas the potentistat recorded the current produced due to the enzyme reaction occurring at the electrode surface (Amperometric Biosensor assembly).

Potentiometric Biosensor assembly

This Biosensor is based on the principle of the metal binding capacity of the metalloenzymes/metallopeptides. Although several biosensors have been reported based on the metal ligating property of the metallothioneins in conjugation with several transducer systems but potential of lyophilized bacterial cells containing metalloenzyme as bioreceptor for potentiometric sensor has been studied here to detect different heavy metals.

Pseudomonas striata cell mass was immobilized in polyvinylchloride (PVC), a neutral carrier to prepare zinc selective membranes using different plasticizers viz., Dibutyl- phthalate, NPOE as the plasticizer. Membranes were prepared using 1, 2, 3, 5 and 7% of bacterial biomass, of which the highest response was obtained for 5%. Use of potassium salt of tetrakis(4-chloro-phenyl) borate KTCIPB showed marginal improvement in the sensitivity of the electrode from 22mV to 26.2 mV/decade possibly due to the exclusion of anionic intereferences. Potentiometric response of the electrode was studied for Zn, Cd and Cu in the concentration range of 10^{-7} to 10^{-1} M. The electrode was found to be fairly selective & sensitive for the zinc ions. It exhibited a linearity range of 10^{-1} to 10^{-5} M with near nernstian slope of 26.2 mV per decade.

Response time and effect of pH

The membrane electrode showed a sharp response time of 6-10 sec and detection limit of 5×10^{-5} M at 25±1ºC in the pH optima of 3-5. The interference was found to arise only from few transition metals such as Hg^{+2}, Ag^{+} and Pb^{+2}. For alkali and alkaline earth metal ions and transition metal ions, except for Pb^{2+} ions the $-\log K^{pot}_{Ag+,B}$ values were in the range of 3, 2. Also the membrane electrode did not show any serious interference from Cu^{2+} ions. It is important to note that the selectivity coefficients for the Zn^{2+} electrode, with reference to most of the alkali and alkaline-earth metal ions, are quite small. The zinc response is seriously interfered with by small amounts of Hg^{2+} and Ag^{+} ions; so these two ions must be removed before the analysis of zinc from the samples.

Amperometric Biosensor assembly:

Urease positive bacterial isolate from soil was immobilized onto polypyrrole-polyvinyl sulphonate films fabricated onto indium-tin-oxide (ITO) coated glass plates for the detection of urea. The urea biosensor fabricated by immobilizing urease producing bacterial cell *Bacillus sp. MD028 (accession FJ005050)* isolated from soil and glutamate dehydrogenase (GLDH) on PPy-PVS composite films. Surface characterization of Ppy-PVS films was done using Scanning electron microscopy (SEM). The amperometric response of bioelectrode towards urea in the absence and presence of heavy metals was studied using square wave voltammetry. These biosensing electrodes have a response time of 210 s. The electrode response is found to be linear in the range of 1.5-18 mM urea concentration range. The value of Michaelis Menten constant Km was estimated using Lineweaver-Burke plot and found to be 9.35 mM. This bioelectrode retains 95% of enzyme activity after 6 months at 4^{o}C.

Simultaneously the above bioelectrode was used for the estimation of heavy metals. The linear range obtained for was similar to that obtained by bioassay except for Cu and Zn which exhibited slightly lower detection limit of 8 μgl^{-1} and 0.7 mgl^{-1}, respectively.

Salient Findings:

1. Two urease producing strains were isolated from soil and identified as *Bacillus* sp. MD028 (FJ005050) and *Bacillus subtilis* strain MD008 (EU780733) using 16S rDNA sequencing. *Bacillus* sp. MD028 (FJ005050) showed good urease activity (12 U/mg (Dry cell weight)) as compared to *Bacillus subtilis* strain MD008 (EU780733) [9 U/mg (Dry cell weight)].

2. Among the various bacterial isolates screened for phosphatase activity, *Pseudomonas striata* procured from IARI, New Delhi showed the maximum alkaline phosphatase activity (3 U/mg (Dry Cell weight)).

3. The effect of three heavy metals viz., Cu, Cd and Zn on the alkaline phosphatase activity of free and immobilized *P. striata* cells showed an inhibition trend of Zn>Cd>Cu. The inhibitory response of heavy metals was linear in the concentration range of 0.01-1 mgl^{-1} for Zn, 0.1-4 mgl^{-1} for Cd and 1-30 mgl^{-1} for Cu.

4. The effect of three heavy metals viz., Cu, Cd and Zn on the urease activity of free and immobilized *Bacillus* sp. MD028 (FJ005050) cells showed an inhibition trend of Cu>Zn>Cd. The inhibitory response of the heavy metals was linear in the concentration range of 0.01-1 mgl^{-1} for Cu, 1-30 mgl^{-1} for Cd and 1-30 mgl^{-1} for Zn.

5. This work presented a novel and efficient strategy for immobilization of lyophilized cell mass of *P. striata* in poly vinyl chloride matrix which upon immobilization retained more than 85 % of the alkaline phosphatase activity which indicated that the protein structure was not damaged after immobilization. An equally efficient and novel strategy of immobilization was used for the immobilization of lyophilized biomass of *Bacillus* sp. MD028 (FJ005050) by covalently attaching the bacterial cells on the conducting polymer matrix of PPy-PVS/ITO electrodes. The *Bacillus* sp. MD028 cells retained approximately 98 % of the urease activity after immobilization. Both these immobilization techniques are novel and are being reported for the first time for the immobilization of bacterial cells.

6. The potentiometric bioelectrode fabricated by immobilizing *Pseudomonas striata* cells in PVC was found to be fairly selective and sensitive for the Zn ions. The present work is the first report on the use of bacterial cell as metal ligands in a sensing device. The

potentiometric response generated by the zinc selective electrode can be attributed to the selective binding of the zinc ions to the zinc ligating sites present in the bacterial biomass immobilized in the PVC membrane. This zinc bioelectrode exhibited a linearity range of 10^{-1} to 10^{-5} M for zinc with near nernstian slope of 26.2 mV per decade. The membrane electrode showed a sharp response time of 6-10 sec and detection limit of 5×10^{-5} M at 25±1ºC in the pH optima of 3-5. The interference was found to arise only from few transition metals such as Hg^{+2}, Ag^{+} and Pb^{+2}. For alkali and alkaline earth metal ions, the $-\log K^{pot}_{Ag+,B}$ values are quite small. Also the membrane electrode did not show any serious interference from Cu^{2+} ions.

7. The urease bioelectrode fabricated by immobilizing bacterial cell mass on PPy-PVS matrix supported on indium tin oxide coated glass had a response time of 210 s and showed linear response towards urea in the range of 1.5-18 mM. The urease bioelectrode was used for the detection of heavy metals ions and showed linearity in the concentration range of 0.008-1 mgl^{-1} for Cu and 1-30 ppm for Cd and 0.7-30 mgl^{-1} for Zn. This bioelectrode retained 95% of enzyme activity after 6 months at 4ºC.
8. Results of the urease bioassay and urease bioelectrode were in good agreement with each other implicating the efficiency of the covalent immobilization which did not cause any interference to the permeability of substrate and metal ions. Therefore the extent of whole cell urease inhibition by heavy metal ions was same for urease bioassay and urease bioelectrode.

List of Research Publications and Conferences attended / Abstracts published

Research Papers

1. Minakshi Datta, Dinesh Goyal, Ravinder Agarwal, Sushil Mittal "*Role of biosensors as analytical tools*" in the "*National Conference on Sensors*" NCS-2005 at Thapar University, Patiala (India) 25-26 November, 2005, pp. 97-102

2. Minakshi Datta, Susheel Mittal, Dinesh Goyal "Potentiometric Zn^{2+} Biosensor Based On Bacterial Cells (2009) *Asian Journal of Biotechnology*, 1(2):67-73 (ISSN: 1996-0700)

3. Minakshi Datta, Susheel Mittal, Dinesh Goyal Sensor based on polyvinyl chloride immobilized *Pseudomonas striata* cells as metal-ionophore (2009) *Indian Journal of Science and Technology*, 2(7): 46-50 (ISSN: 0974-6846)

Conferences Presentation

1. Presented a paper entitled "*Role of biosensors as analytical tools*" by Minakshi Datta, Dinesh Goyal, Ravinder Agarwal, Sushil Mittal in the "*National Conference on Sensors*" NCS-2005 on 25-26 November 2005 at Thapar University, Patiala (India)
2. Presented a poster entitled "Kinetics of alkaline phosphatase produced by *Pseudomonas striata* and effect of heavy metals" by Minakshi Datta and Dinesh Goyal in 47th AMI National annual conference held at Barkatullah University, Bhopal during December 6-8, 2006.
3. Presented a poster entitled "*Potentiometric Response of PVC immobilized bacterial cells towards different heavy metals"* Minakshi Datta, Susheel K. Mittal and Dinesh Goyal in 48th AMI National annual conference held at IIT Madras, Chennai during December 18-21, 2007.

List of Tables

List of Figures

Chapter 1
Introduction

Heavy metal ions constitute a serious environmental problem due to their persistent and non-biodegradable nature. Heavy metals ions are toxic to biological systems even at low concentrations and there is an obvious need to determine them at trace level (Bontidean *et al.*, 1998). Heavy metals tend to accumulate in the upper trophic levels at concentrations surpassing those found in water supplies (Krawczyk *et al*., 2000). The conventional methods used for the determination of the heavy metals based on spectrophotometry, chromatography, mass spectrometry and various hyphenated techniques; require sophisticated and expensive equipments, highly trained staff and are usually time-consuming (Dzyadevych *et al*., 2005; Sherma and Zweig 1983). The total amount of heavy metals detected by such means may not always be related to toxicity of such samples because the original biological availability of the metal ions is not taken into account. Thus need arises for the fast and inexpensive methods for the detection of bioavailable heavy metals. Biosensors are useful analytical devices in this respect. A biosensor is an analytical device, which converts a biological response into a recordable signal. The two main components of a biosensor are bioreceptor and transducer.

Bioreceptor: It is a biomolecule or biocomponent like enzymes, DNA, metalloproteins or microbes etc. that recognizes the target molecule. Enzymes represent the largest class of bioreceptors, which are mainly proteins that catalyze different chemical reactions in cells. Enzymes react only to a specific substrate molecule and produce a reaction that can be measured and is repeatable (i.e., the enzyme is stable). Enzymes/whole cells can be immobilized on to the transducer and their enzymatic activities can be studied electrochemically. These whole cell enzymatic biosensors have the advantage of being more stable as the enzymes are in their natural environment.

Transducer: It a device for converting the recognition event or the interaction/reaction between analyte and bioreceptor into a measurable signal. Transducers can be subdivided into the following four main types.

- Electrochemical Transducers (Potentiometric, Conductometric, Voltammetric, FET-based sensors)

- Optical Transducers
- Piezo-Electric Devices
- Thermal Sensors

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The aim of the present work was to develop whole cell bacterial biosensors for heavy metal detection. Soil bacterial isolates were screened for urease and alkaline phosphatase activity which was used as parameter to monitor the sensitivity of the isolates towards three metal ions Cu^{2+}, Cd^{2+} and Zn^{2+}. The isolated microbes were identified using molecular techniques. Further, the bacterial isolates were characterized based on physiological and biochemical techniques and studied for their efficacy to produce urease and alkaline phosphatase. The metal-microbes interaction studies were conducted at optimized conditions to

determine the linear range and detection limits of zinc, cadmium and copper based on the inhibition bioassays performed. Finally the isolates were immobilized in suitable matrices and integrated with potentiometric and amperometric transducers systems to monitor the metal-microbe interactions. Sensing mechanism of biosensor assemblies was based on the bioassays of enzyme inhibition and metal chelation. The following objectives were framed for the present study:

Approach adopted in present study

1. Two enzymes viz., Urease and Phosphatase were chosen as the parameters to monitor the sensitivity of microbes towards Cu, Zn and Cd. Thus urease and alkaline phosphatase producing bacteria were isolated from soil. Morphological, biochemical and molecular characterization based on 16S rDNA sequence analysis of the isolates was carried out and the isolates P8 and P10 were identified as *Bacillus* sp. MD028 (FJ005050) and *Bacillus subtilis* strain MD008 (EU780733) respectively. Optimization of pH and temperature for the whole cell urease and phosphatase activity and effect of substrate concentration were studied.

2. Threshold concentrations (Linear range and Detection limits) of the three metal ions viz., Cu, Zn and Cd were determined for whole cell enzyme activities (alkaline phosphatase and urease) of *Pseudomonas striata* and *Bacillus sp.* MD028 (FJ005050) respectively. The metal-microbe interaction studies were conducted using lyophilized biomass of *P. striata* and *Bacillus* sp. MD028.

3. The immobilization matrices (polyvinylchloride and polypyrrole-polysulphonate) containing the microbes were integrated with suitable transducer systems (Digital potentiometer, (Equiptronics EQ602) and Autolab Potentiostat/Galvanostat (Eco Chemie, Netherlands) which recorded the chemical changes of enzyme reactions as electrical signals. Potentiometer was used to record the potential difference generated across the PVC membrane containing bacterial cells in response to different metal ion concentrations (Potentiometric Biosensor Assembly), whereas the potentiostat recorded the current produced due to the enzyme reaction occurring at the electrode surface (Amperometric Biosensor assembly).

Chapter 2
Review of Literature

2.1 Heavy Metals

Many definitions of heavy metals have been proposed—some based on density, some on atomic number or atomic weight, and some on chemical properties or toxicity (Duffus, 2002). From chemical point of view, the term heavy metal is strictly ascribed to transition metals with atomic mass over 20 and specific gravity above 5 (Rascio and Navari-Izzo, 2011). It mainly includes the transition metals, some metalloids, lanthanides, and actinides. Unfortunately, a more in-depth consideration reveals a huge amount of problems with this simple definition. This definition is meant to suggest that the density of a heavy metal is high, but this physical property is quite meaningless in the context of plants and other living organisms. Plants do not deal with metals in their elemental (valence state of 0) forms; they are not accessible to plants. Metals are only available to them in solution, and it is necessary for metals to react with other elements and form compounds before they can be solubilised. Once such a chemical compound is formed (e.g. a salt), the density of the metal does not play any role. The correlation between the density of a metal and its physiological or toxicological effects and even the chemical properties of its compounds are not known till date (Appenroth, 2010). In fact the term *heavy metal* has been called a "misinterpretation" in an IUPAC technical report due to the contradictory definitions and its lack of a "coherent scientific basis". There is an alternative term *toxic metal*, for which no consensus of exact definition exists either. Depending on context, heavy metal can include elements lighter than carbon and can exclude some of the heaviest metals. Heavy metals occur naturally in the ecosystem with large variations in concentration.

There are 35 metals that concern us because of occupational or residential exposure; 23 of these are the heavy elements or "heavy metals": antimony, arsenic, bismuth, cadmium, cerium, chromium, cobalt, copper, gallium, gold, iron, lead, manganese, mercury, nickel, platinum, silver, tellurium, thallium, tin, uranium, vanadium, and zinc (Glanze 1996). Some of these heavy metals, such as Co, Cu, Fe, Mn, Mo, Ni and Zn, are essential

elements required for normal growth and metabolism of plants. These elements can easily lead to poisoning when their concentration rises to supra-optimal values (Rascio and Navari-Izzo, 2011). Others, such as As, Cd, Hg, Pb or Se, are not essential, since they do not perform any known physiological functions. For some heavy metals, toxic levels can be just above the background concentrations naturally found in nature. Therefore, it is important for us to inform ourselves about the heavy metals and to take protective measures against excessive exposure. If unrecognized or inappropriately treated, toxicity can result in significant illness and reduced quality of life (Ferner 2001). Table 1 illustrates the toxic levels and permissible limits for the most hazardous and common heavy metals given in the WHO Guidelines for drinking water quality, 2011. Heavy metals become toxic when they are not metabolized by the body and accumulate in the soft tissues. Heavy metals may enter the human body through food, water, air, or absorption through the skin when they come in contact with humans in agriculture and in manufacturing, pharmaceutical, industrial, or residential settings. Industrial exposure accounts for a common route of exposure for adults. Ingestion is the most common route of exposure in children (Roberts, 1999). Children may develop toxic levels from the normal hand-to-mouth activity of small children who come in contact with contaminated soil or by actually eating objects that are not food (dirt or paint chips) (Dupler, 2001). Less common routes of exposure are during a radiological procedure, from inappropriate dosing or monitoring during intravenous nutrition, from a broken thermometer (Smith *et al*., 1997). Heavy metal toxicity can result in damaged or reduced mental and central nervous function, lower energy levels, and damage to blood composition, lungs, kidneys, liver, and other vital organs. Long-term exposure may result in slowly progressing physical, muscular, and neurological degenerative processes that mimic alzheimer's disease, parkinson's disease, muscular dystrophy, and multiple sclerosis. Allergies are not uncommon and repeated long-term contact with some metals or their compounds may even cause cancer (International Occupational Safety and Health Information Centre, 1999).

2.2 Sources of heavy metal contamination

Heavy metals are introduced into the environment either by natural means or by human activities viz.,

- Natural
- Anthropogenic

2.2.1 Natural Sources

Natural sources: In nature excessive levels of trace metals may occur by geographical phenomena like volcanic eruptions, weathering of rocks (Acid rock drainage) and leaching into rivers, lakes and oceans due to action of winds.

2.2.2 Anthropogenic Sources

In modern times, anthropogenic sources of heavy metals, i.e. pollution, have been introduced to the ecosystem. People have always been exposed to heavy metals in the environment. Metals leaching from eating utensils and cookware lead to metallic contamination of food and water. Metallic constituents of pesticides and therapeutic agents are additional sources of hazardous exposure. The burning of fossil fuels containing heavy metals, the addition of tetra-ethyl lead to gasoline, and the increase in industrial applications of metals, such as metal plating factories, mining industries, tanning, dye and chemical manufacturing industries, etc., have made heavy metal poisoning a major source of environmental pollution (Klaassen 1996). Lead, chromium, cadmium, copper, zinc and mercury are among the most frequently observed metal contaminants (Barondeau *et al*. 2002; Liu & Lu 2003). Waste-derived fuels are especially prone to contain heavy metals, so heavy metals are a concern in consideration of waste as fuel. With the industrial revolution excessive mining activities and processing of metal ores have led to heavy metal contamination in the environment.

2.3 Toxicity Mechanism of Heavy Metals

Heavy metal toxicity may result from alterations of numerous physiological processes caused at cellular/molecular level by inactivating enzymes, blocking functional groups of metabolically important molecules, displacing or substituting for essential elements and disrupting membrane integrity. A rather common consequence of heavy metal poisoning is

the enhanced production of reactive oxygen species (ROS) due to interference with electron transport activities, especially that of chloroplast membranes (Pagliano, *et al*., 2006, Rocca *et al*., 2009). This increase in ROS exposes cells to oxidative stress leading to lipid peroxidation, biological macromolecule deterioration, membrane dismantling, ion leakage, and DNA-strand cleavage (Quartacci *et al*., 2001, Navari-Izzo *et al*., 1998, Navari-Izzo *et al*., 1999).

2.3.1 Formation of Reactive Oxygen Species

The bleaching effects of many heavy metals in light have been known for a long time and are connected with the formation of reactive oxygen species (ROS) (Asada, 1999) and methylglyoxal (MG) (Hossain *et al*., 2009). Fig 1 demonstrates the possible biochemical and molecular mechanisms of heavy metal-mediated ROS induction and damage to the development of higher plants (Hossain *et al*., 2012). Heavy metals are known to disturb redox homeostasis by stimulating the formation of free radicals and reactive oxygen species (ROS) such as singlet oxygen ($^1O_2$), superoxide radicals ($O_2^{\bullet -}$), hydrogen peroxide (H_2O_2), and hydroxyl radicals ($^{\bullet}OH$) (Hossain *et al*., 2010). Recently, methylglyoxal (MG), a cytotoxic compound, was also found to increase in response to various stresses including HMs (Hossain *et al*., 2009). The increase in ROS and MG cause the following physiological damages in the cells (Sharma and Agarwal, 2005):

- They directly disturb electron transport, causing electrons to be transferred to oxygen instead of the natural electron acceptors in chloroplasts and mitochondria
- Disturbances to metabolic reactions feedback to electron transport
- Redox-active metals in different oxidation states under physiological conditions can participate in the Fenton and Haber–Weiss reaction (c.f. Shaw *et al*. 2004), producing hydroxyl radicals
- Inactivation and down regulation of enzymes of the antioxidant defence system
- Depletion of antioxidant substrates.

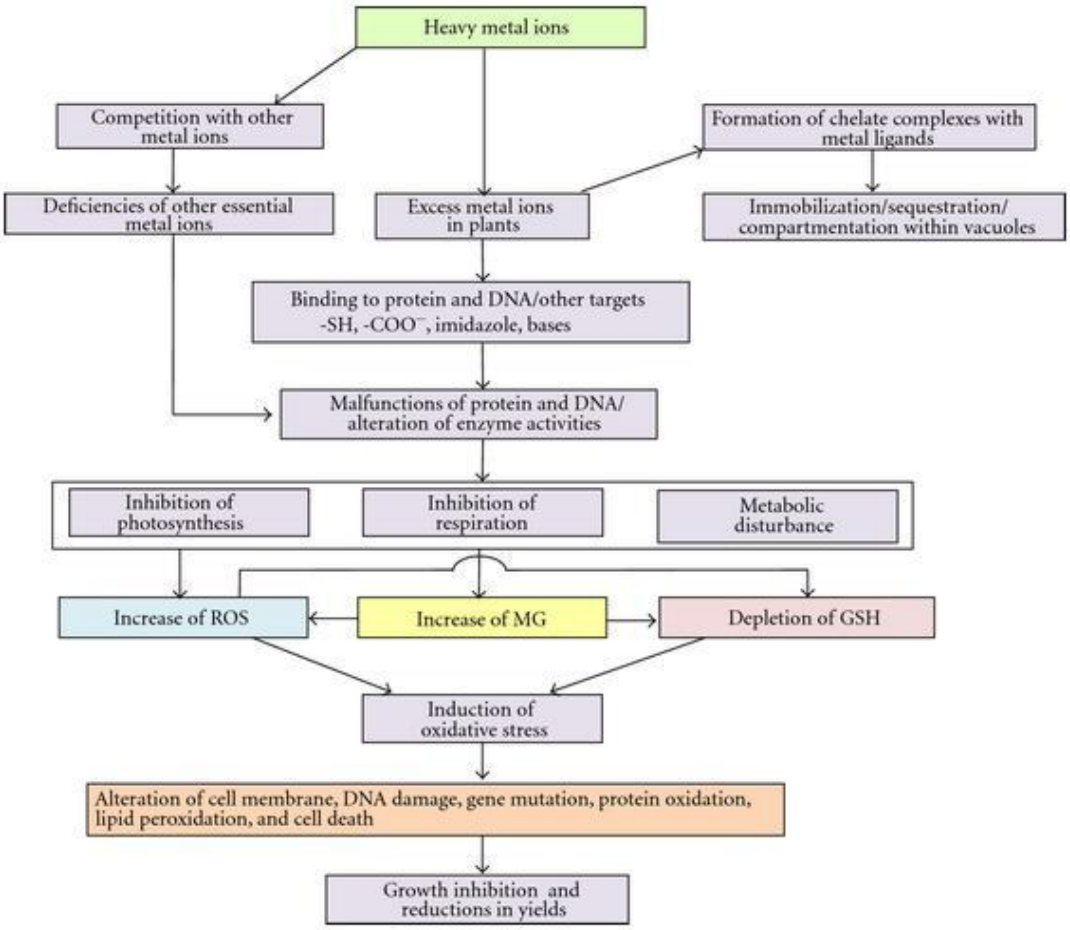

Fig 1 Possible biochemical and molecular mechanisms of heavy metal-mediated ROS induction and damage to the development of higher plants (Hossain *et al*., 2012).

Table 1 Toxic levels and permissible limits for the most hazardous and common heavy metals (WHO Guidelines for Drinking water quality, 2011)

Trace Metal	Guideline value	Occurrence	Tolerable daily intake (TDI))	Limit of detection	Treatment performance	Assessment date	Principal reference
Antimony	0.02 mg/l (20 µg/l)	Concentrations in groundwater less than 0.001 µg/l; concentrations in surface water less than 0.2 µg/l; concentrations in drinking water appear to be less than 5 µg/l	6 µg/kg body weight	0.01 µg/l by electrothermal atomic absorption spectrometry (AAS); 0.1–1 µg/l by inductively coupled plasma mass spectrometry (ICP-MS); 0.8 µg/l by graphite furnace AAS; 5 µg/l by hydride generation AAS	Conventional treatment processes do not remove antimony. However, antimony is not normally a raw water contaminant. As the most common source of antimony in drinking-water appears to be dissolution from metal plumbing and fittings, control of antimony from such sources would be by product control.	2003	WHO (2003) *Antimony in drinking-water*
Arsenic	0.01 mg/l (10 µg/l)	Levels in natural waters generally range between 1 and 2 µg/l, Although concentrations may be elevated (up to 12 mg/l) in areas containing natural sources		0.1 µg/l by ICP-MS; 2 µg/l by hydride generation AAS or flame AAS	It is technically feasible to achieve arsenic concentrations of 5 µg/l or lower using any of several possible treatment methods. However, this requires careful process optimization and control, and a more reasonable expectation is that 10 µg/l should be achievable by conventional treatment (e.g. coagulation).	2011	FAO/WHO (2011) *Evaluation of certain contaminants in food* IPCS (2001) *Arsenic and arsenic compounds* USNRC (2001) *Arsenic in drinking water, 2001 update* WHO (2011) *Arsenic in drinking-water*
Cadmium	0.003 mg/l (3 µg/l)	Levels in drinking-water usually less than 1 µg/l		0.01 µg/l by ICP-MS; 2 µg/l by flame AAS	0.002 mg/l should be achievable using coagulation or precipitation softening	2011	FAO/WHO (2011) *Evaluation of certain food additives and contaminants*

Chromium	0.05 mg/l (50 µg/l)	Total chromium concentrations in drinking-water usually less than 2 µg/l, although concentrations as high as 120 µg/l have been reported		0.05–0.2 µg/l for total chromium by AAS	0.015 mg/l should be achievable using coagulation	1993	WHO (2003) *Chromium in drinking-water*
Copper	2 mg/l (2000 µg/l)	Concentrations in drinking-water range from ≤ 0.005 to > 30 mg/l, primarily as a result of the corrosion of interior copper plumbing		0.02–0.1 µg/l by ICPMS; 0.3 µg/l by ICP–optical emission spectroscopy; 0.5 µg/l by AAS	Copper is not removed by conventional treatment processes. However, copper is not normally a raw water contaminant.	2003	IPCS (1998) *Copper* WHO (2003) *Copper in drinking-water*
Lead	0.01mg/l (10 µg/l) The guideline value is provisional on the basis of treatment performance	Concentrations in drinking-water are generally below 5µg/l, although much higher concentrations (above 100 µg/l) have been measured where lead fittings are present. The primary source of lead is from service connections and plumbing in buildings; therefore, lead should be measured at the tap. Lead concentrations can also vary according to the period in which the water has been in contact with the lead-containing materials.		1 µg/l by AAS; practical quantification limit in the region of 1–10 µg/l	Not a raw water contaminant; treatment not applicable	2011	FAO/WHO (2011) *Evaluation of certain food additives and contaminants* WHO (2011) *Lead in drinking-water*
Mercury	0.006 mg/l (6 µg/l) for inorganic mercury	Mercury is present in the inorganic form in surface water and groundwater at concentrations usually below 0.5 µg/l, although local mineral deposits may produce higher levels in groundwater	2 µg/kg body weight for inorganic mercury	0.05 µg/l by cold vapour AAS; 0.6 µg/l by ICP; 5 µg/l by flame AAS	It should be possible to achieve a concentration below 1 µg/l by treatment of raw waters that are not grossly contaminated with mercury using methods that include coagulation /sedimentation/filtration, PAC and ion exchange.	2004	IPCS (2003) *Elemental mercury and inorganic mercury compounds* WHO (2005) *Mercury in drinking-water*

Nickel	0.07 mg/l (70 µg/l)	Concentration in drinking-water normally less than 0.02 mg/l, although nickel released from taps and fittings may contribute up to 1 mg/l; in special cases of release from natural or industrial nickel deposits in the ground, concentrations in drinking-water may be higher	12 µg/kg body weight	0.1 µg/l by ICP-MS; 0.5 µg/l by flame AAS; 10 µg/l by ICP-AES	20 µg/l should be achievable by conventional treatment (e.g. coagulation). Where naturally occurring nickel is mobilized in groundwater, removal is by ion exchange or adsorption. Where nickel leaches from alloys in contact with drinking-water or from chromium- or nickel-plated taps, control is by appropriate control of materials in contact with the drinking water and flushing taps before using the water.	2004	WHO (2005) *Nickel in drinking-water*
Selenium	0.04 mg/l (40 µg/l)	Most drinking-water contains concentrations of selenium that are much lower than 10 µg/l, except in certain seleniferous areas		0.5 µg/l by hydride generation AAS	Selenium is not removed by conventional treatment processes; significant removals of selenium from water using activated alumina adsorption, ion exchange, reverse osmosis and nanofiltration have been reported.	2010	FAO/WHO (2004) *Vitamin and mineral requirements in human nutrition* WHO (2011) *Selenium in drinking-water*
Zinc	-	In natural surface waters, the concentration of zinc is usually below 10 µg/litre. In groundwaters, 10–40 µg/litre. In tapwater, the zinc concentration can be much higher as a result of the leaching of zinc from piping and fittings	1mg/kg	50 µg/litre using AAS(air–acetylene flame method). Low concentrations can be measured by chelating zinc with ammonium pyrrolidine dithiocarbamate and extracting it with methyl isobutyl ketone (detection limit 0.5–1 µg/litre) (*5*).	Not a raw water contaminant; treatment not applicable	2003	WHO (2003) Zinc in drinking-water

2.4 Determination of Heavy metal

Monitoring of the heavy metals is vital due to the potential health and ecological hazard they present. Laboratory techniques routinely used for metal ion analysis, such as atomic absorption spectrometry (Bannon *et al*., 1994; Parsons and Slavin, 1993; Tahan *et al*., 1994), inductively coupled plasma mass spectrometry (Aggarwal *et al*., 1994; Bowins and McNutt, 1994; Liu *et al*., 1999), anodic stripping voltammetry (Feldman *et al*., 1994; Jagner *et al*., 1994), X-ray fluorescence spectrometry (Blank and Eksperiandova, 1998; Ellis *et al*., 1998; Toeroek *et al*., 1998) and microprobes (Carpenter and Taylor, 1991; Gordon *et al*., 1990; Rindby, 1993; Rivers *et al*., 1992; Sutton *et al*., 1995, 1994; Thompson *et al*., 1988; Wu *et al*., 1990) require sophisticated equipment, sample pretreatment, or skilled operators. Most techniques can detect the total amount of metal ions. However, several studies have established that only certain oxidation states of water-soluble or bioavailable metal ions pose the most risk to human health and the environment. For example, Cr(III) is an essential nutrient required in insulin action and sugar and fat metabolism, while Cr(VI) is believed to be highly toxic and carcinogenic (McCullough *et al*., 1999). Therefore, simple, rapid, inexpensive, selective, and sensitive methods that permit real-time detection of bioavailable metal ions in their different oxidation states are very important in the assessment of concentration, speciation, and stability of these metal ions (Razek *et al*., 1999), In addition, due to the dangers that certain toxic metal ions may pose to operators, remote sensing devices are desirable (Arnold, 1989, 1990, 1992).

Consequently, with the comparable sensitivity and selectivity, the electrochemical methods such as ion-selective electrodes, biosensors, polarography, and other voltammetric techniques are also extensively used as attractive choice to the classical methods, due to their less complex instrumentation and shorter measuring period (Han *et al*., 2001). Also, simple, inexpensive, and portable instruments are attractive and desirable for real-time sampling/measuring and online and continuous analysis/monitoring/control of natural samples (Thompson *et al*., 1998).

2.5 Biosensors- the promising tools

Improvement of “life quality” is one of the most important objectives of global research efforts. Naturally, the quality of life is closely linked to the control of diseases, food quality

and safety, and quality of our environment. In all these fields, a continuous, fast, and sensitive monitoring is required, to control key parameters. Biosensors, combining a biological recognition element and a suitable transducer, represent very promising tools in this context (Castillo *et al*., 2004). Because of their exceptional performance capabilities, which include high specificity and sensitivity, rapid response, low cost, relatively compact size and user-friendly operation, biosensors have become an important tool for detection of chemical and biological components for clinical, food and environmental monitoring (Amine *et al*., 2006).

2.5.1 Definition of Biosensor

According to the International Union of Pure and Applied Chemistry (IUPAC) (namely Physical Chemistry and Analytical Chemistry Divisions) a biosensor is defined as "a self-contained integrated device that is capable of providing specific quantitative or semi-quantitative analytical information using a biological recognition element (biochemical receptor) which is in direct spatial contact with a transduction element" (Pearson *et al*., 2000; Thevenot *et al*., 2001). A typical biosensor construct has three main features: a recognition element (enzyme, antibody, DNA, etc.), a signal transducing structure (electrical, optical, or thermal), and an amplification/processing element (see Fig 2), same models including also, a permselective membrane which controls transport of analyte to the bioreceptor (Velasco and Mottram, 2003). In a biosensor, a biorecognition phase (e.g., enzyme, antibody, receptor, and single-stranded DNA) interacts with the analyte to produce a signal, which may be due to (i) a change in proton concentration, (ii) a release or uptake of gases such as ammonia or oxygen, (iii) a release or uptake of electrons, (iv) a light emission, absorption, or reflectance, (v) a heat emission, or (vi) a mass change, and so forth. According to Gronow, 1984, a biosensor is an analytical device that consists of an immobilized biological material in intimate contact with a compatible transducer, which will convert the biochemical signal into a quantifiable electrical signal. Biosensors are the offspring of the first successful marriage between biotechnology and modern electronics. The biomolecules are responsible for the specific recognition of the analyte whereas the physicochemical transducer supplies an electrical output signal which is amplified by the electronic component (Scheller & Schubert 1992).

2.5.2 Bioreceptors

The specificity of enzymes is the main reason for their use in biosensors. Since most of the enzymes employed for use in sensors have been isolated from microorganisms, it is logical that the organisms themselves should be regarded as potential biocatalysts (Aston & Turner 1984). In microorganisms, the enzymes remain in their natural environment, increasing stability and activity (Guilbault 1984; Corcoran & Rechnitz 1985; Luong *et al*. 1988; D'Souza 2001; Verma & Singh 2003). Cell membranes and organelles can also be used for biosensor construction (Burstein *et al*. 1986, Verma & Malaku 2001). Specific binding between antibody and antigen can be exploited in immunobiosensors. During last years, there has been a huge increase in the use of nucleic acids, as a way in the recognition and monitoring of many toxic compounds of analytical interest, because many of these molecules, and especially heavy metals, show a high affinity for DNA and thus can be used as bioreceptors for heavy metal detection (Turdean, 2011).

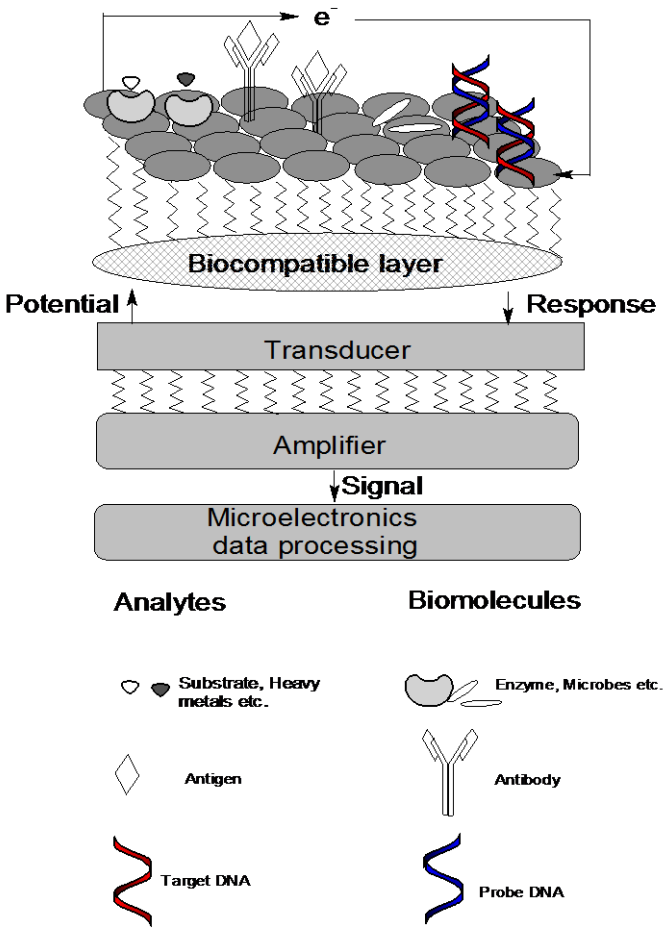

Fig 2 Schematic principle of operation of a biosensor (modified from Turdean, 2011).

2.5.3 Immobilization

The immobilization of the bioreceptor is one of the most important steps involved in the biosensor design. The choice of the technique used for connecting the biological component to the transducer is crucial, since the stability, the longevity and the sensitivity of biosensor largely depend on bioreceptor layer configuration.Various immobilization procedures have been used in biosensor construction. In general, the choice of procedure depends on the nature of the biological element, the type of transducer used, the physicochemical properties of the analyte and the operating conditions in which the biosensor is to function. Perhaps over-riding all the considerations is the necessity for the biological component to exhibit high activity with appropriate specificity in its immobilized microenvironment. The four main approaches to enzyme and microbial immobilization are entrapment and encapsulation, covalent binding, cross linking and adsorption (Brodelius & Vandamme 1987; Kennedy & Cabral 1987; Luong *et al*. 1988; Scheller & Schubert 1992).

The immobilization of the bioreceptor has various advantages (Berezhetskyy *et al*., 2008, Konki *et al*., 2006):

i. Thousands times lower consumption of immobilized enzyme;
ii. Reduction of interferences by the differential mode of operation;
iii. Unnecessary pre incubation;
iv. Rapid analysis procedure, less than 5 min;
v. In the case of reversible inhibition, sometimes the reactivation of enzyme activity is not necessary.

The immobilization methods can be broadly divided into two categories:

- Physical methods
- Chemical methods.

2.5.3a Physical methods

Adsorption and entrapment are the two widely used physical methods for immobilization of microbes and enzymes. Because these methods do not involve covalent bond formation and provide relatively small perturbation of the native structure and function of enzymes and microbes. These methods are preferred when viable cells are required (D'Souza, 1997, Arikawa *et al*., 1998, Simonian *et al*., 1998, Matrubutham and Sayler, 1998, D'Souza,, 2001). Physical adsorption is the simplest method for microbe immobilization. Typically, a

microbial suspension is incubated with the electrode or an immobilization matrix, such as alumina and glass bead (Mikkelsen and Cort´on, 2004, D'Souza,, 1997 , D'Souza,, 2001), followed by rinsing with buffer to remove unadsorbed cells. The microbes are immobilized due to adsorptive interactions such as ionic, polar or hydrogen bonding and hydrophobic interaction. However, immobilization using adsorption alone generally leads to poor long-term stability because of desorption of microbes. The immobilization of microorganisms by entrapment can be achieved by either retention of the cells in close proximity of the transducer surface using dialysis or filter membrane or in chemical/biological polymers/gels such as (alginate, carrageenan, agarose, chitosan, collagen, polyacrylamide, polyvinylachohol, poly(ethylene glycol), polyurethane, etc. (Turner *et al*., 1992, Mulchandani and Rogers, 1998, Tran 1993, Mikkelsen and Cort´on 2004, D'Souza, 1997, Arikawa *et al*., 1998). A major disadvantage of entrapment immobilization is the additional diffusion resistance offered by the entrapment material, which will result in lower sensitivity and detection limit. Acetylcholinesterase (AChE) was encapsulated in sol gel film on a glass cap that could be fixed on an optical fiber (Doong and Tsai, 2001). Sol–gel films have been formed using enzymatic solutions mixed with different fluorescent indicators. The design of such biosensors takes advantage of the ability to entrap large amounts of enzyme and enhance thermal and chemical stability; the techniques offer simplicity of preparation without covalent modification, flexibility in controlling pore size, and geometry and minimal quenching of fluorescent reagents. Reddy and Vadgama, 2002 reported the entrapment of glucose oxidase in non-porous poly (vinyl chloride). The immobilization of the enzyme polyphenol oxidase (PPO) during the anodic electropolymerisation of polypyrrole (PPy) was also reported The enzyme was trapped on the electrode surface during an electrochemical synthesis process (El Kaoutit *et al*., 2004). This biosensor was used for the evaluation of atrazine and provides a rapid and technically simple system for determination at concentrations below the ppm level. Sotiropoulou and Chaniotakis (2005) have used a nanoporous carbon matrix for acetylcholinesterase immobilization and stabilization. They reported that the use of this activated carbon matrix provided both significant enzyme stabilization and a lowering of the detection limit. Using this biosensor the monitoring of the organophosphorus pesticide dichlorvos at picomolar levels was achieved; calculated on the basis of 20% inhibition, they could detect 10^{-12} mol l^{-1} pesticide which was a level 1000 times lower than for other systems reported so far.

Recently, using nanoporous conductive carbon for immobilization of 0.02 pmol of very sensitive acetylcholinesterase from the double mutant E69Y, Y71D of *Drosophila melanogaster*, Sotiropoulou *et al*., (2005) were able to detect dichlorvos at attomolar levels, 10^{-17} M with 40% inhibition. It was shown that in comparison with AChE from *E. electricus* (electric eel), the use of double mutant AChE (E69Y, Y71D) produced a drastic increase of the inhibition constant, *Ki*, value for the dichlorvos pesticide. Malitesta and Guascito, (2005) have described the application of biosensors based on glucose oxidase immobilized by electropolymerisation for heavy metal determination; the investigated enzymatic inhibition appears reversible and in agreement with the data reported for the enzyme in solution. A comparison of several acetylcholinesterase immobilization procedures carried out on the 7,7,8,8 tetracyanoquinonediaminomethane (TCNQ) modified graphite working electrode was presented by Nunes *et al*., (2004). The enzyme immobilization through photopolymerization with polyvinyl alcohol bearing styrylpyridinium groups (PVA-SbQ) produced good results, fast response, good reproducibility, wide working range for pesticides and excellent sensitivity to *N* methylcarbamates. Ivanov *et al*. (2003) evaluated the detection limit and the sensitivity toward some pesticides using different modified screen-printed electrodes. In comparison with the use of unmodified transducers, they concluded that the modification of the sensor surface with 7,7,8,8-tetracyanoquinodimethane (TCNQ) is a powerful tool for the improvement of biosensor performance. According to reports in the literature, the performance of a biosensor device is strongly dependent on its configuration. In a paper that reported the immobilization of enzymes with clay, the influence of the enzyme/clay ratio and the amount of adsorbed coating on cyanide sensing by polyphenol oxidase was investigated (Shan *et al*., 2004). It was reported that, when the enzyme/clay ratio was decreased from 1 to 0.125, the sensitivity of the biosensor decreased sharply from 9130 to 0.5mAM^{-1} cm^{-2} and the detection limit increased from 0.1 nM to 50 µM. Effect of enzyme loading on biosensor response was observed by studying the influence of the amount of deposited coating and hence the thickness of the clay film on the biosensor performance was studied. Higher sensitivity to cyanide and a lower detection limit are observed with thinner coatings. In another paper, Ciucu *et al*. (2003) have confirmed that a lower concentration of paraoxon could be measured using a membrane with a lower amount of immobilized AChE. It has been shown that the sensitivity of enzymes toward heavy metal

ions is a function of enzyme loading (Soldatkin *et al*., 2000). These authors have demonstrated that urease immobilization under a negatively charged polymer induces an increase of the inhibition effect of the heavy metal ions due to cation accumulation in the polymeric matrix. A lower concentration of invertase (2 U) in a tri-enzymatic biosensor matrix resulted in a significant increase of sensitivity, as well as in a decrease of the detection limit of mercury (Mohammadi *et al*., 2005). These results confirm those obtained with the free enzyme, i.e. that a higher percentage of inhibition is often observed with lower enzyme concentration (Evtugyn *et al*., 1998; Mohammadi *et al*., 2002, Sotiropoulou *et al*., 2005) and thus a low enzyme concentration should in general enhance sensitivity to the inhibitor.

2.5.3b Chemical Methods

Chemical methods of immobilization include covalent binding and cross-linking (Turner *et al*., 1992, Mulchandani and Rogers, 1998, Tran 1993, Mikkelsen and Cort´on, 2004, D'Souza 2001). Covalent binding methods rely on the formation of a stable covalent bond between functional groups of the enzyme/microorganisms' cell wall components such as amine, carboxylic or sulfhydryl and the transducer such as amine, carboxylic or epoxy. To achieve this goal, whole cells are exposed to harmful chemicals and harsh reaction condition, which may damage the cell membrane and decrease the biological activity. How to overcome this drawback is still a challenge for immobilization through covalent binding. To our knowledge, this method has therefore not been successful for immobilization of viable microbial cells (Nomura *et al*., 1996, Riedel *et al*., 1998, Arikawa *et al*., 1998, Simonian *et al*., 1998, Matrubutham and Sayler, 1998, D'Souza,, 2001). Cross-linking involves bridging between functional groups on the outer membrane of the cells by multifunctional reagents such as glutaraldehyde and cyanuric chloride, to form a network. Because of the speed and simplicity, the method has found wide acceptance for immobilization of microorganisms. The cells may be cross-linked directly onto the transducer surface or on a removable support membrane, which can then be placed on the transducer (Riedel *et al*., 1998, Arikawa *et al*., 1998, D'Souza,, 2001, Mikkelsen and Cort´on, 2004). The ability to replace the membrane with the immobilized cells is an advantage of the latter approach. While cross-linking has advantages over covalent binding, the cell viability and/or the cell membrane biomolecules can be affected by the cross-

linking agents. Thus cross-linking is suitable in constructing microbial biosensors where cell viability is not important and only the intracellular enzymes are involved in the detection (D'Souza, 1997). In another strategy, acetylcholinestrase was co-immobilised with choline oxidase (ChO) onto a Pt surface using a solution of glutaraldehyde. The activity of immobilized enzymes was evaluated in the presence of dimethyl-2,2-dichlorovinyl phosphate pesticide (DDVP). The cross-linking involving glutaraldehyde significantly increased the attachment of the enzyme to the transducer and thus, the electron exchanges could occur more directly (Kok *et al*., 2002)**.** Gulla *et al*. (2002) reported the immobilization of AChE in nylon net using glutaraldehyde

2.5.4 Tranducers

The function of the transducer is to convert the signal into an appropriate measurable response (e.g., current, potential or temperature change). Through signal processing, this interaction is converted into digital values that relate to the build-up of concentration/activity of the analyte in the environs of the device, which in turn relates to the ambient levels in the bulk investigated sample. A biosensor is not necessarily an individual entity, but is considered as part of a general designed instrumentation (Pearsons *et al*., 2000). Several types of transducers have been used in biosensor construction (Table 2) such as electrochemical (amperometric, potentiometric), thermal, polarimetric, piezoelectric and surface acoustic wave, optical and field effect transducers (Turner *et al*. 1987; Luong *et al*. 1988, 1997; Yim *et al*. 1993; Togawa *et al*. 1997; Tauriainen *et al*. 1998). Some of the major attributes of a good biosensing system are its specificity, reliability, portability, (in most cases) ability to function in optically opaque solutions, real-time analysis and simplicity of operation (D'Souza 2001).

Table 2 Principal transduction systems used in biosensors. (Turdean, 2011)

Transduction system	Measurement/Parameters
Electrochemical	Amperometry/current Potentiometry/voltage at zero current
Electrical	Conductometry/conductance
Optical	Photometry/luminescence Photometry/fluorescence Refractometry/refractive index
Thermal	Calorimetry/temperature
Piezoelectric	Mass-quartz crystal microbalances/mass Mass-surface acoustic waves/velocity and so forth.

2.6 Biosensors for Heavy metals

The bio-recognition element is the main sensing component of any biosensor. Fig 3 illustrates the classification of heavy metal biosensors based on the bio-recognition elements.

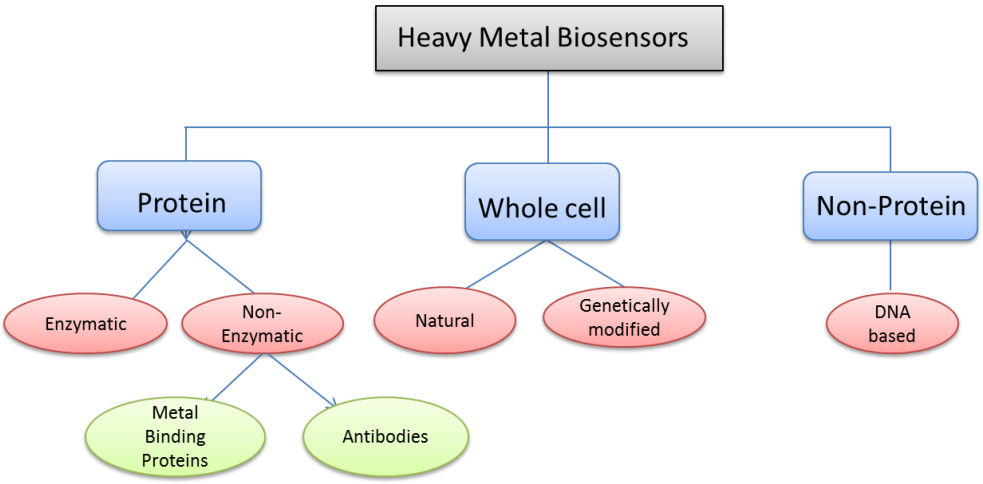

Fig 3 Classification of the heavy metal biosensor based on the bio-recognition Elements

2.6.1 Protein Biosensor

Protein Biosensors are of two types:

1. Enzymatic
2. Non enzymatic

2.6.1.1 Enzymatic Biosensors for heavy metal detection

Enzyme based Heavy metal biosensors are based on the principle of enzyme inhibition. The problem with biosensors based on enzymatic inhibition is that only a few enzymes are sensitive to heavy metals (Garc´ıa *et al*., 2006). Generally, for a bio electrocatalytical scheme the following reactions can be written (Turdean. 2011):

$$S + E_{ox} \xleftrightarrow{K_s} [S - E_{ox}] \xrightarrow{K_{cat}} P + E_{red}, \quad (1)$$

$$M_{ox} + E_{red} \xleftrightarrow{K_M} [M - E_{red}] \xrightarrow{k_{cat}} M_{red} + E_{OX}, \quad (2)$$

$$M_{red} \rightarrow M_{OX} + e^{-}, \quad (3)$$

where S is a substrate; P is a product; E is a enzyme; M_{ox}, M_{red} are the oxidized and reduced forms of a redox mediator molecules (Turdean, 2011).

The "ping-pong" mechanism is proposed for the enzyme reaction described by a reaction rate (v_S), which at steady state is given by (4). The measured current (I_{app}) reflects v_S, as expressed by the approximate equation (Lapenaite *et al*., 2003):

$$v_s = \frac{k_{cat}[E]}{1 + (k_s/[S]) + (K_M/M_{OX})}, \quad (4)$$

$$I_{app} = FA[M]\sqrt{\frac{2n_s n_M D_M k_{cat}[E]}{2K_M + [M]}}, \quad (5)$$

where k_{cat} = catalytic constant; k_S is Michaelis constant for S; K_M is Michaelis constant for M_{ox}; E is enzyme concentration; S is substrate concentration; M_{ox} is mediator concentration; F is Faraday's constant; A is electrode surface area; M is bulk concentration of mediator M; D_M is diffusion coefficient of M; n_S and n_M are the number of electrons involved in (1)–(3), respectively (Turdean, 2011).

2.6.1.2 Enzyme Inhibitor System.

The long-term function of enzyme-based biosensors may be severely limited by the powerful inhibitors which are measured (Turdean, 2011). Because the enzyme-inhibitor reaction is habitually complicated, the inhibition of the enzyme can be either reversible or an irreversible inactivation (Turdean, 2011). Sometimes the effect of an inhibitor can be reversed by decreasing the concentration of inhibitor (e.g., by dilution or dialysis). It is the case of the reversible inhibition (Turdean, 2011). Once inhibition has occurred and there is no reversal of inhibition with decreasing the inhibitor concentration, the inhibition is called

irreversible. The difference between reversible and irreversible inhibition is not absolute and is difficult to do, if the inhibitor binds very strongly to the enzyme and if it is released very slowly. Reversible inhibitors that work in a way that is difficult to distinguish from irreversible inhibition are called tight-binding inhibitors (Copeland, 2000).

2.6.1.3 Reversible Inhibition

Reversible inhibitors are molecules that bind reversibly to enzymes with rapid association by noncovalent interactions and rapid dissociation rates (Turdean, 2011). This chemical equilibrium between the enzyme and the inhibitor can be displaced in favour of the enzyme and so the activity of the enzyme can be regained, by the removal of the inhibitor by dialysis, gel filtration, and so forth (Turdean, 2011). The removal of the inhibitor restores the enzyme activity to its original value. The inhibition process of the immobilized enzyme can be described by the following generally kinetic scheme (Turdean, 2011) (Turdean, 2005, Copeland, 2000, Evtugyn, 1998, Evtugyn, 1999):

$$\begin{array}{ccccccc} E & + & S & \underset{k_{-1}}{\overset{k_1}{\Leftrightarrow}} & E-S & \xrightarrow{k_{cat}} & E + P \\ + Inh & & & & + Inh & & \\ \Updownarrow_{K_i} & & & & \Updownarrow_{K_I} & & \\ E-Inh & + & S & \underset{\alpha k_{-1}}{\overset{\alpha k_1}{\Leftrightarrow}} & E-S-Inh & \xrightarrow{\beta k_2} & E-Inh + P \end{array} \qquad (6)$$

where: E is immobilized enzyme; S is free substrate; P is product; E-S is enzyme-substrate complex; E-Inh is enzyme-inhibitor complex; E-S-Inh is ternary complex containing enzyme-substrate inhibitor; K_I and K_i are equilibrium dissociation constants of the E-S-Inh complex and the E-Inh complex, respectively. In function of the binding site, inhibitors can be: competitive, uncompetitive, noncompetitive and mixed, and their effects on the kinetic parameters (K_M and $v_{\max}$) are resumed in Table 3 (Amine *et al*., 2006, Malitesta and Guascito, 2005)

Table 3 Type of enzyme Inhibitors (Turdean, 2011)

Inhibitor type	Binding site on enzyme	Kinetic effect
Competitive Inhibitor	An inhibitor that is structurally similar to the substrate cannot undergo the catalytic step, so it wastes the enzyme's time by preventing S binding, that is, inhibitor competes with substrate for the enzyme-substrate binding site in a dynamic equilibrium process, thus increasing K_M for substrate. Inhibition is reversible using high concentrations of substrate.	v_{max} is unchanged; K_M is increased.
Uncompetitive Inhibitor	Binds only to ES complexes at locations other than the catalytic site. Substrate binding modifies enzyme structure, making inhibitor-binding site available. Inhibition cannot be reversed by substrate.	v_{max} and K_M decreased with the same factor.
Noncompetitive Inhibitor	If the inhibitor is not only bound to the E, but also to the E-S complex, at a remote site other than at the catalytic site of the enzyme, thus the active centre is usually deformed and its function is thus impaired, affecting k_{cat}. In this case the substrate and the inhibitor do not compete with each other. Substrate binding is unaltered, but ESI complex cannot form products. Inhibition cannot be reversed by substrate.	K_M appears unaltered; v_{max} is decreased proportionately to inhibitor concentration.
Mixed inhibitor	As the noncompetitive, this inhibitor binds at a site other than the active site (E or ES) and causes changes in the overall 3D shape of the enzyme that leads to a decrease in activity. The inhibitor binds to E and ES with different affinity (K_i not equal to K_I). Mixed inhibition cannot be overcome by high substrate concentration.	v_{max} decrease and K_M either increase or decrease.

Dzyadevych *et al*. (2004) reported that the glucoalkaloids are competitive inhibitors of butyrilcholinesterase (BChE). Also Morales *et al*. (2002) showed a competitive inhibition of tyrosinase by benzoic acid.

If the inhibitor is not only bound to the enzyme but also to the enzyme–substrate complex, the active center is usually deformed and its function is thus impaired (Turdean, 2011). In this case the substrate and the inhibitor do not compete with each other (non-competitive inhibition). The inhibition of horseradish peroxidase was apparently reversible and non-competitive in the presence of $HgCl_2$ for less than 8 s of incubation time (Han *et al*., 2001). Cyanide showed non-competitive inhibition versus polyphenol oxidase (Shan *et al*., 2004). The inhibition of immobilized acetylcholinesterase with metal ions (Cu^{2+}, Cd^{2+}, Fe^{3+}, Mn^{2+}) has a reversible and a non-competitive character (Stoytcheva, 2002):

Competitive and non-competitive inhibitions affect the enzyme kinetics differently (Segel, 1976). A competitive inhibitor does not change V_{max} but increases the K_M; on the contrary *a*, non-competitive inhibition results in an unchanged K_M and in a decrease of V_{max}. In the case of mixed inhibition, the inhibitor binds the enzyme and the enzyme–substrate complex with a different affinity. Malitesta and Guascito (2005) demonstrated that the inhibition mechanism of glucose oxidase by heavy metals is reversible and mixed:

For uncompetitive inhibition, the inhibitor binds only when the enzyme–substrate complex is formed. The inhibition of tyrosinase by carbaryl was studied by Kuusk and Rinken (2004); the mechanism of inhibition was found to be analogous to that usually considered for uncompetitive inhibition. Dixon's plot (Segel, 1976) is often used for the evaluation of the inhibition constant and for the differentiation between different types of inhibition.

From the general scheme 6 the value of equilibrium dissociation constants (K_I , K_i) could be calculated from the slope and intercept of the linear plot 1/I versus 1/[S] according to (7) (Gelpi *et al*., 1993, Garcia *et al*., 2003):

$$\frac{1}{I} = \left(1 + \frac{[Inh]}{K_i}\right)\frac{K_M}{I_{max}} + \left(1 + \frac{[Inh]}{K_I}\right)\frac{1}{I_{max}} \qquad (7)$$

2.6.1.4 Irreversible Inactivation

The term irreversible inhibitor means that the decomposition of the enzyme-inhibitor complex results in the destruction of enzyme (e.g., its hydrolysis, oxidation, etc.) or modification of an essential amino acid required for enzyme activity (Turdean, 2011). Frequently, this is due to a covalent bond between the enzyme active site and the inhibitor. These inhibitors are designed to mimic the natural substrate in the recognition phase and to bind to the active site of the enzyme in a second step (Turdean, 2011). Upon binding and some catalytic modification, a highly reactive inhibitor product is formed, that binds irreversibly and consequently inactivates the enzyme (Amine *et al*., 2006). The kinetics of the inhibition depends strongly on the biosensor configuration. Thus, in the case of a thin enzymatic layer, the kinetics observed is similar to that of the enzyme in solution. Also, the inhibition of native enzymes is related directly to the incubation time (Amine *et al*., 2006). An interesting case concerning the inhibition of peroxidase by heavy metals was investigated (Han *et al*., 2001). The conclusion is that there exists an early phase of reversible inhibition (5 s), followed by irreversible inhibition. As consequence, it is hard to accomplish the amperometric measurement of remaining activity (*vi*) so soon after a reversible inhibition. However, irreversible inhibition or inactivation has to be considered, when longer incubation times were used (1–8 min). Also, HM salts at high concentration (>1mM) inactivate all enzymes by co-precipitation, while lower concentrations affect only some enzyme activities by interaction with specific protein groups (Fennouh *et al*., 1998) (Turdean, 2011). Knowing that the irreversible inactivation follows the reaction mechanism described by scheme (8) (Fennouh *et al*., 1998)

$$\begin{array}{ccccc} E + S & \underset{k_{-1}}{\overset{k_1}{\rightleftarrows}} & E-S & \xrightarrow{kcat} & E + P \\ + Inh & & & & \\ \Updownarrow K_i & & & & \\ E-Inh & & \xrightarrow{k_{inac}} & & E-Inh \end{array} \qquad (8)$$

and supposing that the irreversible inactivation process was a first order versus enzyme concentration [E], according to the literature, the reaction rate is described by equation (9)

$$d[E]/dt = -k_{obs}[E] \qquad (9)$$

which become after variable separation:

$d[E]/[E] = -k_{obs}\, dt.$ (10)

After integration

$\ln vi = -k_{obs}t + \text{const},$ (11)

the linear dependence of ln *vi* versus *t* which can be plotted for each incubation time is obtained.

The experimental slope is

$$k_{obs} = \frac{k_{inac}[Inh]}{[Inh] + K_i}, \qquad (12)$$

where [Inh] is inactivator concentration, *Ki* is equilibrium dissociation constant, and *k*inac is rate constant of inactivation.

(i) For [Inh]_*Ki*, equation (12) became

*K*obs = *k*inac[Inh]/*Ki*

(13).

Thus the 1/*k*obs plotted against 1/[Inh] is a straight line with the slope *Ki*/*k*inac (Han *et al*., 2001), which permit also the determination of the detection limit and the efficiency of inactivation.

(ii) For [Inh] around *Ki*, 1/*k*obs plotted versus 1/[Inh] permitted the determination of *k*i and *K*i and calculation of *ki*/*Ki* (Fennouh *et al*., 1998).

Also, when the inhibition reaction 6 is reduced to scheme (8) (i.e., $KI \rightarrow \infty$, $\alpha k1 \rightarrow \infty$, $k2 \rightarrow 0$) the inhibition constant (*Ki*) value can be determined from the slope and intercept of the linear 1/*I* versus1/[*S*] plot of the modified equation (7) (Gelpi *et al*., 1993, Garcia *et al*., 2003):

$$\frac{1}{I} = \left(1 + \frac{[Inh]}{K_i}\right)\frac{K_M}{I_{max}[S]} + \frac{1}{I_{max}}, \qquad (14)$$

2.6.1.5 Degree of Inhibition

Irreversible inhibition is usually quantified in terms of the rate of inhibition (Turdean, 2011). In order to investigate the heavy metals inhibition an experimental method is used consisting in recording the bio-electrode amperometric response to successive additions of substrate, before and after its incubation in an inhibitor solution, for a given period of time (Turdean, 2011). Thus, the method allows to calculate the percent of inhibition (% Inh), defined using the formula (Kok *et al*., 2002, Wilkins *et al*., 2000, Zhang *et al*., 2001)

$$\%Inh = \frac{I_0 - I}{I_0} \quad * \quad 100, \qquad (15)$$

where I_0 is bio-electrode initial response when the inhibitor was absent; I is bio-electrode response after incubation with the inhibitor(Turdean, 2011). Also, it was reported that the degree of inhibition depends on the concentration of the inhibitor and on the exposure time (at a defined pH value and at inhibitor concentration which is in excess with respect to enzyme) (Guerrieri *et al*., 2002, Neufeld *et al*., 2000). There have been some initial efforts at the development and experimental verification of theoretical models for the inhibition of immobilized enzymes using biosensors (Turdean, 2011). When diffusion phenomenon are taken into account, the model predicts that the percentage of enzyme inhibition (% Inh), after exposure to an inhibitor, is linearly related to both the inhibitor concentration [Inh] and the square root of incubation time ($t1/2$) (Amine *et al*., 2006, Zhang *et al*., 2001).

2.6.1.6 Limit of detection

The determination of the inhibitory effect includes the following steps: the determination of initial enzymatic activity, the incubation of a biosensor in a solution that contains an inhibitor, and finally the measurement of the residual activity (activity after exposure of the biosensor to the inhibitor). The limit of detection (LOD) has been defined as the concentration of the species being measured which gives a minimum detectable difference signal (reduction in activity) that is equal to 2 or 3 standard deviations (S.D.) of the mean response of the blank samples (zero concentration of the inhibitor). This simple approach, although widely reported in the literature (Kuswandi, 2003; Del Carlo *et al*., 2004; Suprun *et al*., 2004), is not correct because it does not take into account the confidence interval of

the inhibitor. The true value of LOD can be defined as the concentration of the inhibitor where the confidence interval does not overlap that of the zero concentration of the inhibitor standard. This is shown diagrammatically in Fig 4. Any concentration above the LOD value has a 95% (2 S.D.) or 99% (3 S.D.) probability of being a true positive result. The LOD value generally corresponds to 90–80% of residual activity, that is 10–20% inhibition. Kuswandi (2003) has developed a simple optical biosensor based on immobilized urease for the monitoring of heavy metals. He confirmed that the detection limit depends on the incubation time of the enzyme with the inhibitor; the optimum time of inhibition selected was 6 min. In other work, the residual enzymatic activity was also studied using different incubation times (5, 15, 30 min) with the AChE and ChO bienzymatic system (Kok *et al*., 2002). The degree of the enzyme inhibition increased with increase of the incubation period until reaching a plateau in 15–30 min. The decrease in the enzyme activity could be detected after 5 min, and therefore the incubation time selected was 5 min, and the biosensor could detect 10 ppb aldicarb which gave a 10% inhibition of the initial acethylcholinesterase activity. In another work the same inhibition study has been performed using an incubation time of 30 min (Ciucu *et al*., 2003). In this case, the detection of paraoxon at 10nM has been achieved. Shan *et al*. (2004) have highlighted specific electrostatic interaction of the host matrix that may induce an accumulation of the inhibitor within the anionic clay. This phenomenon improved the sensitivity of the amperometric biosensor toward cyanide (0.1 nM). According to literature it has been shown that the limit of detection of different developed biosensors depends upon several parameters such as pH, temperature and the amount of enzyme loading (in case of irreversible inhibition), the substrate concentration (in case of competitive reversible inhibition), immobilization matrix and the reaction time. Thus, direct comparison of the sensitivity between the different biosensors based on enzyme immobilization is not easy and should take into consideration the cited parameters (Amine *et al*., 2006).

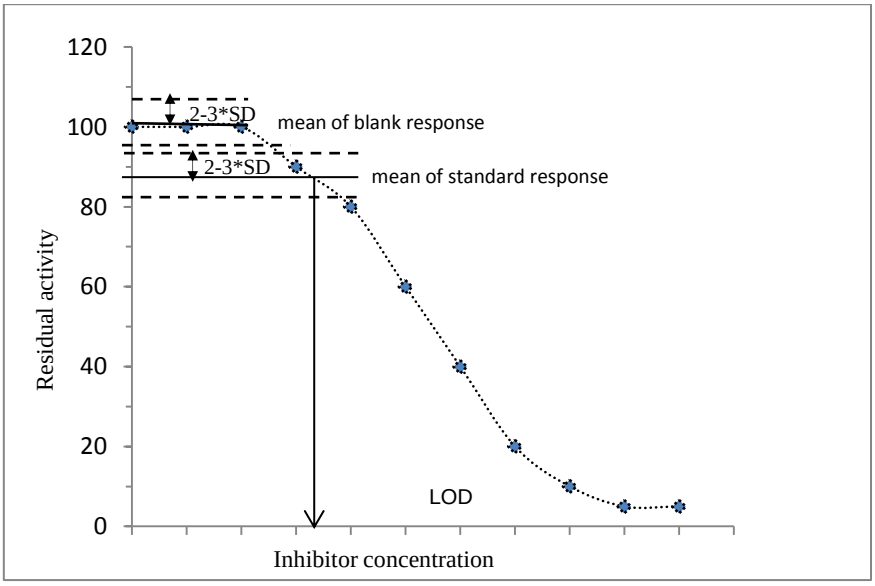

Fig 4 General method to establish LOD for enzyme inhibition assays (Amine *et al*., 2006).

2.6.1.7 Regeneration of Biosensor

Understanding the mechanisms of inhibition and regeneration of enzymes is a general problem of great importance for many biochemists and biotechnologists, especially when using immobilized enzymes. The mode of analyte inhibition of enzymes such as peroxidase, tyrosinase and catalase can occur through blocking of the active sites of these enzymes due to complex formation with copper cofactors and blocking of the electron transfer chain. Organophosphates inhibit acetylcholinesterase (AChE) by blocking the serine in the active site through nucleophilic attack to produce a serine phosphoester (via phosphorylation) (Simonian *et al*., 2001). In any case, the strong inhibition of the enzymes can present a serious problem for practical applications by limiting the reuse of biosensors. To overcome the problem of irreversible enzyme inhibition in the application of AChE based biosensors, reactivation by oximes was investigated (Gulla *et al*., 2002). Further, another phenomenon observed is that of permanent inhibition when the phoshorylated (inhibited) enzyme is left for a period of time without exposing it to the reactivator. This phenomenon is called “ageing”, and is catalyzed by the enzyme itself. After this “ageing”, the inhibited enzyme is even more resistant to hydrolysis and reactivation with oxime so that it becomes permanently inhibited. Reactivation of inhibited AChE was investigated using pyridine-2-aldoxime methyliodide (2-PAM) and 4-formylpyridinium bromide dioxime (TMB-4). TMB-4 was found to be a more efficient reactivator with repeated use,

retaining more than 60% of initial activity after 11 reuses, whereas in the case of 2-PAM, the activity retention dropped to less than 50% after only six reuses. The effect of ageing on the enzyme activity retained has been studied by Gulla *et al*. (2002). It was found that this effect sets in after 15 min when the inhibited enzyme is left without reactivation and increases with time elapsed before exposure of the biosensor to the reactivator (2 PAM and TMB-4). Thus, it was recommended that the pesticide-treated enzyme should be reactivated within 10 min to achieve the maximum reactivation and also to ensure a maximum number of reuses of the immobilized enzyme membrane. This study has demonstrated that TMB-4 is much more effective agent for reactivation of the immobilized AChE enzyme for biosensor applications. In another case of acetylcholinesterase inhibition, 0.4 mmol l−1 sodium fluoride (NaF) was successfully used for 10 min for the reactivation of an inhibited biosensor (Kok *et al*., 2002). Heavy metals act by the binding of the metal salts to protein thiol groups. Depending on the sensing enzyme used, some biosensors can be regenerated after inhibition by use of a metal chelating agent, such as EDTA or thiols (Evtugyn *et al*., 1998). Mohammadi *et al*. (2005) tried to regenerate a 50% mercury-inhibited invertase biosensor by soaking in a cysteine solution; the recovery was 30% of the initial biosensor signal. They also tried to regenerate this biosensor with EDTA solution. Unfortunately, no reactivation has been noted. However, a full and rapid restoration of response has been recently obtained by treatment of Hg^{2+}-inhibited glucose oxidase biosensor with EDTA solution (Malitesta and Guascito, 2005). It was also seen that if exposure time was short enough, the enzymatic activity could be recovered without using reactivators even in the case of inhibition by a high concentration of pollutant (Okazaki *et al*., 2000). Such effectively reversible inhibition can prove an advantage in the analysis by flow injection where the inhibitor is eluted by simple flushing with an electrolyte or a buffer solution (Shan *et al*., 2004). In summary, for irreversible inhibition, the damaged enzyme could often be reactivated using specific regenerating reagents. If the reactivation achieved is not sufficient, the biosensors would have to be assembled for single use, in which case screen-printed electrodes would be recommended.

2.6.1.8 Parameters generally affecting the performance of enzymatic biosensors

2.6.1.8a Effect of pH

The pH of the solutions containing substrates can affect the overall enzymatic activity since, like all natural proteins, enzymes have a native tertiary structure that is sensitive to pH; denaturation of enzymes can occur at extreme pHs. It is well known that the enzyme activity is highly pH dependent and the optimum pH for an enzymatic assay must be determined empirically. It is best to choose a plateau region so that the pH should not have any effect on enzyme activity and will not interfere with the results obtained relative to the inhibition of the enzyme by the inhibitor. The activity of the immobilized acetylcholinesterase as a function of pH has been studied between pH 2 and 9 by Stoytcheva (2002). She has reported a decrease in the activity of approximately 70% at pH 2 compared to of that at pH 7. Mohammadi *et al*. (2005) investigated the effect of pH of a tri-enzymatic biosensor in which the pH-optima of the three enzymes are different (invertase pH, 4.5; glucose oxidase, pH 5.5; mutarotase, pH 7.4). The pH effect on the biosensor response was analysed between pH 4 and 8 and the highest activity was found at pH 6.0. In order to improve the selectivity of the invertase toward mercury and to avoid silver interference, a medium exchange technique has been carried out. The biosensor was exposed to mercury in an acetate buffer solution at pH 4 while the residual activity was evaluated with phosphate buffer solution at pH 6. Dzyadevych *et al*. (2004b) have studied the influence of pH on the analytical performance of a BChE modified pHSFET biosensor for tomatine. In this study the best response was obtained for a buffer solution at pH 7.2, whereas the inhibition level did not depend on the pH of the solution studied in the range 6.0–8.5.

2.6.1.8b Effect of substrate concentration

The substrate concentration can affect the degree of inhibition. Kok *et al*. (2002) concluded that the inhibition level (%) increases with increasing of the substrate concentration and they have worked with a saturating substrate concentration in the case of pesticide inhibition. Joshi *et al*. (2005) have used a concentration of acetylhtiocholine two times higher than the apparent K_M for the determination of the maximum activity of AChE before and after the inhibition by the OP paraoxon which was selected as model pesticide. In the case of competitive inhibition, at high substrate concentrations, the inhibition effect is not

observed since the substrate competes with the inhibitor. Dzyadevych *et al*. showed that the sensitivity of a BuChE biosensor toward tomatine decreases with an increase in the substrate concentration (Dzyadevych *et al*., 2004a).

2.6.1.8c Effect of enzyme concentration

The highest sensitivity to inhibitors was found for a membrane containing low enzyme loading (Shan *et al*., 2004; Mohammadi *et al*., 2005; Sotiropoulou and Chaniotakis, 2005; Sotiropoulou *et al*., 2005). In order to optimize the amperometric biosensor, Ciucu *et al*. (2003) studied a set of five membranes with different amounts of AChE; the response of the biosensors decreases with a decrease of the enzyme concentration. The lowest concentration of paraoxon detected (10^{-8} mol l^{-1}) was achieved by using a membrane with 0.1 IU/cm^2 immobilized AChE. The detection limit of pesticide equal to 10^{-17}M was achieved with very low concentration (0.02 pmol) of engineered acetylcholinesterse enzyme (Sotiropoulou *et al*., 2005).

2.6.1.9 Examples of Enzyme-Based Biosensors for Heavy Metal Detection.

For heavy metals detection, different enzymes such as acetylcholinesterase, alkaline phosphatase, urease, invertase, peroxidase, L-lactate dehydrogenase, tyrosinase, and nitrate reductase, have been used. The inhibition of the immobilized enzyme can be detected via electrochemical (amperometric, potentiometric, and conductometric) or optical measurements. The analytical parameters of the various enzyme-based biosensors are summarized in Table 4.

2.6.1.10 Non- Enzymatic Biosensor for Heavy metal detection

2.6.1.10a Metal- binding protein based biosensors

A variety of non-enzymatic proteins, ranging from naturally occurring metal-binding proteins to various engineered proteins that are constructed to bind specific metal ions, have been utilized in biosensor development (Verma and Singh, 2005, Turdean, 2011). Various examples of the non-enzymatic protein based sensors have been illustrated in Table 5.

Table 4 Enzyme inhibition based biosensors for heavy metals

Inhibitors	Bioreceptors	Immobilisation matrix	Technique	Working range/LOD	Nature of inhibition	Reference
Hg^{2+}, Cu^{2+}, Cd^{2+}	Urease	Entrapment in sol–gel matrix	Optical	LOD= 10 nM, 50 µM, 500 µM	-	Tsai and Doong (2005)
Hg^{2+}, Cu^{2+}	Glucose oxidase	Electropolymerisation in PPD	Amperometric	2.5 µmol l^{-1} to 0.2 mmol l^{-1}, 2.5 µmol l^{-1} to 0.2 mmol l^{-1}	Reversible	Malitesta and Guascito (2005)
Hg^{2+}	Acetylcholinestrase		Amperometric	1×10^{-10} mol/L to 1×10^{-5} mol/L LOD= 10^{-10}MHg^{2+}	Reversible and Non-competitive	Stoytcheva and Sharkova 2002
Hg^{2+}, Cu^{2+}, Cd^{2+}, Zn^{2+}, Ag^{+}	Alkaline Phosphatase	Entrapment in hybrid sol-gel/chitosan film	Amperometric	mg/l Hg^{2+}, Cd^{2+}, Ag^{+}, Zn^{2+}, Cu^{2+}	-	Shyuan *et al*., 2008
$Hg(NO_3)_2$, $HgCl_2$, $Hg_2(NO_3)_2$, phenyl mercury	Urease	Entrapment in sol–gel film	potentiometric	0.05–1.0/0.2, 0.05–1.0/0.2, 0.05–1.0/0.1, 0.1–5.0/0.5µmol l^{-1}	-	Doong and Tsai (2001)
Cd^{2+}	Urease	Self-assembled monolayer on the gold-coated sensor surface	Optical (SPR)	0–10 mg l^{-1} (dynamic range)	-	Lee and Russel (2003)
Hg(II), Ag(I), Cu(II), Ni(II), Zn(II), Co(II), Pb(II)	Urease	Immobilization in ultrabind Membrane	Optical fiber Biosensor	1×10^{-9} to 1×10^{-5}, 1×10^{-8} to 1×10^{-5}, 1×10^{-7} to 1×10^{-5}, 1×10^{-6} to 1×10^{-5}, 2×10^{-5} to 1×10^{-3}, 2×10^{-5} to 1×10^{-3}, 1×10^{-4} to 1×10^{-3} mol l^{-1}	Irreversible	Kuswandi (2003)
Mercury(II), mercury(I), methylmercury, mercury–glutathione complex	HRP	Entrapment in β-cyclodextrin Polymer	Amperometric	LOD= 0.1, 0.1, 1.7 ng ml^{-1}	Reversible in less than 8 s and irreversible in 1–8 min	Han *et al*. (2001)
Ag^{+}, Ni^{2+}, Cu^{2+}	Urease	Deposition onto electrode area and covering with poly(4-vinylpyridine and Nafion)	Potentiometric pH-SFET	LOD= 3.5×10^{-8}, 7×10^{-5}, 2×10^{-6} mol l^{-1}	Irreversible	Soldatkin *et al*. (2000)
Cu^{2+}	AChE	Cross-linking with GA vapour	Amperometric	0.05–4.0 mmol l^{-1}	Reversible inhibition	Evtugyn *et al*. (2003)

Hg^{2+}	GOx	Cross-linking with GA and BSA	Amperometric	2.5–12 ng ml^{-1}, LOD=1ngml^{-1}	-	Mohammadi *et al.* (2002)
$HgCl_2$, $Hg(NO_3)_2$, Hg_2Cl_2, methylmercury, phenyl mercury	Invertase	Cross-linkage with GA and deposition on laponit modified electrode	Amperometric	*I*50 = 0.27, 0.032, 0.27, 0.34, 0.12 ppm	Irreversible	Mohammadi *et al.* (2005)
Hg^{2+}	GOx	Immobilized in a polyvinylpyridine (PVP) in presence of 2-aminoethanethiol mediator	Amperometric	1–100 ppb, LOD= 0.2 ppb	Reversible	Alexander and Rechnitz (2000)
Chromium(VI)	GOx	Cross-linking with GA and covering with aniline membrane	Amperometric	0.49 µg l^{-1} to 8.05 mg l^{-1}, LOD= 0.49µg l^{-1}	-	Guang-Ming *et al.* (2004)
Ag^{+}	Alkaline Phosphatae	Free/Sol-gel Matrix	Flourescence	10.1 µM Ag^{+}	-	Garc´ıa *et al.*, 2003
Hg^{2+}	Glucose Oxidase	Cross linking by glutaraldehyde on electropolymerized aniline membrane on Pt electrode	Amperometric	LOD=0.49 μg/LHg^{2+} and Linear Ranges 0.49–783.21 µg/L and 783.21 µg/L–25.55 mg/L	Reversible	Liu *et al.*, 2009
Hg^{2+}, Ag^{+}, Cu^{2+}, Cd^{2+}, Pb^{2+}, Cr^{3+}, Fe^{3+}, Co^{2+}, Ni^{2+}, Zn^{2+}, Mn^{2+}	Glucose Oxidase	Immobilized in electrosynthesized poly-*o*-phenylenediamine on Pt electrode	Amperometric	2.5 μMHg^{2+}, 0.05μMAg^{+}, 5.0 μMCu^{2+}, 5.0μMCd^{2+}, 12.0 μMFe^{3+}, 8.0μMCo^{2+}, 4.8 μMNi^{2+}, 48μMCrO_2^{-}	Reversible	Guascito *et al.*, 2008
Hg(II), Ag(I), Pb(II) and Cd(II)	Invertase and Glucose Oxidase	entrapment in agarose–guar gum on ultra-microelectrode (UME)	Amperometric	$5*10^{-10}$MHg^{2+}, $3*10^{-8}$MPb^{2+}, $5*10^{-8}$M Ag^{+}, $2.5*10^{-8}$M Cd^{2+}	-	Bagal-Kestwal *et al.*, 2008
Cu2+,Cd2+, Pb2+, Zn2+	Nitrate Reductase	Cross linked on Nafion using Glutaraldehyde-BSA_Methyl-viologen	Conductometry	0.05 μMCu^{2+}, 0.5μM Zn^{2+}, 0.1μMCd^{2+}, 1 μM Pb^{2+}	-	Wang *et al.*, 2009

Cr III	Tyrosinase	A glassy carbon electrode was used as a support for the electropolymerisation of a polypyrrole (PPy) film, in which tyrosinase was immobilised.	Amperometric	$5*10^{-7}MCr^{3+}$	-	Renedo *et al*., 2004
Cu2+	Tyrosinase	Cross linked on a Pt electrode using Glutaraldehyde and BSA	Conductometry	1 ppb Cu2+	-	Anh *et al*., 2006
Hg^{2+}	Urease	Enzyme was immobilized in poly(vinylferrocenium) film electroplymerized on Pt electrode.	Amperometric	7.4 μMHg2+	-	Kuralay *et al*., 2007
Hg^{2+}	Urease	Enzyme immobilized on Gold nanoparticles chemically adsorbed on the PVC-NH_2 matrix	Potentiometric	0.05 μMHg2+	Reversible	Yang *et al*., 2006
Cu^{2+}, Ag^{2+}	Urease	screen-printed electrodes of ruthenium dioxide, urease, graphite and organic polymer.	Potentiometric	Sub ppm levels	-	Ogonczyk *et al*., 2005
$Hg(NO_3)_2$, $HgCl_2$, PhHgCl and $Hg_2(NO_3)_2$	Urease	entrapped in PVC layer at the surface of pH-sensitive iridium oxide electrode	Potentiometric	0.02 μMHg2+	Reversible	Krawczyk *et al*., 2000
Cu2+,Cd2+, Pb2+, Hg2+	Urease	Entrapped in sol-gel	Conductometric	0.005mM Hg2+, 0.02mM Cu2+, 0.1mMCd2+, 0.9mM Pb2+	Irreversible	Lee and Lee 2002
Hg2+, Cu2+, Cd2+	Urease/GLDH	Screen printed electrodes	Amperometric	7.2 μg/L Hg2+, 8.5μg/L Cu2+, 0.3mg/L Cd2+, 0.2mg/L Zn2+	-	Rodriguez *et al*., 2004
Cu^{2+}	Alcohol oxidase	Quantam dots–enzyme hybrid system	QDs fluorescence	0.176 ng/mL (2.75 nM)	-	Guo *et al*., 2012

ALP = alkaline phosphatase; Au-NP = gold nanoparticles; BSA = bovine serum albumin; C-Rh = rhodinised carbon electrode; ENFET = enzyme field effect transistor; Fc = ferrocene; MB = methylene blue; GA = glutaraldehyde; GdA = glutaric dialdehyde; GCE= glassy carbon electrode; GLDH = glutamate dehydrogenase; Gox = glucose oxidase; Inv = invertase; Mu = mutarotase; NR = nitrate reductase; PA = polyaniline; PPD = poly(*o* phenylenediamine); pPy = polypyrrole; PQQ-GDH = pyrroloquinoline quinine dependent glucose dehydrogenase; PVC = poly(vinyl chloride); PVC-NH2 = ethylenediamine poly(vinyl chloride); PVF = poly(vinylferrocenium); SCE = saturated calomel electrode; SPE = screen-printed electrode; Ty = tyrosinase.

Table 5 Examples of non-enzymatic protein based heavy metal biosensor

Metal-binding protein	Metal ions	Detention Limit	Transducer	References
Synthetic phytochelatin (EC)/Au	Hg^{2+}, Cd^{2+}, Pb^{2+}, Cu^{2+}	1 fM–10mM	Capacitance	Bontidean *et al*., 2003
GST-SmtA/Au	Hg^{2+}		Capacitance	Bontidean *et al*., 2004
GST-SmtA/Au	Hg^{2+}, Cd^{2+}, Cu^{2+}, Zn^{2+}	10^{-15}M	Capacitance	Bontidean *et al*., 1998, Bontidean *et al*., 2000
*Mer*R/Au	Hg^{2+}, Cd^{2+}, Cu^{2+}, Zn^{2+}	10^{-15}M	Capacitance	Bontidean *et al*., 1998, Bontidean *et al*., 2000
Cytocrome c3 from *Desulfomicrobium norvegicum*/GCE	Cr^{6+}	0.2 mg/L	Amperometry/−0,53 V versus SCE	Michel, *et al*., 2006
Glutathione-S-transferase	Hg^{2+}, Cd^{2+}, Cu^{2+}, Zn^{2+}	10^{-15} M	Capacitance	Corbisier *et al*., 1999
Human carbonic anhydrase II variant	Cu^{2+}	0.1pM	Fibre optic	Zeng *et al*., 2003

GCE = glassy carbon electrode;

2.6.1.10b Antibody-based biosensors

Immunoassays have emerged as an alternate approach for metal ion detection since they offer significant advantages over traditional detection methods such as high sensitivity, selectivity and species-specificity and are theoretically applicable to any pollutant for which a suitable antibody can be generated (Blake 1995). Monoclonal antibodies have been generated that recognize metal–EDTA complexes of cadmium, mercury, copper, nickel, lead, cobalt and silver, besides many other metal ions. The developed antibodies had maximum binding affinity for Cd(II) and were studied at a concentration of 100 ppm Cd–EDTA–bovine serum albumin (BSA) complex (Blake *et al*. 1996). An inhibition immunoassay that is insensitive to the presence of interfering metal ions has been employed for the analysis of cadmium using anti-cadmium (2 A8 1G5) monoclonal antibodies that bind tightly to Cd– EDTA complex but not to metal-free EDTA. These antibodies are able to detect Cd(II) in the range of 70–500 ppb (0.06–4.45 µM) (Khosraviani *et al*. 1998). Useful monoclonal antibodies have been purified that recognize zinc, cobalt and nickel diethylenetriamine pentaacetic acid (DTPA) complexes and a monoclonal antibody that recognizes lead (II) – cyclohexyldiethylenetriamine- pentaacetic acid (CHXDTPA) (Blake *et al*. 1998). More recently, monoclonal antibodies for complexes of cadmium–EDTA, cobalt– DTPA

and lead–CHXDTPA with enhanced sensitivities of 0.25, 10 and 6.0 nM, respectively have been developed (Blake *et al*. 2001). Table 3 lists the antibodies developed for various metal ions.

2.6.2 Whole Cell Biosensor

Enzymes are the most widely used biological sensing elements in the fabrication of biosensors (Turner *et al*., 1992, Mikkelsen and Cort´on, 2004). Although purified enzymes have very high specificity for their substrates or inhibitors, their application in biosensors construction may be limited by the tedious, time-consuming and costly enzyme purification, requirement of multiple enzymes to generate the measurable product or need of cofactor/coenzyme. Microorganisms provide an ideal alternative to these bottle-necks (Arikawa, 1998). The many enzymes and co-factors that co-exist in the cells give the cells the ability to consume and hence detect large number of chemicals; however, this can compromise the selectivity. They can be easily manipulated and adapted to consume and degrade new substrate under certain cultivating condition (Jain *et al*., 1994, Leung *et al*., 1997). Additionally, the progress in molecular biology/ recombinant DNA technologies has opened endless possibilities of tailoring the microorganisms to improve the activity of an existing enzyme or express foreign enzyme/protein in host cell (Rensing and Maier, 2003, Belkin, 2003). All of these make microbes excellent biosensing elements.

Table 6 Advantages and disadvantages of whole cell based biosensors (Turdean, 2011).

Advantages	Disadvantages
1. React only to the available fraction of metal ions 2. Are fast, less expensive, and less intensive labour 3. Are compatible with and comparable to chemical analysis; 4. Are more sensitive than chemical methods; 5. Produces real-time data and can be applied in field work or in situ analysis; 6. does not involve the bulky, fragile equipment, or specialized training; 7. They are more tolerant of suboptimal pH and temperatures than purified enzymes; 8. Are cheaper to use because the active biological component does not have be isolated and because microorganisms are living, unlimited quantities can be prepared relatively inexpensive; 9. Can provide information about the bioavailability of the analyte; 10. May perform multi-step reactions since all reactions are conveniently packaged within the cell and thus, efficiently carried out.	1. The limited understanding of the biochemistry involved. 2. Lack of genetic stability and short lifetime 3. Cells require relatively long incubation time (usually longer than 30 min) 4. Difficult reversibility of the signal 5. Experimental conditions (temperature, pH, incubation time, buffer, and reagents) can effects the luminescence production and thus the biosensor performances 6. Less/limited of selectivity

Whole cell biosensors can be divided into following two categories:

- Genetically modified microbes (Table 7)
- Natural microbes (Table 8)

Table 7 Examples of recombinant bacteria for specific heavy metal detection.

Promoter/ reporter gene	Host microorganism	Metal ion/ detection limit	Linear range	Detection method	References
	Bacteria				
Ars pR773/*lac*Z	*Escherichia coli*	As^{3+}/50 μM; Sb^{3+}/1 fM	μM-mM; fM-μM	Chemiluminescence	Ramanathan *et al*., 1997
CUP 1/*lac*Z	*Saccharomyces Cerevisiae*	Cu^{2+}	16.0-32.0mg/L	Amperometry/ −0.6 V versus Ag/AgCl	Tag *et al*., 2007
pMOL 90 + Tn4431/*lux*CDABE	*Alcaligenes eutrophus* (AE1239)	Cu^{2+}/1 μM	0–250 μM	Bioluminescence	Leth *et al*., 2002
*cad*A and *cad* C/*luc*FF	*Staphylococcus aureus* (RN4220)	Cd^{2+}/10 nM; Pb^{2+}/33 nM; Sb^{2+}/1 nM	10 nM1μM 33 nM–330 μM 1 nM–330 nM	Bioluminescence	Tauriainen *et al*., 1998
mer /lux	*Escherichia coli* (CM 2624)	Hg^{2+}	-	Bioluminescence	Bontidean, 2004
*mer*R/luxFF	*Escherichia coli* (S30)	Hg^{2+}	-	Luminescence	Pellinen, 2004
*mer*R/luxFF	*Escherichia coli* (MC 1061)	Hg^{2+}	-	Luminescence	Barrocas, 2010
−/DsRed-GFP	*Escherichia coli* (DH5α)	Cu^{2+}/45 nM	-	Fluorescence	Sumner, 2006
−/lux	*Burkholderia sp* (RASC c2)	Zn^{2+}/1.7 μg/mL Cu^{2+}/0.09 μg/mL	-	Bioluminescence	Chinalia, 2008
cad/*rs-GFP*	*Escherichia coli* (DH5α)	Cd^{2+}/0.1 nmol/L; Pb+/10 nmol/L; Sb^{3+}/0.1 nmol/L	-	Fluorescence	Liao *et al*., 2006
−/eGFP205C	*Escherichia coli (*K12), *Caenorhabditis Elegans*	Hg^{2+}	-	Fluorescence	Chapleau and Sagermann, 2009
copA/lux	*Escherichia coli* (W3110)	Cu^{2+}, Ag^{+}, Au^{3+}	-	Bioluminescence	Stoyanov *et al*., 2003

pczcR3GFP	*Pseudomonas putida* X4	Zn^{2+}	-	Fluorescence	Liu *et al*., 2012
*phi*YFP	*E. coli* DH5α	As^{3+}, As^{5+}	-	Fluorescence	Hu *et al*., 2010

CPE = carbon paste electrode; CV = cyclic voltammetry, DPSV = differential pulse stripping voltammetry, GCE = glassy carbon electrode; ISE = ion selective electrode; ITO = indium-tin-oxide glass electrode; lucFF = firefly luciferase; PAH = poly(allylamine hydrochlorure); PSS = poly(styrene sulfonate); *rs-GFP* = red-shifted green fluorescent protein; SCE = saturated calomel electrode, SPE = screen-printing electrode.

Table 8 Examples of unmodified microbes (Whole cells) for specific heavy metal detection

Microbes	Metal ions	Detention Limit/ Linear Range	Transducer	Reference
Tetraselmis chuii (Prasinophyceae)/CPE	Cu^{2+}	$4.6*10^{-10}$M,$5*10^{-8}$–10^{-6}M	Amperometry/ −0.4 V versus Ag/AgCl	Alpat *et al*., 2007
Chlorella vulgaris	Cd^{2+}	16ppb	Synchronous-scan Spectrofluorimetry	Nguyen-Ngoc *et al*., 2009
AlkP from *Chlorella vulgaris*/Pt	Cd^{2+}, Zn^{2+}	10 ppb	Conductometry	Chouteau *et al*., 2005
Chlorella sp./GCE	Hg^{2+}	10^{-14}-10^{-6}M	Conductometry	Singh and Mittal 2012
Phormidium sp./CPE	Pb^{2+}	$2.5*10^{-8}$M , $5*10^{-8}$–$2*10^{-5}$M	CV, DPSV	Y¨uce *et al*., 2010
Rhodotorula mucilaginosa/CPE	Cu^{2+}	10^{-7}–10^{-5}M	CV, DPSV	Y¨uce *et al*., 2010
Rhizopus arrhizus/CPE	Pb^{2+}	$0.5*10^{-8}$M ,10^{-7}–$1.25*10^{-5}$M	CV, DPSV	Y¨uce *et al*., 2010
Escherichia coli (NCIMB 8277)/SPE	Hg^{2+}	1 ppm	Conductometry	Bhatia *et al*., 2003
Acidithiobacillus ferrooxidans/O2 Clark electrode	Cr^{3+}	$2*10^{-5}$–$40*10^{-5}$M	Amperometry/—	Zlatev *et al*., 2006
Circinella sp./CPE	Cu^{2+}	$5.4*10^{-8}$ M(0.0034 mg/L), $5*10^{-7}$ – $1*10^{-5}$M Cu^{2+}(0.032–0.635 mg/L)	DPSV	Alpat *et al*., 2008
Escherichia coli (K-12)-(PAH-PSS)3—ITO	Hg^{2+}	10^{-12}M , 10^{-12}–10^{-3}M	Electrochemical impedance spectroscopy	Souiri *et al*., 2009
Bacillus sphaericus MTCC 5100/ NH^{+4} –ISE	Ni^{2+}	0.044 ppm in food , 0.03–0.68nM	Potentiometry	Verma and Singh, 2006
Sulfur oxidizing bacteria	Cr^{6+}	5 ppb	Electrical conductivity	Oh *et al*., 2011

CPE = carbon paste electrode; ISE = ion selective electrode; ITO = indium-tin-oxide glass electrode; GCE = glassy carbon electrode; CV = cyclic voltammetry, DPSV = differential pulse stripping voltammetry

2.6.3 DNA based Metal Biosensor

During last years, there has been a huge increase in the use of nucleic acids, as a way in the recognition and monitoring of many toxic compounds of analytical interest, because many of this molecules, and especially HMs, show a high affinity for DNA and they can interact with nucleic

acids. The interaction between metal ions and DNA is important in living organisms, because it could have, either favourable, or adverse effects in life science reported to the damage, replication and transcription of DNA in *vivo*, mutation of gene, action mechanism of some synthetic chemical nucleases, and molecular analysis (Tencaliec *et al*., 2006), which often lead to the change of the structure and function of genetic materials, by development of malignant tumours (Oliveira *et al*., 2008). Thus, the ability to monitor and quantify the levels of HMs (such as Pb, Cd, and Ni) that interact with DNA is widely studied (Wong *et al*., 2007), because these ions have been detected in different sources: foods, beverages, soil, plants, natural waters, and so forth. Also, the International Agency for Research on Cancer (IARC) lists some HMs (as Pb and Cd) as possible human carcinogens, while the carcinogenic properties of Ni are related to tumour promotion (Oliveira *et al*., 2008). Usually, for HMs studies, the DNA was native (from *Calf Thymus*, double-stranded DNA, ds-DNA) or denaturated (single-stranded DNA, ss-DNA) (Wong *et al*., 2007, Babkina *et al*., 2004). The electrochemical techniques (chronopotentiometric or voltammetric methods), especially the pulse techniques, are appropriate for studying the biological systems, and also, for DNA heavy metal interactions, because they improve the selectivity and the signal-to-noise ratio, are fast, of low cost, and have high sensitivity (Tencaliec *et al*., 2006, Oliveira *et al*., 2008). An electrochemical DNA biosensor is an integrated receptor-transducer device that uses DNA as a biomolecular recognition element to measure specific binding processes with DNA, through electrochemical (especially carbon electrodes) transduction (Tencaliec *et al*., 2006). As for other biosensors, the most important factor for the construction of efficient DNA-based electrochemical biosensors is the immobilization of the DNA or its components (such as nucleotides, nucleosides, purine, and pyrimidine bases) on the electrode surface. Different adsorption immobilization procedures, such as electrostatic adsorption or evaporation, adsorption of a monolayer, or multilayer DNA films have been widely used (Oliveira *et al*., 2008). Because DNA has four different potential sites for binding of metal ions: (1) the negatively charged phosphate oxygen atoms, (2) the ribose hydroxyls, (3) the base ring nitrogens, and (4) the exocyclic base keto groups (Oliveira *et al*., 2008), in the case of DNA biosensors, there are two possibilities to detect pollutants: one is to detect the hybridization of nucleic acid sequences from infectious microorganisms, and the other one is to monitor the interaction of small pollutants with the immobilized DNA (drugs, mutagenic pollutants, etc.) (Rodriguez-Mozaz, 2004). Most HM ions interact with more than two different sites and their interactions with DNA are more complex. Under chosen conditions, the following methods of

metal-DNA interactions are proposed: (i) indirect chelation between the nitrogen *N*7 atom of the DNA purine base with the oxygen atom of the DNA phosphate backbone and (ii) direct coordination bond with the nitrogen *N*7 of guanine base (rarely of adenine). Two additional models have been identified: (a) intrastrand chelating between *N*7 and *O*6 atoms of guanine and (b) intrastrand bindings formation with the *N*7 guanine atoms of ss-DNA (Oliveira *et al*., 2008, Wong *et al*., 2007, Babkina and Ulakhovich, 2004). Several examples and the obtained results/conclusions are synthesized in Table 9.

Table 9 Examples of DNA-based electrodes

Electrode	Immobilisation technique	Toxic effect	References
GCE/ds-DNA	Deposition	Pb^{2+} interacts with ds-DNA preferentially at adenine containing segments, leading to modifications in the double-helical structure.	Oliveira *et al*., 2008
SMFE/ss-DNA	Inclusion in a CN Membrane	Pb^{2+} and Cd^{2+} covalent bind during complexation with ss-DNA. Constants of binding (estimated from Scatchard graphs): *K*bind (Pb^{2+}– ssDNA) = $(12 \pm 0.3) * 10^5\ M^{-1}$; *K*bind (Fe3+ – ssDNA) = $(1.4 \pm 0.3) * 10^5\ M^{-1}$; *K*bind ($Cd^{2+}$ – ssDNA) = $(0.6 \pm 0.2) * 10^5\ M^{-1}$. Limits of detection: 10^{-10} MPb^{2+}, 10^{-9} M Cd^{2+} and 10^{-7} M Fe^{3+}	Babkina and Ulakhovich 2004
Au/ss-DNA.	Self assembled Method	$Cd2^{+}$ has the ability to be deposited at underpotential conditions onto gold substrates when it is electrochemically reduced. Constant of binding *K*bind (Cd^{2+}-ssDNA) = $8.33 * 10^5 M^{-1}$. Limit of detection: 10pM Cd^{2+}.	Wong *et al*., 2007
SPE/ds-DNA and SPE/MWCNT-ds-DNA	Deposition on SPE	Sn^{2+} and As^{3+} was studied	Ferancov´ *et al*., 2007

GCE = glassy carbon electrode; SMFE = stationary mercury-film electrode; CN = cellulose nitrate; MWCNT= multi-wall carbon nanotubes; SPE = screen printed electrode.

2.7 Conclusion and scope

The last decade has witnessed tremendous improvement in the sensitivity and specificity of the heavy metal biosensors. The alliance between biotechnology and microelectronics has brought forward numerous opportunities to build highly sensitive and compact biosensors. A wide spectrum of biosensing elements like enzymes, whole cells (natural/genetically modified), DNA, and antibodies have been used in conjugation with different transducers systems viz., conductometric, potentiometric, calorimetric, piezoelectric etc. to develop highly integrated biosensing systems. In addition to them novel immobilization matrices like conducting polymers and carbon nano tubes have resulted in drastic improvement in the sensitivity and selectivity of biosensors by providing better signal conductivity and improved bioreceptor stability. Despite the considerable research activity devoted to the development of biosensors, analytical applications are still limited for real-time monitoring. It has been observed that all the biosensing elements and mechanisms have their sets of advantages and disadvantages viz., in whole cell biosensors, the diffusion of a substrate and product through the cell wall or membrane results in a slower response than that of enzyme-based sensors. But enzyme sensors lack stability since they are more vulnerable to immobilization startegies and harsh sample environment as compared to whole cells. The major limitation of a whole-cell biosensor made with recombinant DNA technology involves the time and cost required for the reporter gene to be transcribed and translated. Similarly, the enzyme inhibition based sensors are not usually able to discriminate various toxic compounds in the same sample. In particular, the simultaneous presence of heavy metals and pesticides in contaminated samples provides a challenge for their use for purely regulatory purposes. Thus seeing all these limitations a consolidated recommendation of any one's result cannot be accepted as universally applicable.

The goal of the present work is to provide an in-depth application of the different methodologies based on literature to fill the gap of current research towards enhancement of the stability and sensitivity of the biosensor and also make the process cost effective.

Chapter 3
Materials and Methods

3.1 Screening, isolation and identification of microbes sensitive to Cu, Zn and Cd ions

For isolation of microbes with high urease and phosphatase activity soil samples were collected from sites within Thapar University Campus, Patiala. Urease and alkaline phosphatase were chosen as the parameters to monitor the sensitivity of microbes towards Cu, Zn and Cd. Morphological, biochemical and molecular characterization based on 16S rDNA sequence analysis of the isolates was done. Production of the respective enzymes at different growth phases of isolates was studied and optimization of pH and temperature for maximal urease and alkaline phosphatase production for the isolates was carried out.

3.1.1 Isolation of phosphate solubilizing bacteria

The phosphate-solubilizing bacteria were screened and isolated on Pikovskya media (Pikovskya, 1948).

Pikovskya medium (g l^{-1}) pH 7±0.2

Glucose	10.0g
Tri calcium phosphate	5.0g
$(NH_4)_2 SO_4$	0.5g
NaCl	0.2g
$MgSO_4.7H_2O$	0.1g
KCl	0.2g
Yeast extract	0.5g
$MnSO_4$	0.025g
$FeSO_4.7H_2O$	0.020g
Agar	15g

Procedure

1. 1g of soil was added to a 10 ml water blank and shaken well.
2. 1 ml from this was added to a test tube containing 9 ml water making a dilution corresponding to 10^{-1}.
3. Further dilutions were prepared in a similar way upto 10^{-6}.

4. 100 µl inoculum was taken from dilution 10^{-3} and $10^{-4}$and added to nutrient agar plates and the inoculum was spread with the help of a spreader.
5. The plates containing inoculum were incubated at 30ºC for 24 hours and observations recorded.
6. The single colonies were streaked repeatedly on nutrient agar and Pikovskya medium for isolation of phosphate-solubilizing bacteria.
7. Bromophenol blue (0.01%) was added to the Pikovskya medium and the colonies were streaked on petri plates for obtaining zones of P-solubilization after 24 hour incubation at 30ºC.

3.1.2 Selection of alkaline phosphatase producing bacterial isolates

Single colony isolation was carried out using streak plate technique. Culture grown in liquid medium was streaked onto agar plates aseptically and incubated at 28±2°C. Repeated streaking of each well-isolated colony was carried out to obtain a pure culture (Madigan *et al*., 1997).

3.1.3 Whole cell alkaline phosphatase activity (Barnes & Morris, 1957)

Requirements:

1. *p*-nitrophenol: 100 ppm stock solution was prepared in 0.04 N NaOH for standard curve.
2. *p*-nitrophenyl phosphate: 1000 ppm stock solution was prepared in distilled water.
3. Tris-HCl: 0.1 M Tris-HCl was made and the pH adjusted to 8
4. Nurient broth for the growth of the bacteria
5. 1 N NaOH solution
6. 0.04 N NaOH

Procedure:

Standard curve:

100 ppm stock solution of *p*-nitrophenol was prepared by dissolving 0.01 g of *p*-nitrophenol in 0.04 N NaOH. Further dilutions of the stock solution were also prepared in 0.04 N NaOH and the absorbance values of these dilutions were taken at 420 nm.

Bacterial cell suspension: The bacterial strain was grown in Nutrient broth. Overnight culture (1 O.D) was used as inoculum and the culture media was incubated for overnight on a rotary shaker (Orbitek Scigenis Biotech) at 120 rpm in medium at 28±ºC. The bacterial cells from overnight

culture were harvested by centrifugation at 5500 x g for 5 min. The harvested cell pellet was suspended in 0.1 M Tris-HCl buffer (pH 8). O.D. of the cell suspension was adjusted to 1 at 600 nm.

Effect of pH and temperature on whole cell alkaline phosphatase activity of the isolates

The enzyme activity was determined for different concentrations of p-nitrophenyl phosphate and bacterial cell suspension varying one at a time. The reaction volume was made up to a final volume of 2 ml by adding required amount of distilled water. Test tubes were incubated in a water bath for 30 min. at 37ºC. After the incubation reaction was stopped by adding 1 ml of 1 N NaOH. The yellow color produced due to the formation of p-nitrophenol was measured at 420 nm. The alkaline phosphatase bioassay was performed at different pH (6-10) of 0.1 M Tris-HCl buffer to optimize the pH and at was incubated at different temperatures (25-50°C) to optimize the temperature.

Calculations

Using the standard curve the concentration of p-nitrophenol (*p*-NP) could be found for unknown samples.

The enzyme activity in terms of production of *p*-NP micromoles per minute was calculated as follows:

Enzyme activity = µmol (p-NP)/min

Mol.Wt (*p*-NP) =139

x ppm (*p*-NP) = x*1000/(139*30 min) µmol (*p*-NP)/min

(value of x ppm can be found from the standard curve)

OR

x ppm (*p*-NP) = x*0.239 µmol (*p*-NP)/min

Further experiments were done with lyophilized bacterial biomass of the selected isolates.

Lyophilization of microbial biomass

Pseudomonas striata was cultured in a 1L flask in nutrient broth to late log phase and bacterial cells were harvested by centrifuging the culture broth at 5500 x g for 10 min to obtain a cell pellet which was lyophilized at -50ºC under vaccuum using a freeze dryer (Modulyod, ThermoElectron

Corporation) to obtain dry cell mass. The lyophilized biomass of *Pseudomonas striata* was checked for alkaline phosphatase activity and stored at −20 ◦C in small vials.

Effect of substrate concentration

Effect of substrate concentration (para nitrophenyl phosphate) on whole cell alkaline phosphatase activity of *Pseudomonas striata* was studied by varying the amount of substrate from 0 to 36 mM at a constant whole cell concentration achieved by suspending 1 mg of the lyophilized bacterial biomass in the reaction mixture containing 0.1M Tris-HCl at pH 8 and optimum temperature of 37°C.

Enzyme Kinetics Parameters

Michaelis–Menten plot

This plot is a graphical representation of the effect of substrate concentration on the enzyme velocity (µmol product formed per min) and was made by ploting substrate concentration Vs. Enzyme reaction velocity

Lineweaver Burke Plot

The Lineweaver–Burk plot (or double reciprocal plot) between inverse of substrate concentration (1/[S]) Vs inverse of enzyme velocity (1/V). The Lineweaver–Burk plot is used to determine important enzyme kinetics parameters, such as Km and Vmax. The y-intercept of Lineweaver burke is equivalent to the inverse of Vmax and the x-intercept of the graph represents −1/Km

3.1.4 Isolation of urease producing bacteria

1. To make the media selective, urea (25g/l was added to Nutrient Agar (Stanier *et al*., 1987).
2. 1g of soil was added to 10 ml water blank and shaken well.
3. 1 ml from this was added to a test tube containing 9 ml water making a dilution corresponding to 10^{-1}.
4. Further dilutions were prepared in a similar way upto 10^{-6}.
5. 100µl inoculum was taken from dilution 10^{-3} and $10^{-4}$and added to nutrient agar plates and the inoculum was spread with the help of a spreader.
6. The plates containing inoculum were incubated at 37ºC for 24 hours and observations recorded.

7. The isolated colonies were carefully picked and inoculated in 50 ml Nutrient broth containing urea (25g/l) and incubated for 24 h.

3.1.5 Phenol red indicator test (Prescott *et al*., 1993)

Phenol red is a pH indicator. The release in NH_3 is accompanied by an increase in pH, leading to a change in colour. Phenol red (0.01 %) indicator was added to the nutrient agar media and slants were prepared. The indicator imparts yellow colour to the media. On inoculating the media with the isolated culture, the change in colour from yellow to violet, if any was observed over a period of time.

3.1.6 Whole cell urease activity at different pH & temperature

The whole cell urease activity was determined for all the bacterial isolates by measuring the amount of ammonia released from urea according to the phenol-hypochlorite assay method (Natarajan, 1995). Ammonium chloride (50-1000 μM) was used as the standard. Bacterial isolates were grown in nutrient broth media and 1% of overnight grown cultures of OD 1 were re-inoculated into culture media and incubated at 37°C under shaking condition (120 rpm). The bacterial cells from the overnight grown culture were harvested by centrifugation at 5500 x g for 5 minutes. The cell pellet was suspended in 50 mM potassium phosphate buffer (pH 8.0) and the optical density of the cell suspension was adjusted to $O.D_{600}$ 1. 2.5 ml of 0.1 M urea was added to 1 ml of the cell suspension and incubated at 37°C for 5 min followed by addition of phenol nitroprusside and alkaline hypochlorite, 1 ml each and incubated at 37°C for 25 min. Optical density was measured at 626 nm. One unit of urease is defined as the amount of enzyme hydrolyzing one μmole urea per min. The urease bioassay was performed at different pH (6-10) of 50 mM potassium phosphate buffer to optimize the pH and at was incubated at different temperatures (25-50°C) to optimize the temperature.

Effect of substrate concentration

Effect of substrate concentration (urea) on whole cell urease activity of *Bacillus* sp. MD 028 was studied by varying the amount of substrate from 0 to 32 mM at a constant whole cell concentration achieved by suspending 1 mg of the lyophilized bacterial biomass in the reaction mixture containing 50 mM potassium phosphate buffer at pH 8 and optimum temperature of 37°C.

3.1.7 Morphological characterization of isolates

Gram staining

Gram staining of phosphate solubilizing bacterial isolates was done and the strains were analysed microscopically (Gram, 1884).

Requirements

1. **Aqueous Crystal Violet (1%)** 1 g crystal violet in 100 mL distilled water.
2. **Gram's Iodine:** 2 g potassium iodide in 100 mL distilled water.
3. **Decolorizer** acetone (50%) and alcohol (50%).
4. **Aqueous safranin (2%)** 2g safranin in 100 mL distilled water.

Procedure

1. Preparation of a fixed bacterial smear: A drop of bacterial culture was placed on a slide with the help of an inoculation needle spread evenly in the center of the slide. The smear was dried and heat-fixed.
2. The slide was placed on a staining rack and flooded with crystal violet for about 1 min.
3. The stain was washed gently with iodine solution and stained with fresh iodine solution for 1 min followed by washing in tap water or by dipping in a beaker containing water.
4. A few drops of decolorizer were added and continued until colour ceased to come out of the preparation. This took 5 seconds to 1 minute.
5. Washing repeated gently with water as in step 4.
6. Counter-stained with dilute carbol fuchsin or safranin for 10-30 seconds.
7. Again the slide was washed with water and dried with absorbent paper and left for drying by evaporation.
8. The dry slide is a permanent preparation, which was examined under the microscope directly without a cover slip first under low power and then under higher magnification.

Confirmation of the Gram character of the isolates was done by carrying out KOH test.

3.1.8 Biochemical characterization of isolates

Following biochemical tests (Cappucino *et al*., 1987) were performed on the alkaline phosphatase and urease producing bacterial isolates.

Catalase test

Procedure

1. One drop of 3% H_2O_2 was placed on a glass slide.
2. With a sterile inoculating loop, transferred several isolated colonies of the organism to the H_2O_2 on the microscope slide.
3. The slide was observed for the formation of gas bubbles for upto 1 minute.

Oxidase test

Procedure

1. Added 1-2 drops of freshly prepared 3% tetramethyl-p- phenylene diamine solution on a filter paper.
2. Bacterial isolate was lifted with the help of sterile cotton swab and rubbed on the presoaked tetramethyl-*p*-phenylenediamine filter paper.
3. A colour change to purple within 30 seconds was looked for.

Nitrate reduction test

Procedure

1. Nitrate agar plates were inoculated with the cultures and incubated at 37 °C until growth was observed.
2. To the grown bacterial colony, added few drops of sulphanilic acid and few drops of alphanaphtalamine, each.
3. The appearance of a red colour was noted.

3.1.9 Molecular characterization of isolates

Isolation of genomic DNA (Sambrook *et al*., 1989)

1. 2ml bacterial culture was grown overnight.
2. This culture was taken in eppendorf tubes and centrifuged at speed of 13,000 rpm for 5 min.
3. The bacterial pellet was dissolved in 800 µl of saline EDTA.
4. To this, 50 µl of lysozyme was added and incubated at 37ºC in a shaker for 30 min.
5. 200 µl of 10 % SDS was added and mixed thoroughly by inversion.
6. The solution was maintained at 65 ºC for 15 minutes in a water bath.

7. 1000 µl of Phenol-$CHCl_3$-isoamyl alcohol (25:24:1) was added and centrifuged at 10000 r.p.m for 10 minutes.
8. The upper aqueous layer was removed in a fresh tube and equal volume of isopropanol was added and mixed gently. It was, then centrifuged at 13,000 r.p.m for 10 minutes.
9. To the precipitated DNA equal volume of 70 % ethanol was added and centrifuged at 13,000 r.p.m for 10 minutes.
10. The supernatant was discarded and the pellet was air dried.
11. The pellet was suspended in 25-50 µl of TE buffer and electrophoresed on) 7% agarose gel.

16 s rDNA-PCR

The 16s rDNA reaction mixture was prepared in 25 µl as mentioned below.

Primers used in 16s rDNA-PCR

Primer Name	Type	Sequence (5'----->3')
E8F	Forward	AGAGTTTGATCCTGGCTCAG
E1492R	Reverse	GGTTACCTTGTTACGACATT

Reaction mixture prepared for 16s rDNA-PCR

Reagent	Volume (µl)
Buffer (10X)	2.5
Magnesium Chloride (25 mM)	2
4 dNTPs (10 mM)	2
2 Primers (10 mM)	0.5+0.5
Taq polymerase (5U/ µl)	0.15
DNA	1
Water	16.35
Total Volume	25.0

Taq polymerase and dNTPs were procured from Fermantas Life Sciences, whereas primers of Qiagen Ltd. were used. The PCR reaction was run in the thermocycler (Applied Biosystems) as per the following program

Thermocycler Program for 16s-rDNA-PCR

Reaction	Temperature (ºC)	Duration (Seconds)
Initial Denaturation	92	180
Denaturation	92	70
Annealing	48	30
Extension	72	130
Final Extension	72	370
Number of Cycles:36		

The 16s rDNA-PCR products were analysed on 0.7% agarose gel.

Ligation of the desired PCR product into *E.coli* vector

The PCR products were directly cloned into the pGEM-T Easy vector system using the Promega Cloning Kit. The pGEM-T Esay vector multiple cloning region is Flanked by recognition sites for the restriction enzymes EcoR1, BstZ1 and Not1, thus providing three single-enzyme digestions for the release of the insert. The ligated vectrors were incubated at 4ºC for 16 hrs and transferred to refrigerated conditions for further storage.

Reaction mixture prepared for the ligation of PCR product in pGEM-T Easy vector

Reagents	Volume (µl)
Ligation Buffer	5
T4 DNA Ligase	1
Vector pGEM T Esay	1
DNA	4

Transformation using $CaCl_2$ treatment and Heat Shock method

1. *E. Coli* DH5α cells were taken from the glycerol stock and straked on Luria Agar (LA) Plates and incubated at 37 ºC.
2. Isolated colonies were inoculated in luria broth (LB) and incubated overnight at 37 ºC.

3. 100 µl of the overnight grown culture was inoculated in 50 ml LB and incubated in a shaker at 180 rpm for 5 hrs and then the culture was centrifugrd at 5500 x g for 15 mins at 5 ºC in autoclaved oakridge tube.
4. The supernatant was discarded and 10 ml of the filter sterilized 0.1 M $CaCl_2$ was added and the tubes were incubated in the ice for 15 mins.
5. The cells were again centrifuged and the supernatant was discarded and 1 ml of 0.1 M $CaCl_2$ was added and the tubes were incubated in ice for 3 hrs to make competent cells.
6. To 100 µl of the competent cells 5 µl of the ligated product was added and mixed gently and kept in ice for 30 mins for binding of the plasmid to the cells.
7. Then the cells were given a heat shock treatment for exactly 2 mins at 42 ºC in a still watr bath.
8. The cells were then kept in ice for 2-3 mins and then 1 ml of the LB+Ampicillin (50mg/ml) was added to each tube and the tubes were then kept at 37 ºC for 1 hr for expression of Amp^r gene of thr transformd cells.
9. Meanwhile on LA + amp (50 mg/ml) plates X-Gal and IPTG were plated and kept for incubation for 30 mins at 37 ºC. (50 mg/ml stock of X-Gal was prepared by adding 100mg of X-Gal to 2ml of N,N'-dimethylformamide.).1 M stock of IPTG was prepared by adding 1.2 gms of IPTG to 50 ml of mQ water (20 µl of X-Gal and 100 µl of IPTG were plated on each plate).
10. 75 µl, 100 µl, 200 µl and 300 µl of the the transformed cells were palted on LA+Amp+X-Gal+IPTG plates.
11. The plates were incubated at 37ºC for 16-20 hrs and checked for appearance of white and blue colonies, and kept them in refrigerator overnight to intensify the blue colour of the colonies to differentiate between recombinants and non-recombinants.

Screening of the positive clones

Plasmid Isolation

1. The plasmid of the recombinant cells was isolated using the alkali lysis method to confirm the presence of the plasmid in the cells.
2. The recombinant white colonies were picked up from the plates with the help of autoclaved toothpicks and inoculated in 2ml of LB+ Amp and also a patch of the same

colony was made on LA+Amp plates with proper naming/numbering and incubated overnight at 37 ºC.

3. 1.5 ml of the culture was taken in a tube and centrifuged the cells were dissolvd in the solution by vortexing and kept at room temperature for 5 mins.
4. 200 µl of freshly prepared solution 2 was added to the cells and mixed the contents by inverting the tubes 10-12 times rapidly and kept for 2 mins.
5. 50 µl of the solution 3 was added and the tubes were inverted slowly 10-12 times and kept in ice for 10-15 mins.
6. 400 µl of phenol:chloroform:isoamylalcohol (24:25:1) was added to the contents and the tubes were inverted for 2 mins and later centrifuged at 12,000g for 10 mins.
7. The upper aqueous layer was transferred to a fresh tube and added 800 µl of 95% ethanol.
8. After Proper vortexing the tubes were kept at room temperature for 5 mins and centrifuged at 12000g for 5 ins for precipitation of DNA.
9. The supernatant was discarded and the pellet was washed with 400 µl of 70% ethanol and centrifuged 12,000g for 5 mins.
10. The supernatant was removed and the pellet was air-dried for 20 mins and dissolved in 50 µl of autoclaved mQ water
11. 0.7% agarose gel electrophoresis (50 volts) was done for 1:5 diluted DNA sample.

Tests for confirming the right recombinant plasmid

There are two tests that were carried out to confirm the presence of the right insert in the isolated plasmid.

Restriction digestion of the plasmid with EcoR1 endonuclease

The plasmid has two sites for EcoR1 endonuclease one on each side of the insert. So the plasmid when cut with EcoR1 was expected to produce at least two bands one of plasmid size (3kb) and the other of the insert size, least the insert itself does not have the a site for the enzyme, otherwise insert might break into segments. Ribonuclease helps in removing non specific RNA molecules from the reaction mixture.

Reagents	Volume (µl)
Buffer (10 X)	2
Enzyme	0.5
DNA sample (Plasmid)	10
Ribonuclease	0.5
Water	7

The total reaction mixture was made up to 20 µl. The restriction enzyme used was provided by Fermantas Life Sciences. The buffer used for this process was provided with the enzyme for the increased activity of the enzyme. All the samples were incubated at 37ºC for 3 hrs. The enzyme reaction was stopped by keeping the samples at -20 ºC and the samples were loaded in 1% agarose gel and observed under UV transilluminator.

PCR amplification with T7 and SP6 primers

The plasmid has T7 and SP6 primers on either side of the insert. In order to confirm the presence of the insert these primers can be used. Likewise, the primers were used to carry out PCR amplification with the plasmids. This would result in amplification of the DNA of size approximately 200 bp larger than the original size of the insert in the plasmid. The high-copy-number pGEM-T Easy vector contains T7 and SP6 RNA polymerase promoters flanking a multiple colony region within the α-peptide coding region of the enzyme β-galactosidase.

Reaction Mixture prepared for the PCR

Reaction mixture (25 µl)	Volume (µl)
PCR Buffer (10X)	2.5
MgCl2 (50mM)	1.5
dNTPs (2mM)	2
T7 primer	1
SP6 primer	1
Taq DNA polymerase- 5U/ml	0.15
DNA – plasmid (10 ng)	1.5
Water	15.35

Sequence of T7 and SP6 primers

Primer	Type	Sequence
T7	20 mer	TAATACGACTCACTATAGGG
SP6	19 mer	GATTTAGGTGACACTATAG

Restriction enzymes, Taq polymerase and dNTPs were procured from fermantas Life sciences, whereas primers of Qiagen Ltd. were used. The PCR reaction was run in the thermocycler (Applied Biosystems) as per the following program

Thermocycler program followed for PCR

Reaction	Temperature (ºC)	Duration (Sec)
Initial Denaturation	94	180
Denaturation	94	60
Annealing	52	60
Extension	72	120
Final Extension	72	320
NUMBER OF CYCLES:36		

3.1.10 Sequencing of 16srDNA insert

The transformed colonies were streaked on agar slants and were sent to Labindia DNA analysis services, Gurgaon for sequencing of the 16srDNA insert.

3.1.11 Phylogenetic analysis

The sequences of all the 16s rDNA segments of isolates were BLASTed and compared with already existing sequences in the database to identify the most probable similarity with high expect value. Multiple sequence alignment of all the sequences obtained with existing sequences in databank was carried out using the program MULTALIN (http://prodes.toulouse.inra.fr/multalin). Then a Neighbor-joining tree was constructed using the program MEGA3 version. 16s rDNA sequences were submitted to NCBI database with the help of software Sequin and the corresponding accession numbers were received.

3.2 Metal-microbe interaction studies to determine threshold concentration of metal ions

Threshold concentrations (Linear range and Detection limits) of the three metal ions viz., Cu, Zn and Cd were determined for whole cell enzyme activities (alkaline phosphatase and urease) of *Pseudomonas striata* and *Bacillus sp.* MD028 (FJ005050) respectively. The metal-microbe interaction studies were conducted using lyophilized biomass of *P. striata* and *Bacillus* sp. MD028. The urease activity was measured using phenol-hypochlorite method (Natarajan, 1995) and phosphatase activity was determined spectrophotometrically at 420 nm with p-NPP (para nitrophenyl phosphate) as the substrate (Barnes and Morris, 1957).

3.2.1 Interactions of *Pseudomonas striata* and Heavy metals

The *in-vivo* inhibitory effect of the three heavy metals viz., Zn, Cu and Cd on the whole cell alkaline phosphatase activity of the *Pseudomonas striata* was studied using a colorimetric reaction between para nitro phenyl phosphate (p-NPP) and the *P. striata* alkaline phosphatase. The reaction was determined spectrophotometrically (420 nm) by monitoring the yellow coloured product p-NP (para nitrophenol). The reaction mixture consisted of 1 mg of lyophilized *P.striata* biomass suspended in Tris-HCl buffer (0.1 mM, pH 8) as the source of enzyme and the substrate (p-NPP) 30 mM. The three heavy metals viz., Zn, Cu and Cd were separately added to each reaction mixture such that one particular reaction mixture contained only one heavy metal at a particular concentration ranging from 1-50 mg/l. A control test without heavy metals was conducted in parallel. The reaction mixture was incubated in water bath at a temperature of 37ºC for 30 min. After 30 min the reaction was stopped by adding 1 ml of 1 N NaOH to each reaction mixture and the absorbance was taken at 420 nm.

3.2.2 Interactions of *Bacillus sp.* MD028 (Acc. No. FJ005050) and Heavy metals

The *in-vivo* inhibitory effect of the three heavy metals viz., Zn, Cu and Cd on the whole cell urease activity of the *Bacillus* sp. (FJ005050) was studied using phenol-hypochlorite method (Natarajan, 1995). The reaction was determined spectrophotometrically (625 nm) by monitoring the blue coloured product indophenol. The reaction mixture consisted of 1 mg of lyophilized *Bacillus sp. MD028 (FJ005050)* biomass suspended in 50 mM potassium phosphate buffer as the source of enzyme, and the substrate (Urea) 20 mM. The three heavy metals viz., Zn, Cu and Cd in the concentration range of 1-50 mg/l were separately added to the reaction mixture such that

one particular reaction mixture contained only one heavy metal at a particular concentration. A control test without heavy metals was conducted in parallel. The reaction mixture was incubated at 37°C for 5 min followed by addition of phenol nitroprusside and alkaline hypochlorite, 1 ml each and incubated at 37°C for 25 min. After 25 min the absorbance values of the reaction mixtures were taken at 625 nm using a UV-Vis spectrophotometer.

Calculation

$I = 100 (V_C - V_I)/V_C$

Where I: Inhibition (in %), V_I: Reaction velocity in inhibition test after incubation time (min), and V_C : observed velocity in the control test after incubation time (min).

3.3 Immobilization and process optimization for whole cell-electrode assembly and its application for detection of heavy metals.

Chemicals and Reagents

Pyrrole (Py), PVS (polyvinyl sulphonic acid), potassium monohydrogen phosphate and potassium dihydrogen phosphate, glutamate dehydrogenase (GLDH), nicotinamide adenine dinucleotide (NADH), α-ketoglutarate (α -KG) were procured from Sigma–Aldrich, Milwankee, USA. Indium-tin-oxide (ITO) coated glass plates were obtained from Balzers UK. All the chemicals and reagents used in the present studies were of molecular biology (MB) grade. These reagents were prepared in de-ionized water (Milli Q 10 TS) and the solutions and glasswares were autoclaved prior to being used.

3.3.1 Electrochemical preparation of PPy-PVS/ITO electrodes (Prabhakar *et al*., 2007)

PPy-PVS/ITO electrodes were prepared by polymerization of pyrrole on the surface of indium tin oxide (ITO). ITO glass plate was cut into several small pieces with uniform size of 1x2 cm^2. Before polymerization the surface of the ITO glass plate was cleaned with acetone in order to remove any dirt and debris. The ITO glass surface should not be scratched as it can cause the indium tin oxide coating to come out and effect the conducting property of the ITO glass.

Pyrrole distillation Prior to polymerization distillation of the pyrrole was done to remove the stabilizers, which are added by the manufacturers to prevent self-polymerization. Distillation also removes impurities and oxidized pyrrole which is brown in colour. Upon distillation pyrrole becomes colorless.

Electrochemical polymerization of PPy-PVS films

PPy-PVS films were polymerized chrono-potentiometrically in a three-electrodes electrochemical cell having Ag/AgCl as reference electrode (cathode), platinum as a counter and ITO (indium tin oxide) glass plate (1×2 cm^2) as a working electrode (anode) using Potentiostat/Galvanostat (Model 273A, Princeton Applied Research). Monomer solution containing 0.1M Py, 0.1M PVS was subjected to constant current (200 µA) for about 15 min to obtain PPy-PVS films (conductivity ~ 120 S cm^{-1}) onto ITO glass plates with a working area of about 1 cm^2.

3.3.2 Optimization of the glutamate dehydrogenase

Optimum amount of glutamate dehydrogenase (GLDH) was worked out by covalently immobilizing different concentrations of GLDH on the PPy-PVS/ITO electrodes. Different quantities of GLDH ranging from 0.05-0.3 mg were suspended in 10 µl of milli Q water in an eppendorf. This was mixed with 3 µl each of EDC (15 mM) and NHS (30 mM) and kept for 2 hours. After 2 hours 4 µl milli Q water was added and the dilution was spread on PPy-PVs/ITO electrode. 5 µl of MQ was spread over the electrode surface and was covered with petridish to keep it moist and was refrigerated for overnight. Effect of copper (1.5 mgl^{-1}), cadmium (30 mgl^{-1}) and zinc (30 mgl^{-1}) was studied on different concentrations of GLDH using sqaure wave voltammetry in phosphate buffered saline (50 mM, 0.9% NaCl) containing 5 mM $[Fe(CN)_6]^{3-/4-}$) in the presence of 30 µl nicotinamide adenine dinucleotide (NADH, 0.5 mM), 70 µl α-Keto glutarate (2.5 mM) and 5 mM NH_4Cl. NH_4Cl was used as the source of NH_4^+ ions instead of urease and urea since the inhibition of only GLDH had to be monitored.

3.3.3 Immobilization of lyophilized bacterial biomass of *Bacillus* sp. MD028

The lyophilized biomass of *Bacillus sp*. MD028 was immobilized on to conducting polymer matrix of polypyrrole polysulfonate. Two types of immobilization techniques were used:

1. Physical entrapment
2. Chemical cross-linking using EDC-NHS (EDC [*N*-(3-dimethylaminopropyl)-*N*-ethyl-carbodiimide hydrochloride], NHS [*N*-hydroxy-succinimide]

Physical immobilization

The physical entrapment of the bacterial biomass was achieved by suspending the lyophilized bacterial biomass (10-60 mg) in 10 ml monomer solution containing 0.1M Py (pyrrole) and 0.1M PVS (poly vinyl sulphonate), which was subjected to constant current of 200 µA for about 900 seconds to obtain a Ppy-pVS film with entrapped bacterial biomass on the surface of the working electrode (ITO glass plate (1X2 cm^2)).

Covalent immobilization

The covalent immobilization on the surface of PPy-PVS/ITO electrode was done by chemically cross linking the lyophilized biomass of *Bacillus* sp. MD028 and GLDH on the surface of the PPy-PVS/ITO electrode using EDC-NHS solutions.

Stock solutions required

EDC (15 mM)

NHS (30 mM)

Lyophilized biomass of *Bacillus* sp. MD028 (0.2 g/ml)

GLDH (0.05 g/ml) (optimized amount)

5 µl each of the above stocks were mixed in an eppendorf tube and kept for 2 hours. After 2 hours the above mixture (total volume 20 µl) was spread on the surface of a PPy-PVS/ITO electrode. 5 µl of MQ was spread over the electrode surface and was covered with petridish to keep it moist and refrigerated for overnight. Several such electrodes were prepared as per the requirement.

3.3.4 Effect of pH on the *Bacillus*-GLDH/PPy-PVS/ITO bioelectrodes

The effect of pH in the range of 2-9 on *Bacillus*/PPy-PVS/ITO bioelectrodes was studied using square wave voltammetry recorded in phosphate buffer saline (PBS, 50 mM, 0.9% NaCl) containing 5mM $[Fe(CN)_6]^{3-/4-}$ and in the potential range, -0.6 to 1.4 V at scan rate of 20mV/s.

3.3.5 Electrochemical response studies of *Bacillus*-GLDH/PPy-PVS/ITO bioelectrode

To determine the linear range and detection limit of urea using *Bacillus*-GLDH/PPy-PVS/ITO bioelectrode electrochemical response studies of *Bacillus*-GLDH/PPy-PVS/ITO bioelectrode were carried out as a function of urea concentration in the presence of 30µL of nicotinamide

adenine dinucleotide (NADH, 3.7 mg/dL) and 70µL of α-Keto glutamate (α -KG, 47.5 mg/dL) using square wave voltammetry in PBS solution (50mM PBS (pH 7, 0.9%NaCl) containing 5mM $[Fe(CN)_6]^{3-/4-}$). Urea concentrations in the range of 1-40 mM were used. Response of the bioelectrode was recorded using square wave voltammetry in the potential range of -0.6 to 1.4 V at a scan rate of 20 mV/s.

3.3.6 Electrochemical detection of heavy metals using *Bacillus*-GLDH/PPy-PVS/ITO bioelectrode

Inhibition of the whole cell urease activity of *Bacillus sp*. MD 028 in the presence of heavy metal was studied using square wave voltammetry technique recorded on Autolab Potentiostat/Galvanostat (Eco Chemie, Netherlands) in the potential range of -0.6 to 1.4 V at a scan rate of 20mV/s. 10 ml of the reaction mixture contained 30µL of nicotinamide adenine dinucleotide (NADH, 3.7 mg/dL) and 70µL of α-Keto glutamate (α -KG, 47.5 mg/dL) in PBS solution (50mM PBS (pH 7, 0.9%NaCl) containing 5mM $[Fe(CN)_6]^{3-/4-}$) and 20 mM urea. Copper, cadmium and zinc were separately added to each reaction mixture such that one particular reaction mixture contained only one heavy metal at a particular concentration ranging from 1-2 mg/l, 1-30 mg/l, 1-40 mg/l for copper, cadmium and zinc respectively.

3.3.7 Shelf life of *Bacillus*-GLDH/PPy-PVS/ITO bioelectrode

The *Bacillus*-GLDH/PPy-PVS/ITO bioelectrodes were stored under dry conditions at 4°C. The urease activity of the bioelectrodes was monitored by measuring the spectrophotometric response using Phenol hypochlorite method (Natranjan 1995) with respect to time at regular interval of 2 week for 24 weeks.

3.3.8 Zinc selective potentiometric electrode based on *Pseudomonas striata* immobilized in PVC

Preparation of the Electrode and EMF Measurements (Mittal *et al*., 2007)

Membranes of ~ 0.2 mm thickness were obtained by pouring a solution of the membrane components of PVC 33%, ligand (lyophilized biomass of *Pseudomonas striata*) 2-6%, and o-nitro phenyl octyl ether 63% dissolved in 2-3 mL of tetrahydrofurane (THF). The viscous solution of polymer thus, obtained was poured in a glass ring of 30 mm diameter placed on a dust free Pyrex glass plate. The solvent was allowed to evaporate slowly for about 24 h at room

temperature. To obtain membranes with similar characteristics, viscosity of the casting solution and the rate of solvent evaporation were controlled so that thickness and morphology of membranes remained almost unchanged. Membranes were then removed from the glass ring and circular pieces of 1.25 cm diameter were cut and mounted on the ground end of a Pyrex glass tube with araldite and conditioned with the metal ion solution for 24 h.

EMF Measurements

All the emf measurements were carried out using the following cell assembly:

Ag/AgCl, KCl (sat.) ‖0.1 M $Zn^{+2}/Cd^{+2}/Cu^{+2}$ |membrane| $Zn^{+2}/Cd^{+2}/Cu^{+2}$ test solu‖ KCl (sat.), AgCl/Ag

Salt bridges containing KNO_3 were used to provide electrical links between KCl and Ag^+ solutions on both sides of the membrane. A digital potentiometer having a sensitivity of 0.1 mV (Equiptronics EQ602, India) was used for the potential measurements. Activities were calculated according to the Debye-Hückle equation (Kielland, 1937). Standard metal nitrates solutions were obtained by gradual dilution of a 0.1 M metal salt solution, and their emf measurements were performed.

Temperature - It is important that temperature be controlled as variation in this parameter can lead to significant measurement errors. A single degree (C) change in sample temperature can lead to measurement errors greater than 4%. The experimental set up was placed in a thermostat temperature of which was maintained up to ±0.1 °C.

Agitation - When carrying out selective ion measurements, it is important to have a good agitation. This allows a fresh supply of ions to be exposed to the sensing portion of the ISE. An agitation of the solution was done by stirring it using a telfon-coated magnetic bead and a magnetic stirrer at a moderate speed.

Response Time - ISEs require a much longer time for the reading to stabilize. Sufficient time was allowed for equilibrium to be established while measuring standard solutions. The response time was measured when the mV reading remained stable for about 30 seconds.

Rinsing - It is necessary to rinse the ISE between measurements to insure accurate readings. A steady stream of deionized or distilled water was used. The electrode was wiped off the excess water by shaking it gently.

Conditioning - The ISE needs to remain moist at all times even when not in use. It was kept immersed in standard solution of primary ion when not in use.

Slope - It is the linear part of the calibration curve of the electrode. The theoretical value according tothe Nernst equation is: 59.16 [mV/log(a_x)]at 298 K for a single charged ion or 59.16/2 = 29.58 [mVperdecade] for a double charged ion.

Range of Linear Response - At high and very low target ion activities there are deviations from linearity. Typically, the electrode calibration curve exhibits linear response in a range between 10^{-5}M and 10^{-1}M.

Detection Limit - The detection limit is defined by the cross-section of the two extrapolated linear parts of the ion-selective calibration curve (Mittal *et al*., 2007). Detection limit of the order of 10^{-5}-10^{-6}M was measured for most of ion-selective electrodes.

Selectivity

Selectivity is one of the most important characteristics of an electrode, as it often determines whether a reliable measurement in the sample is possible or not. The selectivity coefficient (K_{xy}) has been introduced in the Nikolski-Eisenman equation. Negative values indicate a preference for the target ion relative to the interfering ion. Positive values of log K_{xy} indicate a preference of the electrode for an interfering ion. The experimental selectivity coefficients depend on the activity and the method of their determination. Different methods of the selectivity determination can be found in the literature. The IUPAC suggests two methods (Umezawa *et al*., 1995): separate solution method (SSM) and fixed interference method (FIM). There is also an alternative method of the selectivity determination called matched potential method (MPM). Each of them has got advantages and drawbacks, and there are not general rules pointing, which method gives the true result. Selectivity coefficients were determined by using FIM & MPM methods (Umezawa *et al*., 1995).

Mixed Solution Method - There are various measurement methods using mixed solutions of the two ions. The fixed interference method is commonly used. The emf of a cell comprising an ion-selective electrode and a reference electrode is measured with solutions of constant activity of

interfering ion, a_B, and varying activity of the primary ion. The emf values obtained are plotted against the logarithm of the activity of the primary ion a_A. The intersection of the extrapolation of the linear portions of this plot indicates the value of a_A which is to be used to calculate K_{AB} from the Nikolski-Eisenman equation

$$K_{AB} = \frac{a_A}{a_B^{Z_a/Z_B}}$$

Matched Potential Method - This method involves measuring potentials of a pure solution of the primary ion, E_A and of a mixed solution containing the primary and interfering ions, E_{A+B}. Activities of the primary ion are the same in both solutions. And the selectivity co-efficient is calculated by inserting the values of potential difference, $\Delta E = (E_{A+B} - E_A)$, a_A and a_B into the following equation

$$K_{AB}^{Pot} = \frac{a_A(10^{\Delta E Z_A F / 2.303})}{a_B^{Z_A/Z_B}} - 1$$

In this method, primary ions (A) of a specified activity (concentration) are added into a reference solution, and the potential is measured. In a separate experiment, interfering ions (B) are successively added to an identical reference solution until the measured potential matches with that obtained before adding the primary ions. In the matched potential method, the selectivity coefficient K_{AB} is then given by the ratio of the resulting activities (concentrations) of primary ions versus interfering ions. The selectivity coefficient, K_{AB}, is determined as:

$K_{AB} = \Delta A / a_B$

Here $\Delta A = a_A - a\bullet_A$; $\mathbf{a}_A$ is the initial activity of primary ions A and $a\bullet_A$ is the activity of A in the presence of interfering ions at $\mathbf{a}_B$.

Chapter 4
Results

4.1 Screening, isolation and identification of microbes sensitive to Cu, Zn and Cd ions

For isolation of microbes with high urease and phosphatase activity soil samples were collected from sites within Thapar University Campus, Patiala. Urease and alkaline phosphatase were chosen as the parameters to monitor the sensitivity of microbes towards Cu, Zn and Cd. Morphological, biochemical and molecular characterization based on 16S rDNA sequence analysis of the isolates were carried out and the isolates P8 and P10 were identified as *Bacillus* sp. MD028 (FJ005050) and *Bacillus subtilis* strain MD008 (EU780733) respectively. Optimization of pH and temperature for maximal urease and alkaline phosphatase activity by the isolates was done and effect of substrate concentration was studied.

4.1.1 Isolation of alkaline phosphatase producing bacteria

The phosphate-solubilizing bacteria were screened and isolated on Pikovskya media (Pikovskya, 1948). Formation of clear halo zones around the colonies confirmed the solubilization of the insoluble phosphates by the phosphatases released by the bacterial colony. Five bacterial isolates with clear halo zones and different colony morphology were streaked on separate agar slants and studied for morphological and biochemical characterization.

4.1.1.1 Morphological and biochemical characterization of bacterial isolates

Bacterial isolates were characterized for colony shape, size and elevation Gram character and for the presence of spores, capsule and biochemical characters like catalase, oxidase and nitrate reductase activity as per the standard protocol (Table 10) (Cappuccino *et al*., 1987). All the isolates formed opaque creamy colonies without any pigmentation on nutrient agar media. Colonies of the four isolates (AP4, AP7, AP10, AP11) were circular and convex whereas the Isolate AP5 was found to have wavy and flat colony morphology.

Table 10 Morphological and biochemical characterization of alkaline phosphatase +ve bacterial isolates

Bacterial isolates	Morphological						Biochemical			
	Colony Shape	Colony Elevation	Cell shape	Gram Staining	Capsule staining	Spore Staining	Alkaline phosphatase	Catalase	Oxidase	Nitrate Reductase
AP4	Circular	Convex	Short Rod	-	-	-	+	+	+	-
AP5	Wavy	Flat	Long Rod	+	-	-	+	+	-	+
AP7	Circular	Convex	Long rod	-	-	-	+	-	+	+
AP10	Circular	Convex	Long Rod	+	-	-	+	+	-	-
AP11	Circular	Convex	Short Rod	+	-	-	+	-	+	-

+: positive reaction; –:negative reaction;

AP5 and AP10 were Gram positive long rods, AP4 was Gram negative with short rods AP7 was gram negative with long rods while AP11 was gram positive with short rods. None of the isolates were found to have capsule or spores. All the isolates showed positive alkaline phosphatase activity. Three isolates (AP4, AP5, AP10) tested positive for catalase activity and whereas among the 5 isolates only AP5 and AP10 did not show any oxidase activity. Only two isolates (AP5 and AP7) showed positive nitrate reductase activity.

4.1.1.2 Quantitative estimation of alkaline phosphatase activity of isolates

Quantitative estimation of the alkaline phosphatase activity of the isolates was done by a colorimetric method using *p*-NPP as substrate (Barnes and Morris, 1957). Along with the selected bacterial isolates, *Pseudomonas striata* procured from Division of Microbiology, IARI, New Delhi was also checked for alkaline phosphatase activity. The isolates (AP4, AP5, AP7, AP10 and AP11) showed alkaline phosphatase activity of 1.5, 1.3, 1.4, 2 and 1.7 U/ml respectively. However, *Pseudomonas striata* showed the maximum activity of 2.3 U/ml among all the isolates and was selected for further enzymatic assay (Fig 5).

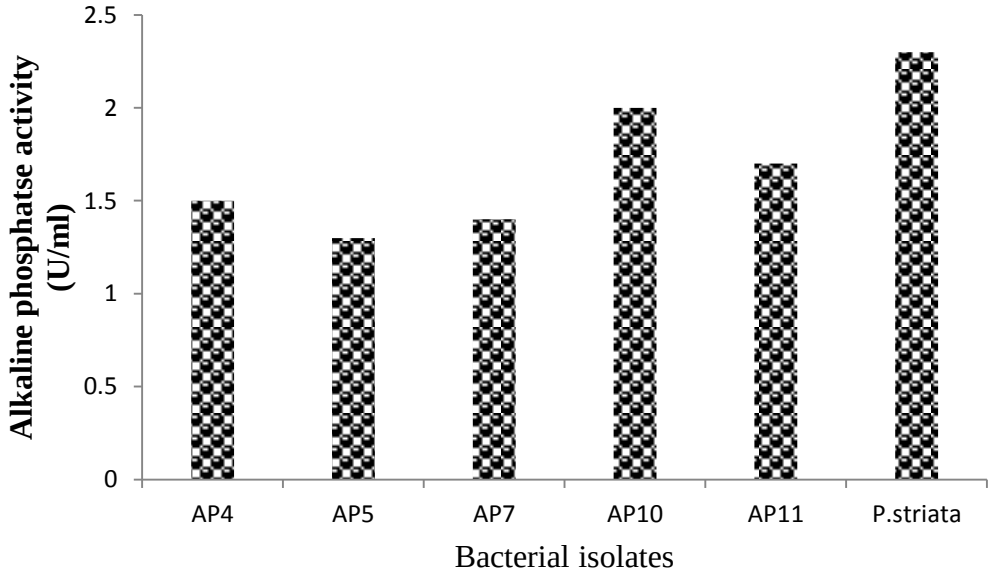

Fig 5 Alkaline phosphatase activity of bacterial isolates

4.1.1.3 Optimization of parameters for maximum alkaline phosphatase activity

Parameters such as temperature and pH were optimized to achieve the maximum alkaline phosphatase activity.

Temperature

The effect of temperature on the alkaline phosphatase activity produced by bacterial isolates is presented in Table (11). The maximum alkaline phosphatase activity was observed at 37º C by all isolates (Fig 6). Generally above 40-50ºC the enzyme activity decreased because denaturation of proteins occurs at these temperatures. Alkaline phosphatase activity of all isolates was lower at 50 º C.

Table 11 Optimization of temperature for maximum alkaline phosphatase activity (U/ml) by different bacterial isolates (Values are mean ± SD (n =3))

Temp ℃	AP4	AP7	AP10	AP11	*P. striata*
25	1.23±0.07	0.92±0.02	1.15±0.08	1.4±0.04	1.7±0.07
30	1.5±0.06	1.2±0.02	1.4±0.04	1.7±0.07	1.9±0.02
37	11.4±0.04	1.7±0.06	1.7±0.07	1.6±0.03	2.1±0.05
45	1.1±0.05	0.77±0.05	1±0.05	1.3±.01	1.6±0.06
50	0.84±0.04	0.7±0.07	0.5±0.06	0.5±0.06	0.7±0.01

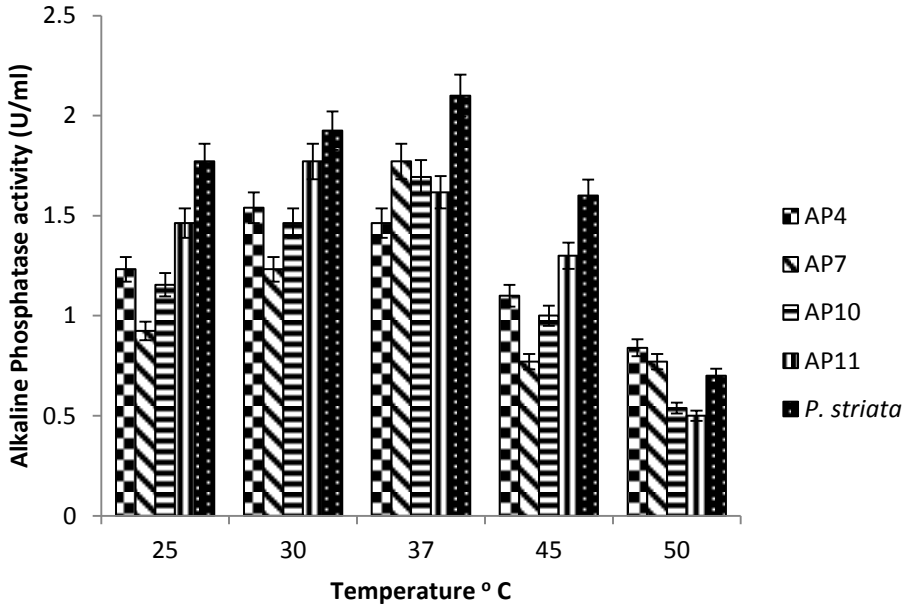

Fig 6 Effect of temperature on the alkaline phosphatase activity (U/ml) by different bacterial isolates. Values are mean ± SD (n =3)

pH

The effect of pH on the alkaline phosphatase activity is shown in Table 12; Fig (7). Tris-HCl buffer of different pH was used to measure enzyme activity. The optimum pH for the maximum alkaline phosphatase activity was observed at pH 8 by all isolates and the maximum alkaline phosphatase activity was observed for *Pseudomonas striata*. A significant improvement in alkaline phosphatase activity was found when incubated at pH 8.0 and after that alkaline phosphatase activity reduced drastically.

Table 12 Optimization of pH for maximum alkaline phosphatase activity (U/ml) by different bacterial isolates (Values are mean ± SD (n =3))

pH	AP4	AP7	AP10	AP11	*P.striata*
6	1±0.02	0.9±0.07	0.7±0.02	1±0.05	1.1±0.05
7	1.4±0.05	1.7±0.05	1.7±0.04	1.6±0.04	2±0.06
8	1.7±0.08	1.7±0.08	1.6±0.09	2±0.07	2.3±0.07
9	1.4±0.08	1.6±0.04	1.5±0.06	2.1±0.03	2.1±0.01
10	1.1±0.06	1±0.05	1.3±0.06	1.9±0.04	1.8±0.08

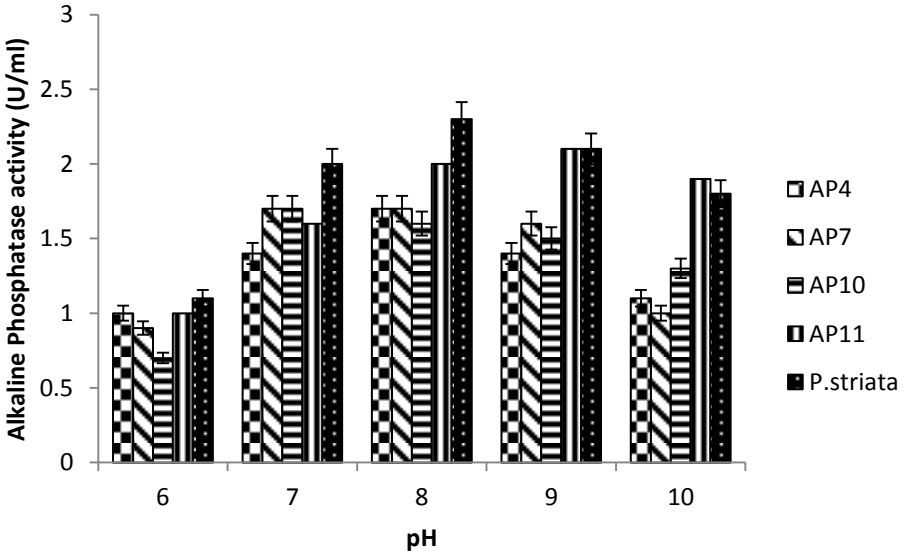

Fig 7 Effect of pH on the alkaline phosphatase activity (U/ml) by different bacterial isolates. Values are mean ± SD (n =3)

Effect of substrate concentration on alkaline phosphatase activity of *P. striata*

Effect of substrate concentration (para nitrophenyl phosphate) on whole cell alkaline phosphatase activity of *Pseudomonas striata* was studied by varying the amount of substrate from 0 to 36 mM at a constant whole cell concentration achieved by suspending 1 mg of the bacterial biomass in the reaction mixture containing 0.1M Tris-HCl at pH 8 and optimum temperature of 37°C. As can be inferred from Fig 8 the reaction velocity initially increased almost linearly till the substrate concentration of 22 mM. Thereafter the increase in reaction velocity became smaller till 28 mM of *p*-NPP and beyond this concentration the increase in reaction velocity was vanishingly small. Thus 30 mM substrate concentration was selected as the optimum concentration for achieving maximum alkaline phosphatase activity. The enzyme kinetics parameters Vmax and Km were calculated using Lineweaver Burke plot (Fig 9). The Km and Vmax values were 14.28 mM and 4.1 µmol. min^{-1}

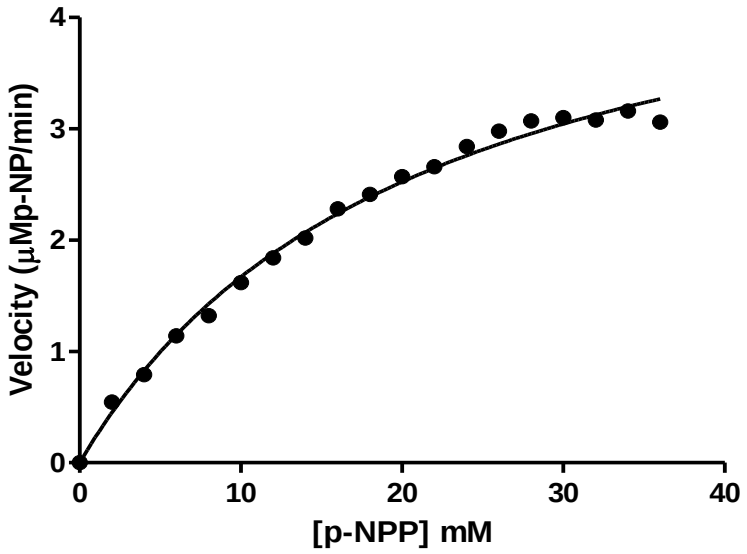

Fig 8 Michaelis-Menten Plot for whole cell alkaline phosphatase activity of *P. striata*

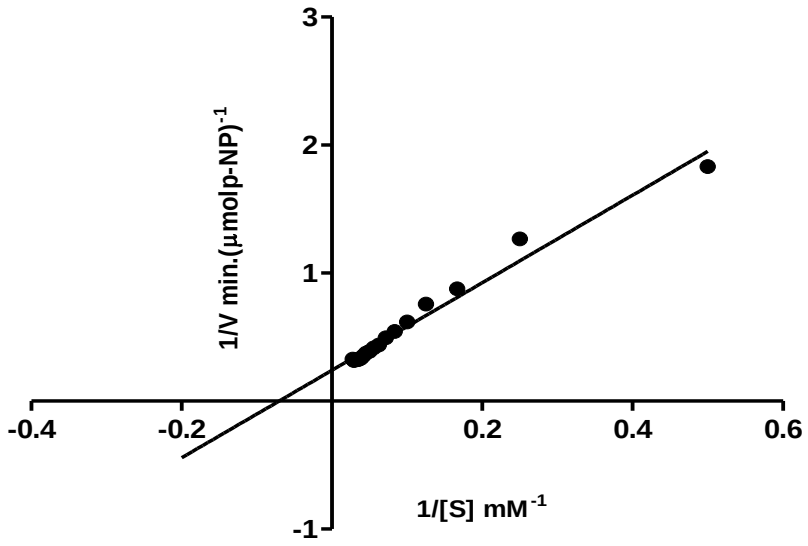

Fig 9 Lineweaver burke plot for alkaline phosphatase activity of *P. striata*

4.1.2 Isolation of urease producing bacteria

Urease producing bacteria were isolated from the soil samples collected from agricultural fields of Thapar University Campus, Patiala (India) using urea enriched nutrient Agar (Stanier *et al.*, 1987). The isolated bacteria were subjected to primary screening for the selection of efficient strains. The primary screening of the bacteria included qualitative tests for detecting the ability of the isolates to utilize urea as an energy source. Qualitatively the isolates were screened for urease activity using phenol red indicator media. Urease producing isolates were further quantitatively screened for urease production using the colorimetric estimation by phenol-hypochlorite method (Natarajan, 1995).

4.1.2.1 Selection of urease producing isolates

Out of the various colonies obtained on the urea enriched nutrient agar plates 10 bacterial colonies with different morphology were selected for the estimation of the urease activity using phenol red indicator test. The urease activity was confirmed using phenol red indicator media slants (Fig 10). Change in the colour of phenol red indicator media from yellow to purple was observed for all the 10 isolates with varying intensity of purple colour. Maximum colour change was observed for 4 isolates P2, P6, P10 and P8 (Fig 10). Further these 4 isolates were studied for morphological and biochemical characterization.

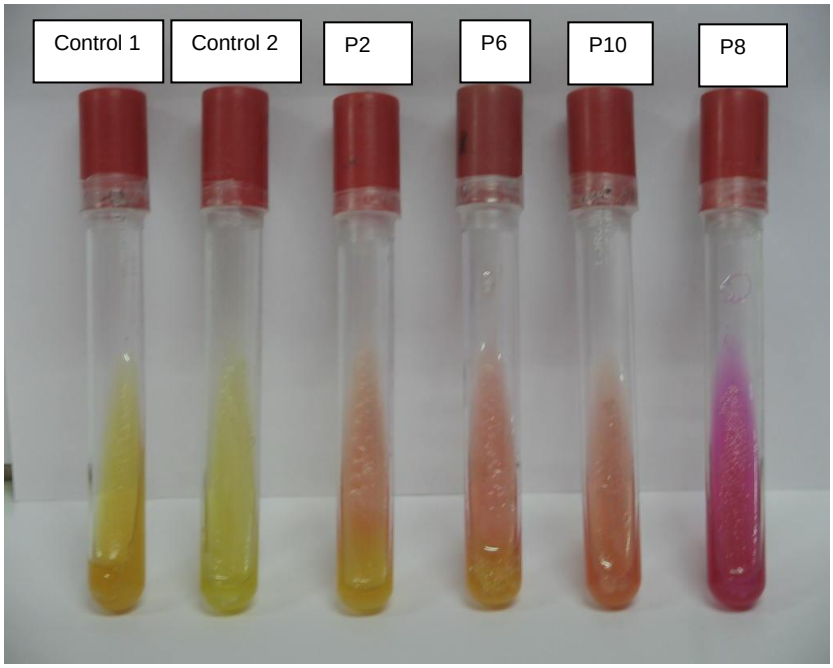

Fig 10 Phenol red indicator test for the urease +ve bacterial isolates

4.1.2.2 Morphological and biochemical characterization of bacterial isolate

Bacterial isolates were characterized for colony shape, size and elevation and for the presence of spores, capsule, catalase, oxidase and Gram character as per the standard protocol (Table 13) (Cappuccino *et al*., 1987). All the isolates formed opaque creamy colony without any pigmentation on nutrient agar media except for P10 which formed translucent colonies. The colonies of all the four isolates were circular and convex. All the isolates were gram positive and rod shaped. None of the isolates were found to have capsule or spores. All the isolates showed positive urease, catalase and oxidase activity and only two isolates P6 and P8 tested positive for nitrate reductase activity.

Table 13 Morphological and biochemical characterization of urease +ve bacterial isolates

Bacterial isolates	Morphological						Biochemical			
	Colony Shape	Colony Elevation	Cell shape	Gram Staining	Capsule staining	Spore Staining	Urease	Catalase	Oxidase	Nitrate Reductase
P2	Circular	Convex	Short Rod	+	-	-	+	+	+	-
P6	Circular	Convex	Long Rod	+	-	-	+	+	+	+
P8	Circular	Convex	Long rod	+	-	-	+	+	+	+
P10	Circular	Convex	Long Rod	+	-	-	+	+	+	-

+: positive reaction; –: negative reaction;

4.1.2.3 Optimization of parameters for maximum urease activity

Parameters such as temperature, pH, and substrate concentrations were optimized to achieve the maximum urease activity.

Temperature

The effect of temperature on the urease activity of bacterial isolates is presented in Table (14); Fig (11). The maximum urease activity was observed at 37º C by all isolates. Generally above 40-50ºC the enzyme activity decreased because denaturation of proteins occurs at these temperatures. Urease activity of all isolates was lower at 50 º C.

Table 14 Optimization of temperature for maximum urease activity (U/ml) by different bacterial isolates (Values are mean ± SD (n =3))

Temp °C	P2	P6	P10	P8
25	5.5±0.19	5.6±0.18	5.8±0.35	5.8±0.21
30	6±0.16	6.3±0.31	6.7±0.26	7.6±0.09
37	6.3±0.09	6.7±0.39	7.2±0.12	8.3±0.13
45	5.8±0.21	6±0.25	6±0.29	7±0.18
50	4.3±0.38	4.1±0.31	3.5±0.17	3.8±0.23

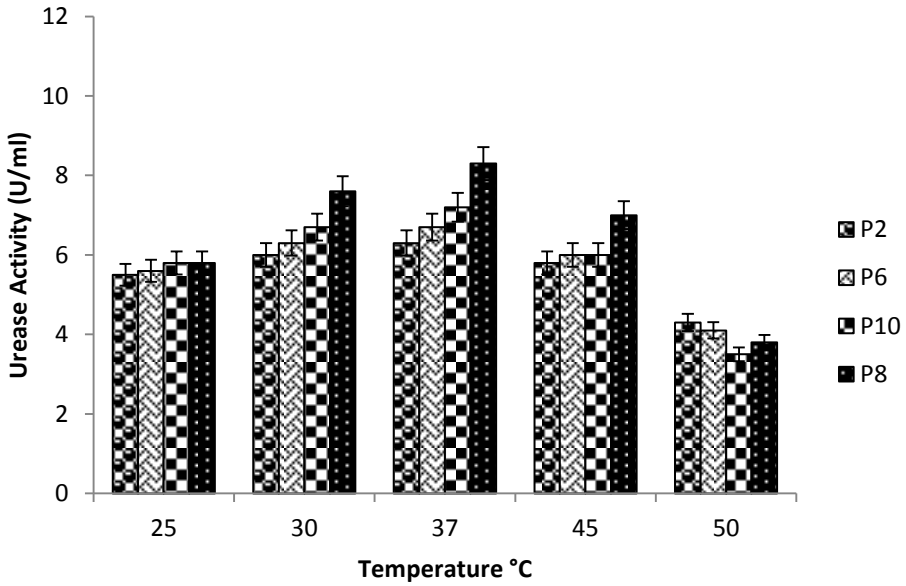

Fig 11 Effect of temperature on the urease activity (U/ml) by different bacterial isolates. Values are mean ± SD (n =3)

pH

The effect of pH on the urease activity is shown in Table 15, Fig (12). Different pH of potassium phosphate buffer was used to measure urease activity. The urease activity of all the isolates was maximum and comparable at pH 8 and pH 9, thus pH 8 was chosen for further experimental studies. Maximum urease activity was observed in P8 followed by P10.

Table 15 Optimization of pH for maximum urease activity (U/ml) by different bacterial isolates (Values are mean ± SD (n =3))

pH	P2	P6	P10	P8
6	5.4±0.12	5.3±0.31	5.8±0.26	6.3±0.14
7	6.3±0.22	6±0.29	6.6±0.32	8.3±0.28
8	6.6±0.18	6.3±0.13	6.9±0.21	9.2±0.32
9	6.7±0.15	6.6±0.34	6.9±0.22	9.2±0.33
10	6.1±0.18	6.3±0.36	6.3±0.31	8±0.25

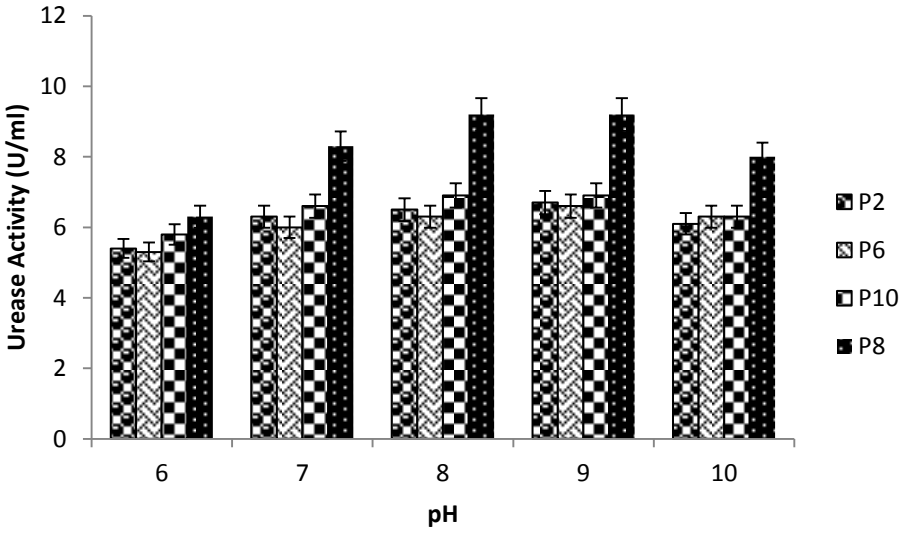

Fig12 Effect of pH on the urease activity (U/ml) by different bacterial isolates. Values are mean ± SD (n =3)

Effect of substrate concentration on urease activity of *Bacillus* sp. MD028

Effect of substrate concentration (urea) on whole cell urease activity of *Bacillus* sp. MD 028 was studied by varying the amount of substrate from 0 to 32 mM at a constant whole cell concentration achieved by suspending 1 mg of the bacterial biomass in the reaction mixture containing 50 mM at pH 8 and optimum temperature of 37°C. As can be inferred from the Fig 13 the reaction velocity initially increased almost linearly up till the substrate concentration of 20 mM. Thereafter the increase in reaction velocity was very small. Thus 20 mM substrate concentration was selected as the optimum concentration for achieving maximum urease activity. The enzyme kinetics parameters Vmax and Km were calculated using Lineweaver-Burke plot (Fig 14). The Km and Vmax values were 8.13 mM and 16.6 µmol. min^{-1}

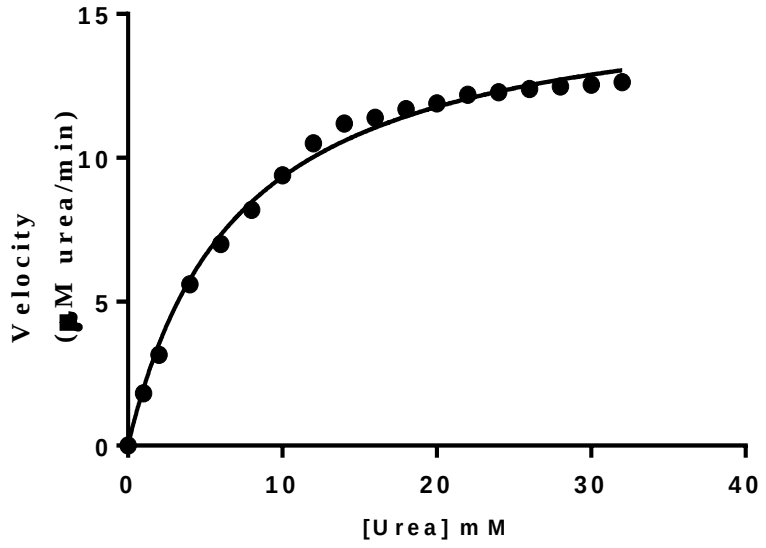

Fig 13 Michaelis-Menten Plot for whole cell urease activity of *Bacillus* sp. MD 028

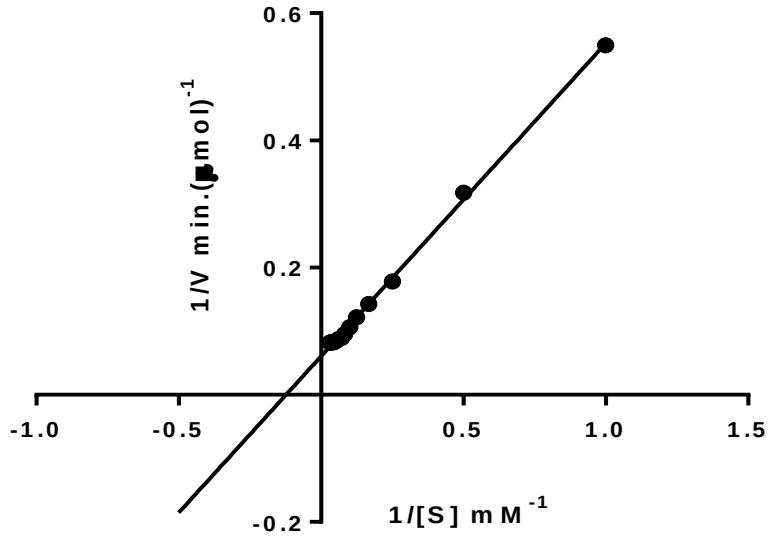

Fig 14 Lineweaver burk plot for whole cell urease activity of *Bacillus* sp. MD 028

4.1.2.4 Molecular identification of the urease +ve bacterial isolates

The isolates P8 and P10 which showed the maximum urease activity were subjected to 16S rRNA amplification using universal primers and about 1.5 Kb amplicon was observed in both isolates (Fig.15). 16S rDNA PCR products were cloned into pGEM-T Easy Vector (Promega Inc., USA) System. The plasmid DNA was extracted from different clones and amplified with T7 and SP6 primers. The 16S rRNA products from selected clones were sent for sequencing to Labindia DNA analysis services, Gurgaon. Sequencing reactions were performed with T7 and SP6 primers.

16S rDNA sequences of the bacterial isolates were obtained from Labindia DNA analysis services, Gurgaon. Sequences were compared for the similarity in the GenBank DNA database using BlastN (NCBI) (Altschul *et al*., 1997). The partial sequences of all the 16S rDNA of bacterial isolates were BLAST compared with already existing sequences in the database to identify the most probable similarity with high expect value. Multiple sequence alignment of all the sequences obtained with existing sequences in databank was carried out using the program MULTALIN (http://prodes.toulouse.inra.fr/multalin). The phylogenetic tree was constructed based on the sequences of 16S rDNA gene and those obtained from the GenBank database after BLAST analysis using the Neighbor-joining method of MEGA4 software (Fig 17). Branch lengths and scale bar corresponding to the evolutionary distances (% 100) was assigned by MEGA4, measured by the number of nucleotide

substitutions between sequences. A similarity of 50.3% was found between these two isolates Pairwise alignment revealed that 16S rDNA of bacterial isolates had 88 to 99% similarity with the sequences of NCBI database (Tables 16 & 17, Fig 16).

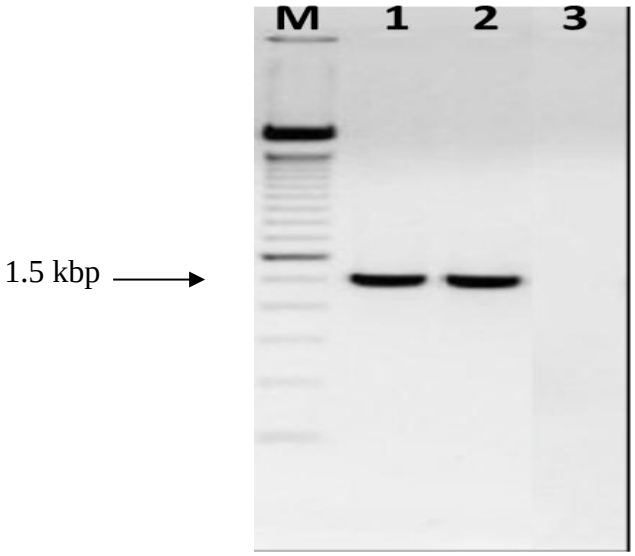

Fig 15 16S rDNA amplification of bacterial isolates. Lane 1-2: P8 and P10; Lane 3: Control and Lane M: 1 Kb marker (Fermentas)

```
#=======================================
# Aligned_sequences: 2
# 1: P8
# 2: P10
# Matrix: EDNAFULL
# Gap_penalty: 10.0
# Extend_penalty: 0.5
#
# Length: 1454
# Identity:     731/1454 (50.3%)
# Similarity:   731/1454 (50.3%)
# Gaps:         687/1454 (47.2%)
# Score: 3365.5
#=======================================

P8                 1 ------------------------------------------------
0

P10                1 gagtttgatcctggctcaggacgaacgctggcggcgtgcctaatacatgc
50

P8                 1 ------------------------------------------------
0

P10               51 aagtcgagcggacagatgggagcttgctccctgatgttagcggcggacgg
100
```

```
P8                1 ----------------------------------------------------
0

P10             101 gtgagtaacacgtgggtaacctgcctgtaagactgggataactccgggaa
150

P8                1 ----------------------------------------------------
0

P10             151 accggggctaataccggatggttgtttgaaccgcatggttcaaacataaa
200

P8                1 ----------------------------------------------------
0

P10             201 aggtggcttcgactaccacttacagatggacccgcggcgcattagctagt
250

P8                1 ----------------------------------------------------
0

P10             251 tggtgaggtaatggctcaccaaggcaacgatgcgtagccgacctgagagg
300

P8                1 ----------------------------------------------------
0

P10             301 gtgatcggccacactgggactgagacacggcccagactcctacgggaggc
350

P8                1 ----------------------------------------------------
0

P10             351 agcagtagggaatcttccgcaatggacgaaagtctgacggagcaacgccg
400

P8                1 ----------------------------------------------------
0

P10             401 cgtgagtgatgaaagttttcggatcgtaaagctctgttgttagggaagaa
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P8                1 ----------------------------------------------------
0

P10             451 caagtaccgttcgaatagggcggtaccttgacggtacctaaccagaaagc
500

P8                1 ----------------------------------------------------
0

P10             501 cacggctaactacgtgccagcagccgcggtaatacgtaggtggcaagcgt
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P10             551 tgtccggaattattgggcgtaaagggctcgcaggcggtttcttaagtctg
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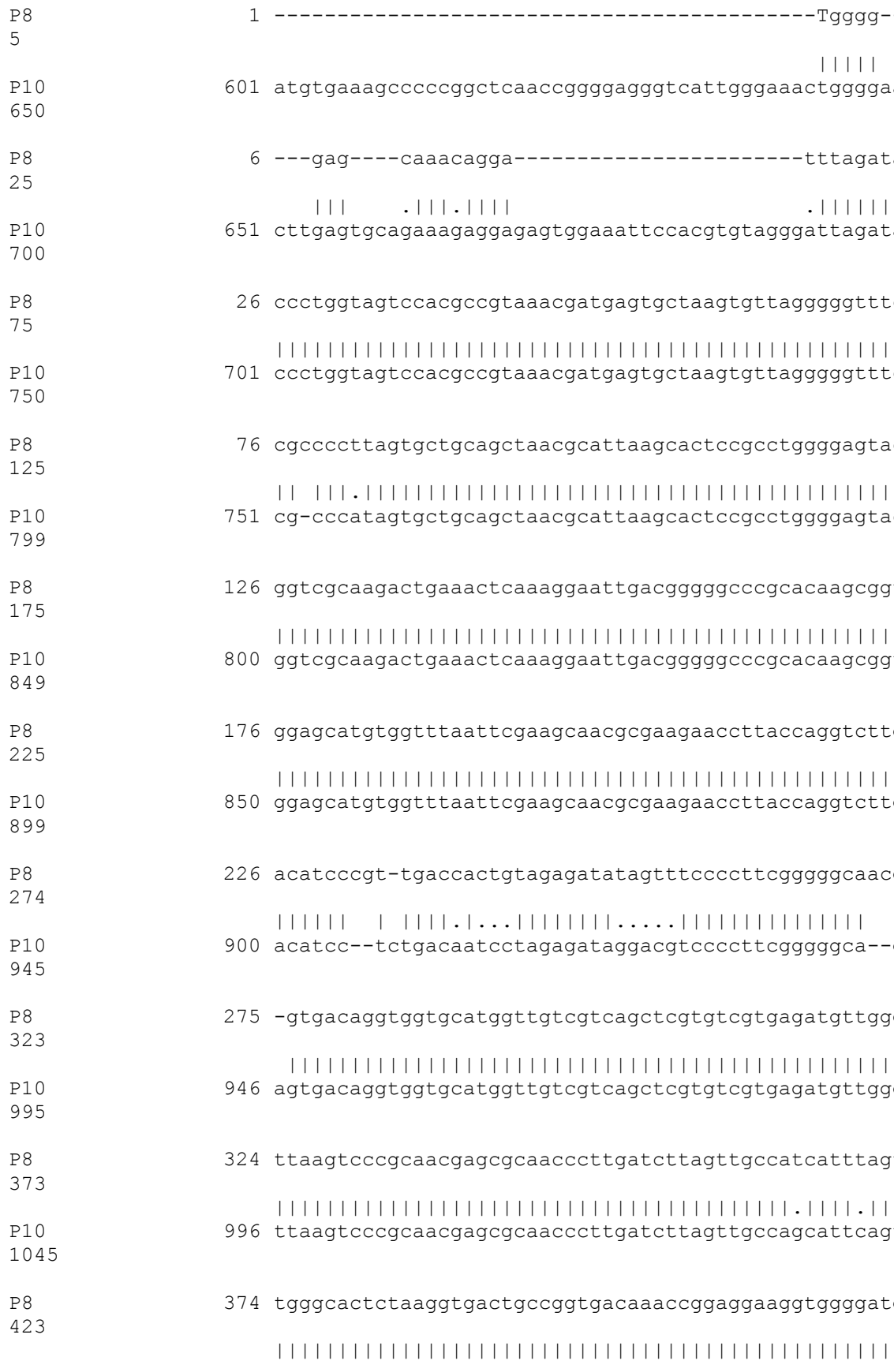
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|||||
P10 601 atgtgaaagcccccggctcaaccggggagggtcattgggaaactggggaa
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P8 6 ---gag----caaacagga----------tttagata
25
||| .|||.|||| .|||||||
P10 651 cttgagtgcagaaagaggagagtggaaattccacgtgtagggattagata
700
P8 26 ccctggtagtccacgccgtaaacgatgagtgctaagtgttagggggtttc
75
|||||||||||||||||||||||||||||||||||||||||||||||||||
P10 701 ccctggtagtccacgccgtaaacgatgagtgctaagtgttagggggtttc
750
P8 76 cgccccttagtgctgcagctaacgcattaagcactccgcctggggagtac
125
|| |||.||||||||||||||||||||||||||||||||||||||||||||
P10 751 cg-cccatagtgctgcagctaacgcattaagcactccgcctggggagtac
799
P8 126 ggtcgcaagactgaaactcaaaggaattgacgggggcccgcacaagcggt
175
|||||||||||||||||||||||||||||||||||||||||||||||||||
P10 800 ggtcgcaagactgaaactcaaaggaattgacgggggcccgcacaagcggt
849
P8 176 ggagcatgtggtttaattcgaagcaacgcgaagaaccttaccaggtcttg
225
|||||||||||||||||||||||||||||||||||||||||||||||||||
P10 850 ggagcatgtggtttaattcgaagcaacgcgaagaaccttaccaggtcttg
899
P8 226 acatcccgt-tgaccactgtagagatatagtttccccttcgggggcaacg
274
|||||| | ||||.|...||||||||.....|||||||||||||| |
P10 900 acatcc--tctgacaatcctagagataggacgtcccccttcgggggca--g
945
P8 275 -gtgacaggtggtgcatggttgtcgtcagctcgtgtcgtgagatgttggg
323
|||||||||||||||||||||||||||||||||||||||||||||||||||
P10 946 agtgacaggtggtgcatggttgtcgtcagctcgtgtcgtgagatgttggg
995
P8 324 ttaagtcccgcaacgagcgcaacccttgatcttagttgccatcatttagt
373
|||||||||||||||||||||||||||||||||||||.||||.|||
P10 996 ttaagtcccgcaacgagcgcaacccttgatcttagttgccagcattcagt
1045
P8 374 tgggcactctaaggtgactgccggtgacaaaccggaggaaggtggggatg
423
|||||||||||||||||||||||||||||||||||||||||||||||||||

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1095

P8               424 acgtcaaatcatcatgccccttatgacctgggctacacacgtgctacaat
473
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P10             1096 acgtcaaatcatcatgccccttatgacctgggctacacacgtgctacaat
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P8               474 ggac-gatacaaacggttgccaactcgcgagagggagctaatccgataaa
522
                     |||| || |||||.||..||.||..||||||....|||.|||||.|.|||
P10             1146 ggacaga-acaaagggcagcgaaaccgcgaggttaagccaatcccacaaa
1194

P8               523 gtc-gttctcagttcggattgtaggctgcaactcgcctacatgaagccgg
571
                      || ||||||||||||||.|.||.||||||||||.||.|.||||||.||
P10             1195 -tctgttctcagttcggatcgcagtctgcaactcgactgcgtgaagctgg
1243

P8               572 aatcgctagtaatcgcggatcagcatgccgcggtgaatacgttcccgggc
621
                     ||||||||||||||||||||||||||||||||||||||||||||||||||||
P10             1244 aatcgctagtaatcgcggatcagcatgccgcggtgaatacgttcccgggc
1293

P8               622 cttgtacacaccgcccgtcacaccacgagagtttgtaacacccgaagtcg
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P10             1294 cttgtacacaccgcccgtcacaccacgagagtttgtaacacccgaagtcg
1343

P8               672 gtgaggtaacctttt-ggagccagccgccgaaggtgggatagatgattgg
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P10             1344 gtgaggtaacctttaggagccagccgccgaaggtgggacagatgattgg
1393

P8               721 ggtgaagtcgtaacaaggtagccgtatcggaaggtgtggctggatcacct
770
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1443

P8               771 cctt    774
                     ||||
P10             1444 cctt   1447
```

Fig. 16 Pairwise sequence alignment data of both isolates (P8 and P10) using EMBOSS Needle program

Table 16 Sequences of P8 (*Bacillus* sp.) which showed homology to the 16S rDNA gene of other bacteria

Accession No.	Description	Max score	Total score	Query coverage	E value	Max identity
GU566326.1	*Bacillus* sp. JU2(2010) 16S ribosomal RNA gene, partial sequence	1413	1413	99%	0.0	99%
FJ528593.1	*Lysinibacillus sphaericus* strain G10 16S ribosomal RNA gene, partial sequence	1413	1413	99%	0.0	99%
GU384236.1	*Bacillus cereus* strain ZQN6 16S ribosomal RNA gene, partial sequence	1408	1408	99%	0.0	99%
FJ418643.1	*Lysinibacillus fusiformis* strain WH22 16S ribosomal RNA gene, complete sequence	1408	1408	99%	0.0	99%
AY548956.1	*Bacillus fusiformis* strain S10 16S ribosomal RNA gene, complete sequence	1408	1408	99%	0.0	99%

Table 17 Sequences of P10 (*Bacillus subtilis*) which showed homology to the 16S rDNA gene of other bacteria

Accession No.	Description	Max score	Total score	Query coverage	E value	Max identity
JF412545.1	*Bacillus subtilis* strain TUL322 16S ribosomal RNA gene, partial sequence	1384	2617	99%	0.0	99%
EF423598.1	*Bacillus subtilis* strain BCRC 17435 16S ribosomal RNA gene, partial sequence	1384	2613	99%	0.0	99%
JQ435698.1	*Bacillus subtilis* strain CE1 16S ribosomal RNA gene, partial sequence	1382	2620	100%	0.0	99%
HQ687501.1	*Bacillus subtilis* strain KJB06-35 16S ribosomal RNA gene, partial sequence	1382	2620	100%	0.0	99%
EF105451.1	*Geobacillus* sp. A9.17 16S ribosomal RNA gene, partial sequence	1557	1557	93%	0.0	88%

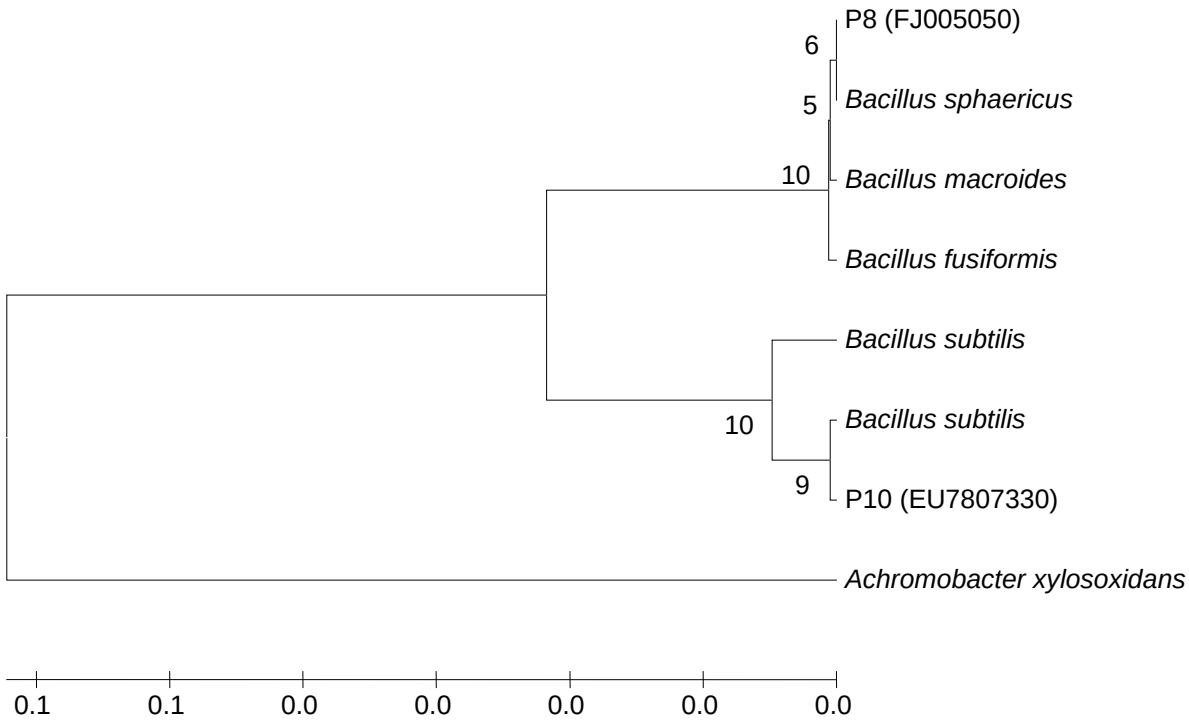

Fig (17) Neighbor-joining tree based on bacterial 16S rRNA sequence data from different isolates of current study along with sequences available in GenBank database. Numerical values indicate bootstrap percentile.

The nucleotide BLAST analysis showed that both isolates belong to phylum Firmicutes and family Bacillaceae. Phylogenetic analyses of the 16S rDNA region showed a reasonable degree of correlation with the morphological classification schemes of species within the genus. Six sequences were included in the dataset, *Achromobacter xylosoxidans* was included as outgroup taxon for rooting purposes. The phylogenetic analysis grouped P8 into *Bacillus sphaericus*, *B. macroides* and *B. fusiformis* with similarity ranging from 53 to 100%. The isolate P10 showed 99% similarity with *Bacillus subtilis*. Phylogenetic analysis revealed that isolate P8 is *Bacillus* sp., while isolate P10 is *B. subtilis*.

The 16S rDNA gene sequences of isolates P8 and P10 determined in this study were deposited in the GenBank of NCBI data library under accession numbers FJ005050 and EU7807330, respectively.

GenBank flat file: (P10)

Bacillus subtilis strain MD008 16S ribosomal RNA gene, partial sequence

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VERSION     EU780733
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            Bacillus.
REFERENCE   1 (bases 1 to 1447)
AUTHORS     Datta,M. and Goyal,D.
TITLE       Bacterial strains isolated from soil of Patiala, Punjab
JOURNAL     Unpublished
REFERENCE   2  (bases 1 to 1447)
AUTHORS     Datta,M. and Goyal,D.
TITLE       Direct Submission
JOURNAL     Submitted (31-MAY-2008) Biotechnology, Thapar University,
            Bhadson Road, Patiala, Punjab 147004, India
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Bacillus sp. MD028 16S ribosomal RNA gene, partial sequence

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REFERENCE   1  (bases 1 to 774)
AUTHORS     Datta,M. and Goyal,D.
TITLE       Diversity of bacteria isolated from soil of Patiala, Punjab
JOURNAL     Unpublished
REFERENCE   2  (bases 1 to 774)
AUTHORS     Datta,M. and Goyal,D.
TITLE       Direct Submission
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4.2 Metal-microbe interaction studies to determine threshold concentration of metal ions

Threshold concentrations (Linear range and Detection limits) of the three metal ions viz., Cu, Zn and Cd were determined for whole cell enzyme activities (alkaline phosphatase and urease) of *Pseudomonas striata* and *Bacillus sp.* MD028 (FJ005050) respectively. The urease activity was measured using phenol-hypochlorite method (Natarajan, 1995) and phosphatase activity was determined spectrophotometrically at 420 nm with *p*-NPP (para nitrophenyl phosphate) as the substrate (Barnes and Morris, 1957).

4.2.1 Interactions of *Pseudomonas striata* and heavy metals

The *in-vivo* inhibitory effect of the three heavy metals viz., Zn, Cu and Cd on the whole cell alkaline phosphatase activity of the *Pseudomonas striata* was studied using a colorimetric reaction between para nitro phenyl phosphate (*p*-NPP) and the *P. striata* alkaline phosphatase. The reaction was determined spectrophotometrically (420 nm) by monitoring the yellow coloured product *p*-NP (para nitrophenol). The reaction mixture consisted of 1 mg of lyophilized *P.striata* biomass suspended in Tris-HCl buffer (0.1 mM, pH 8) as the source of enzyme, the three heavy metals viz., Zn, Cu and Cd ranging from the concentration range of 1-50 mg/l and the substrate (*p*-NPP) 30 mM. A control test without heavy metals was conducted in parallel.

Inhibitory effect of zinc on the whole cell alkaline phosphatase activity of *P. striata*

The optical assays for alkaline phosphatase inhibition were carried out at different conentrations of Zn(II). It was observed that the rate of substrate (*p*-NPP) hydrolysis decreased with increase in Zn^{2+} ion concentration until a constant absorbance was obtained indicating the maximum alkaline phosphatase inhibition (%Inhibition (I) = 93.9%) an effect that occurred at 1.7 mg l^{-1} (Fig 18). The decrease in the APA activity of *P. striata* was linear for zinc concentration in the range of 0.01-1 mg l^{-1} (r^2= 0.9931) (Fig 19), and the limit of detection for the determination of Zn^{2+} ions was 10 µg l^{-1} (Table 18).

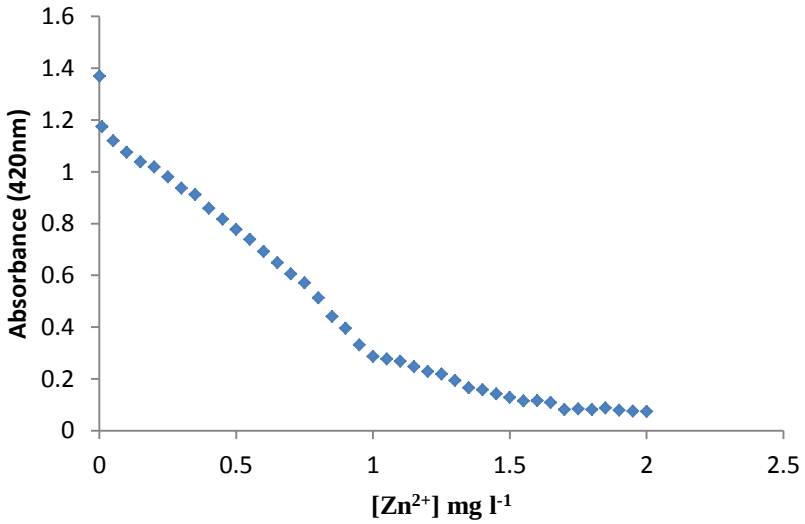

Fig 18 Inhibitory effect of Zn^{2+} (mg l^{-1}) on the alkaline phosphatase activity of *P. striata*

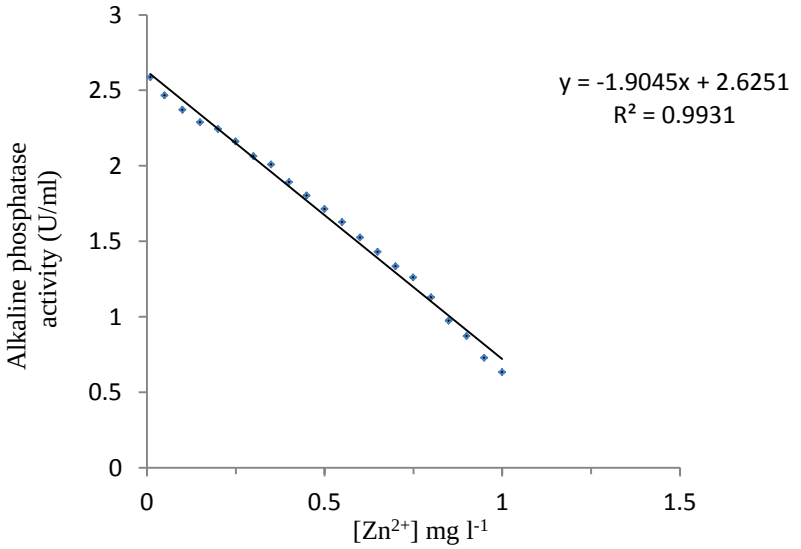

Fig 19 Linear range for detection of zinc based on the inhibition of whole cell alkaline phosphatase activity of *P. striata*

Inhibitory effect of cadmium on the whole cell alkaline phosphatase activity of *P. striata*

The concentration range of cadmium (mg l^{-1}) used for the optical assays was broader than that of zinc. This implied that cadmium was comparitively less toxic than zinc. Hydrolysis

of the substrate (p-NPP) catalysed by alkaline phosphatase was inversely related with the Cd^{2+} ion concentration. The maximum inhibition percentage (I%) of APA observed was 86.3 % at a concentration of 4 mg l^{-1} (Fig 20). Two linear ranges for cadmium detection were observed from 0.1-2.5 mg l^{-1} (r^2=0.9813) (Fig 21) and from 2.5-4 mg l^{-1} (r^2=0.9903) (Fig 22). The detection limit for cadmium was found to be 100 µg l^{-1}.

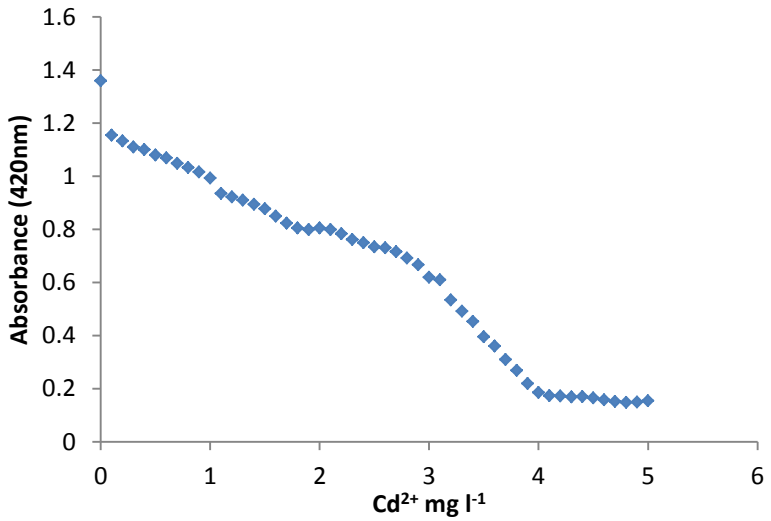

Fig 20 Inhibitory effect of Cd^{2+} (mg l^{-1}) on the alkaline phosphatase activity of *P. striata*

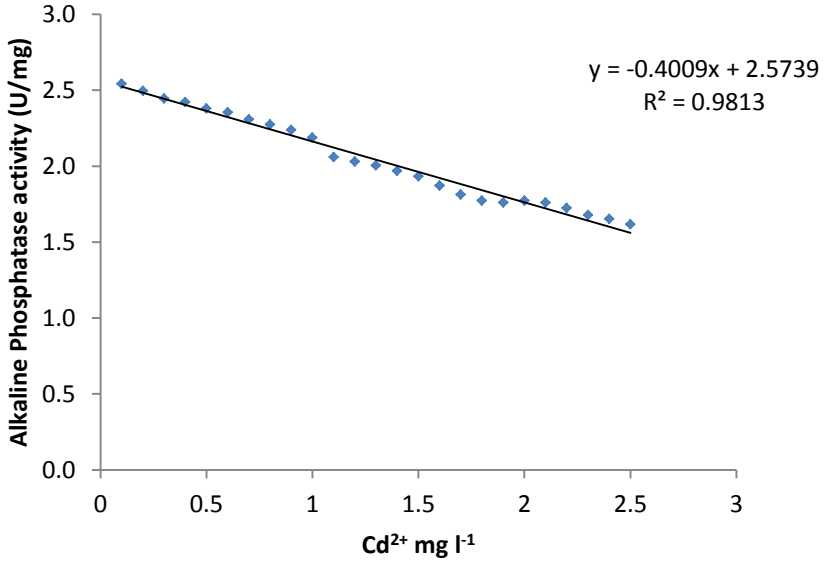

Fig 21 Linear range for detection of cadmium based on the inhibition of whole cell alkaline phosphatase activity of *P. striata*

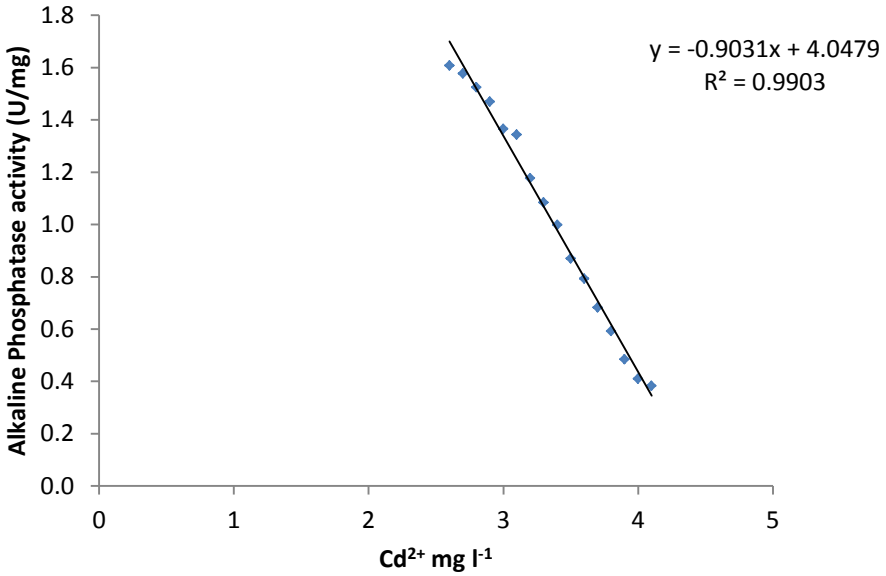

Fig 22 Linear range for detection of cadmium based on the inhibition of whole cell alkaline phosphatase activity of *P. striata*

Inhibitory effect of copper on the whole cell alkaline phosphatase activity of *P. striata*

Copper was found to be least toxic towards APA inhibition of all the three heavy metals studied. The inhibitory effect of copper on the APA activity was studied at a much wider range (1-40 mgl^{-1}) as compared to zinc and cadmium attributing to its less toxic nature. The maximum inhibition percentage (I) of 65.6% was observed at Cu^{2+} ion concentration of 34 mg l^{-1} (Fig 23). The decrease in the APA activity of *P. striata* was linear for copper concentration in the range of 1-30 mg l^{-1} (r^2= 0.9926) (Fig 24), and the limit of detection for the determination of Cu^{2+} ions was 1 mg l^{-1}.

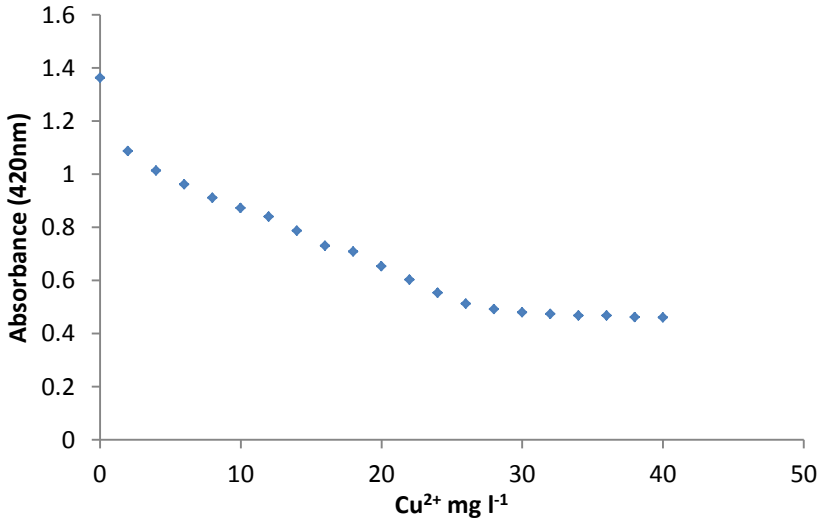

Fig 23 Inhibitory effect of Cu^{2+} (mg l^{-1}) on the alkaline phosphatase activity of *P. striata*

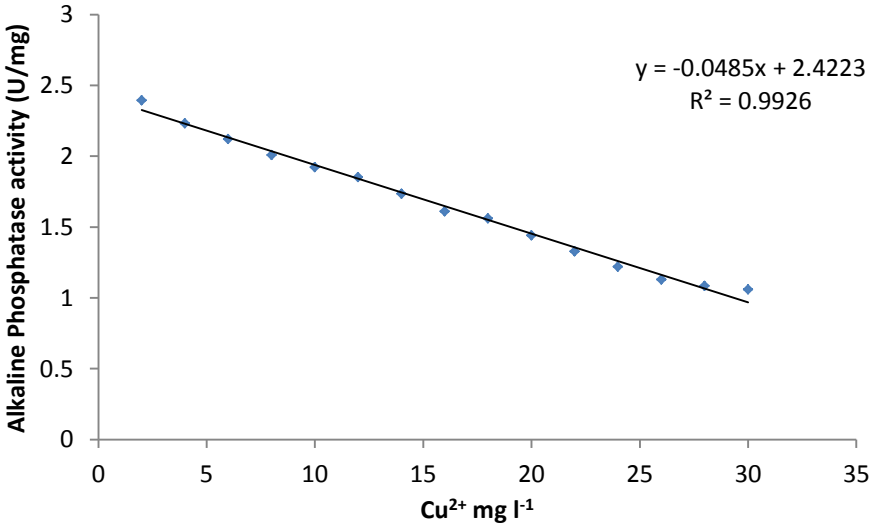

Fig 24 Linear range for detection of copper based on the inhibition of whole cell alkaline phosphatase activity of *P. striata*

4.2.2 Interactions of *Bacillus sp*. MD028 and heavy metals

The *in-vivo* inhibitory effect of the three heavy metals viz., Zn, Cu and Cd on the whole cell urease activity of the *Bacillus sp*. MD028 was studied by measuring the amount of

ammonia released from urea according to the phenol-hypochlorite assay method (Natarajan, 1995). This reaction leads to the formation of a coloured compound called indophenol, optical density of which is measured at 625nm. The reaction mixture consisted of bacterial cell suspension (OD_{600} =1) in K-phosphate buffer (0.1 mM, pH 8) as the source of enzyme, the three heavy metals viz., Zn, Cu and Cd ranging from the concentration range of 1-30 mg/l and the substrate (urea). A control test without heavy metals was conducted in parallel.

Inhibitory effect of copper on the whole cell urease activity of *Bacillus sp*. MD028

The optical assays for whole cell urease inhibition of *Bacillus sp*. MD028 were carried out at different conentrations of Cu(II). It was observed that higher the copper concentration, the lower the substrate (urea) hydrolysis until a constant absorbance ($O.D._{600nm}$) was obtained indicating the maximum urease inhibition (%Inhibition (I) = 91.4%) an effect that occurred at 1.5 mg l^{-1} (Fig 25). The decrease in the urease activity of *Bacillus sp*. MD028 was linear for copper concentration in the range of 0.01-1 mg l^{-1} (r^2= 0.9968) (Fig 26), and the limit of detection for the determination of Cu^{2+} ions was 10 µg l^{-1}.

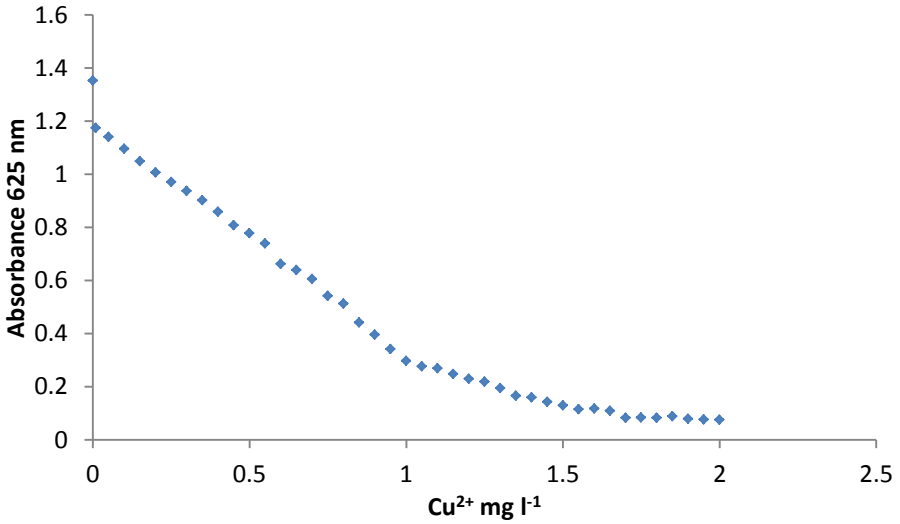

Fig 25 Inhibitory effect of Cu^{2+} (mg l^{-1}) on the urease activity of *Bacillus sp*. MD028

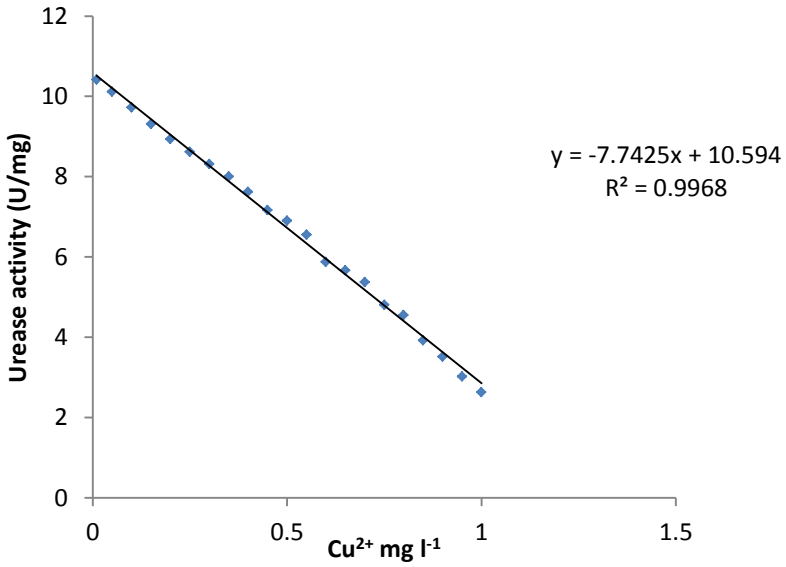

Fig 26 Linear range for detection of copper based on the inhibition of whole cell urease activity of *Bacillus sp.* MD028

Inhibitory effect of cadmium on the whole cell urease activity of *Bacillus sp*. MD028

The concentration range of cadmium (mg l^{-1}) used for the urease inhibition optical assays was broader than that of copper. This implied that cadmium was comparitively less toxic than copper. The rate of hydrolysis of the substrate (urea) was inversely related with the Cd^{2+} ion concentration. The maximum inhibition percentage (I%) of urease observed was 54.2 % at a concentration of 30 mg l^{-1} (Fig 27). The linear range for cadmium detection was observed from 1-30 mg l^{-1} (r^2=0.9967) (Fig 28). The detection limit for cadmium was found to be 1 mg l^{-1}.

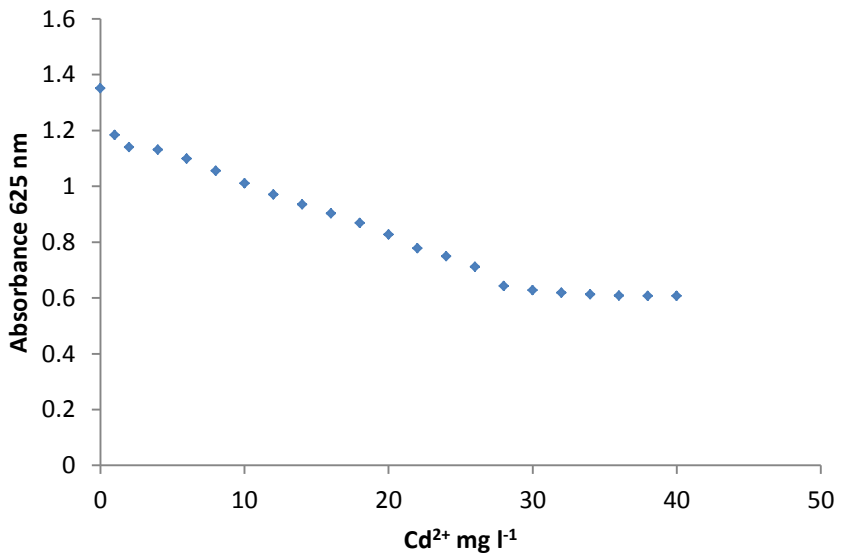

Fig 27 Inhibitory effect of Cd^{2+} (mg l^{-1}) on the urease activity of *Bacillus sp.* MD028

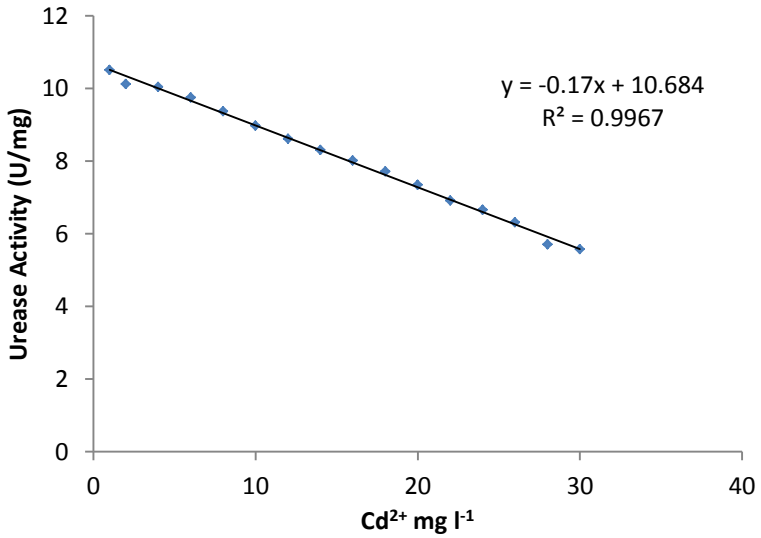

Fig 28 Linear range for detection of cadmium based on the inhibition of whole cell urease activity of *Bacillus sp.* MD028

Inhibitory effect of zinc on the whole cell urease activity of *Bacillus sp*. MD028

The toxic effects of zinc on the urease acivity of *Bacillus sp. MD028* was comparable to that of cadmium. The inhibitory effect of zinc on the urease activity was also studied at a wide concentration range of (1-40 mg l^{-1}) same as of cadmium attributing to its less toxic nature. The maximum inhibition percentage (I) of 58.7% was observed at Zn^{2+} ion

concentration of 30 mgl^{-1} (Fig 29). The decrease in the urease acivity of *Bacillus sp. MD028* was linear for zinc concentration in the range of 0.7-30 mg l^{-1} (r^2= 0.9987) (Fig 30), and the limit of detection for the determination of Zn^{2+} ions was 0.7 mg l^{-1} (Table 18).

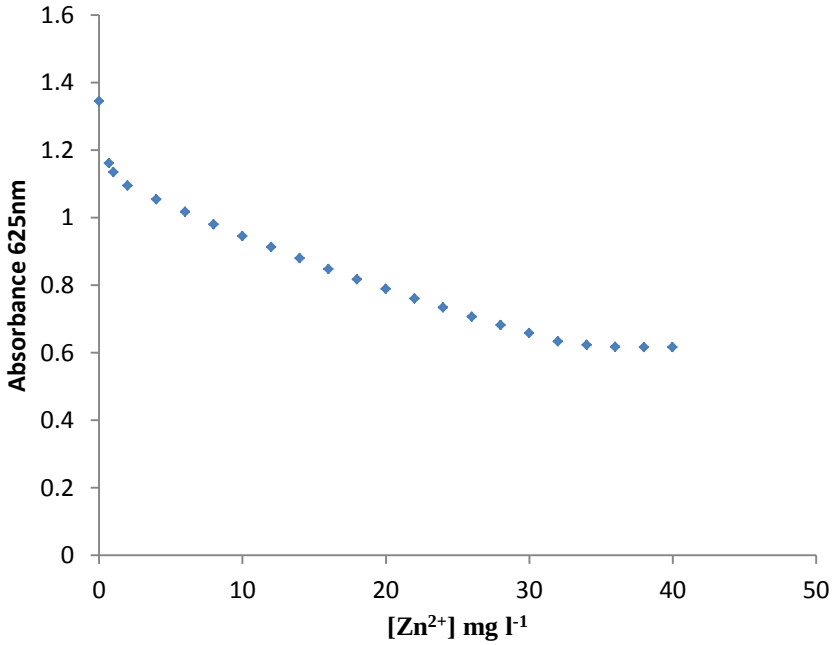

Fig 29 Inhibitory effect of Zn^{2+} (mg l^{-1}) on the urease activity of *Bacillus sp.* MD028

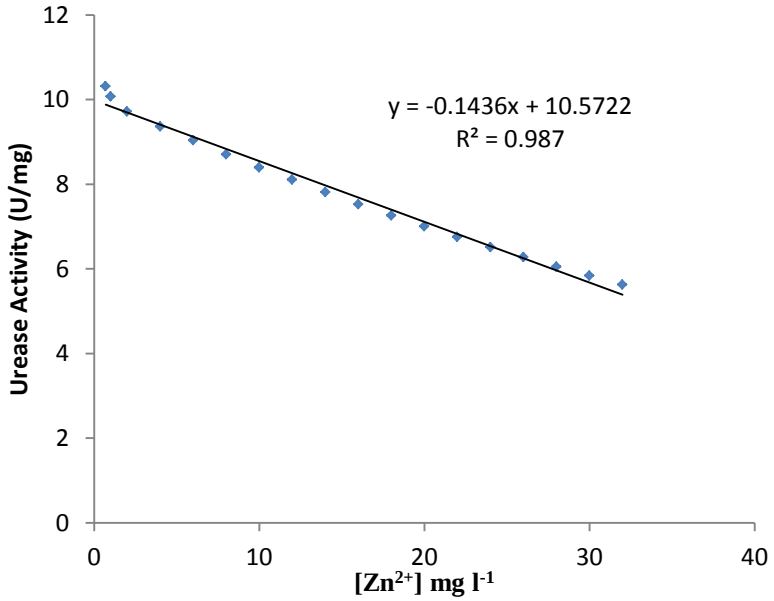

Fig 30 Linear range for detection of zinc based on the inhibition of whole cell urease activity of *Bacillus sp.* MD028.

Table 18 Inhibitory effect of heavy metals on the whole cell alkaline phosphatase activity of *P. striata* and whole cell urease activity of *Bacillus* sp. MD 028

Bacterial Whole cells	Enzyme	Inhibitors	Maximum inhibition (%) & corresponding metal concentration	Linear range (mgl^{-1})	Limit of Detection
Pseudomonas striata	Alkaline Phosphatase	Zn^{2+}	93.9%, 1.7 mgl^{-1}	0.01-1	10 μgl^{-1}
		Cd^{2+}	86.3%, 4 mgl^{-1}	0.1-2.5, 2.5-4	100 μgl^{-1}
		Cu^{2+}	65.6%, 34 mgl^{-1}	1-30	1 mgl^{-1}
Bacillus sp. MD028	Urease	Cu^{2+}	91.4%, 1.55 mgl^{-1}	0.01-1	10 μgl^{-1}
		Zn^{2+}	58.7%, 32 mgl^{-1}	0.7-30	0.7 mgl^{-1}
		Cd^{2+}	54.2%, 32 mgl^{-1}	1-30	1 mgl^{-1}

4.3 Immobilization and process optimization for whole cell-electrode assembly and its application for detection of heavy metals

The biomass of *Pseudomonas striata* and *Bacillus sp. MD028* was lyophilized at -50ºC under vacuum using a freeze dryer (Modulyod, ThermoElectron Corporation) to obtain dry cell mass, which was immobilized in polyvinyl chloride (PVC) membranes and electropolymerised films of polypyrrole-polyvinylsulphonate (Ppy-PVS) respectively. The work is a novel approach for immobilization of bacterial cells in PVC and Ppy-PVS and their use in integration with suitable transducers systems to develop a biosensor. The immobilization matrices (polyvinylchloride and polypyrrole-polysulphonate) containing the microbes were integrated with suitable transducer systems (Digital potentiometer, (Equiptronics EQ602) and Autolab Potentiostat/Galvanostat (Eco Chemie, Netherlands), which recorded the chemical changes of enzyme reactions as electrical signals. The digital potentiometer (Equiptronics EQ602) was used to record the potential difference generated across the PVC membrane containing bacterial cells in response to different metal ion concentrations (Potentiometric Biosensor Assembly), whereas the Autolab Potentiostat/Galvanostat (Eco Chemie, Netherlands) recorded the current produced due to the enzyme reaction occurring at the electrode surface (Amperometric Biosensor assembly).

4.3.1 Electrochemical preparation of PPy-PVS/ITO electrodes

PPy-PVS films were polymerized chrono-potentiometrically in a three-electrodes electrochemical cell having Ag/AgCl as reference, platinum as a counter electrode and ITO (indium tin oxide) glass plate (1×2 cm^2) as a working electrode using Potentiostat/Galvanostat (Model 273A, Princeton Applied Research). Monomer solution containing 0.1M Py, 0.1M PVS was subjected to constant current (200 µA) for about 15 min to obtain PPy-PVS films (conductivity ~ 120 S cm^{-1}) onto ITO glass plates at working area of about 1 cm^2. (Fig 31 & 32)

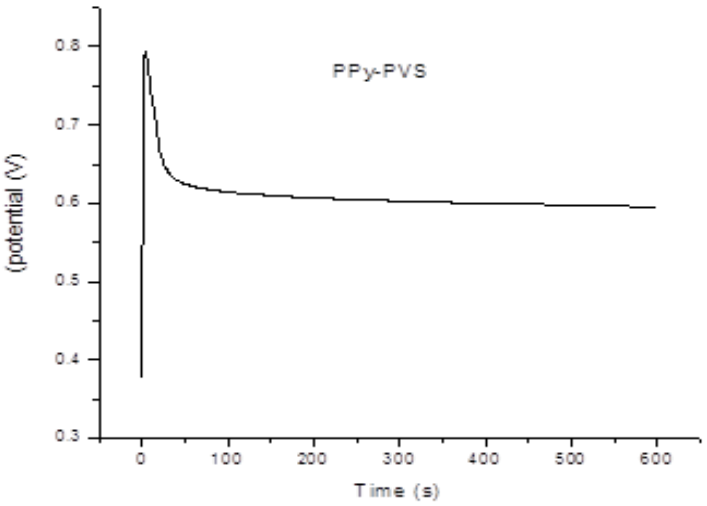

Fig 31 Chrono-potentiometric deposition of PPy-PVS on ITO (indium tin oxide) glass plate at 200 μA of constant current.

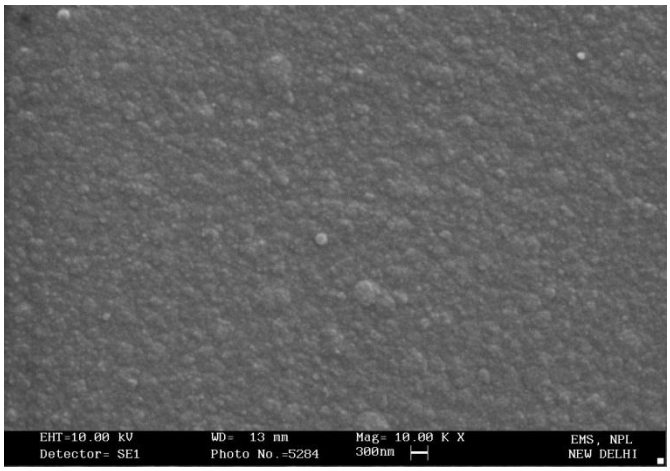

Fig 32 Scanning Electron Micrograph (10 KX) of chrono-potentiometrically deposited polypyrrole film on ITO (indium tin oxide) glass plate.

4.3.2 Immobilizaion of the lyophilized biomass of *Bacillus sp*. MD028 on PPy-PVS electrodes

The lyophilized biomass of *Bacillus sp*. MD028 was immobilized on to conducting polymer matrix of polypyrrole polysulfonate. Two types of immobilization techniques were used:

1. Physical entrapment
2. Chemical cross-linking using EDC-NHS (EDC [*N*-(3-dimethylaminopropyl)-*N*-ethyl-carbodiimide hydrochloride], NHS [*N*-hydroxy-succinimide)

Physical immobilization

The physical entrapment of the bacterial biomass was achieved by suspending the lyophilized bacterial biomass (10-60 mg) in 10 ml monomer solution containing 0.1M Py (pyrrole) and 0.1M PVS (poly vinyl sulphonate), which was subjected to constant current of 200 µA for about 900 seconds to obtain a Ppy-PVS film with entrapped bacterial biomass on the surface of the working electrode (ITO glass plate (1X2 cm^2)) (Fig 33). The SEM image of the electrode shows the rod shaped bacteria entrapped in the PPy-PVS matrix. Approximately 0.3 mg of bacterial biomass was entrapped when 60 mg of lyophilized bacterial biomass was suspended in 10 ml of monomer solution. The amount of the bacterial cells entrapped varied according to the concentration bacterial cell suspension and was estimated by measuring the urease activity of the bacterial biomass containing PPy-PVS film using phenol hypochlorite method (Natarajan, 1995). The estimation of *Bacillus* sp. MD028 biomass entrapped in PPy-PVS film by colorimetrically measuring the urease activity of the film was not very accurate as the entrapment procedure must have caused some loss in the urease activity which could not be predicted accurately. Also, a large quantity of biomass (60 mg) suspended in monomer solution resulted in the entrapment of relatively a small quantity ≈ 0.3 mg. Therefore the physical immobilization was not used for the preparation of *Bacillus*-GLDH/PPy-PVS/ITO bioelectrodes.

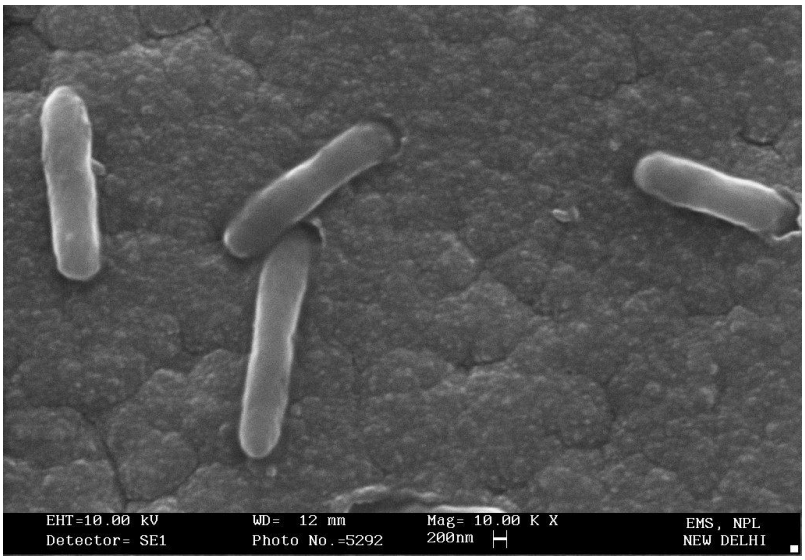

Fig 33 Scanning Electron Micrograph (10 K X) of chrono-potentiometrically entrapped lyophilized biomass of *Bacillus* sp. MD028 and GLDH on PPy-PVS/ITO electrode.

Covalent Immobilization

The covalent immobilization on the surface of PPy-PVS/ITO electrode was done by chemically cross linking the bacterial biomass on the surface of the PPy-PVS/ITO electrode using EDC-NHS solutions. (Fig 34). These whole cell/PPy-PVS/ITO electrodes were characterized using Spectrophotometry, CV (cyclic voltammetry), SWV (Square wave voltammetry) and scanning electron microscopy. Two major advantages of covalent immobilization over the entrapment method were that a known amount of the biomass was immobilized and after immobilization the biomass retained more than 98% urease activity (Table 19).

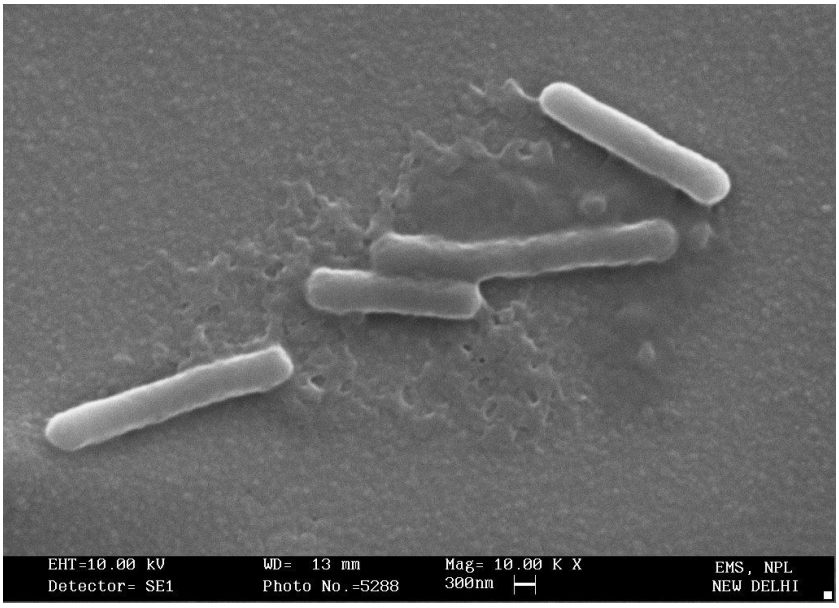

Fig 34 Scanning Electron Micrograph (10 K X) of covalently immobilized lyophilized biomass of *Bacillus* sp. MD028 and GLDH on PPy-PVS/ITO electrode.

Table 19 Residual enzyme activities of lyophilized bacterial biomass of *Pseudomonas striata* and *Bacillus* sp. MD 028 after immobilization in polyvinyl chloride and polypyrrole-polyvinyl sulphonate matrices

Microbe	Enzyme	Enzyme activity of unimmobilized bacterial biomass (U/mg DCW)	Immobilization matrix	Immobilized biomass replicates	Enzyme activity	Residual activity (%)	Average Residual activity (%)
Pseudomonas striata	alkaline phosphatase	3±.07	PVC (entrapment)	1	2.56	85.3	84.9
				2	2.49	83	
				3	2.6	86.6	
Bacillus sp. MD 028	Urease	12±.21	PPy-PVS (Cross-linking)	1	11.52	98.8	98.7
				2	11.38	98.3	
				3	11.56	99	

DCW**:** Dry cell weight, Ppy-PVS: polypyrrolre polysulphonate, PVC: polyvinyl chloride, U (enzyme activity units) = µmol product formed per ml

4.3.3 Optimization of glutamate dehydrogenase

Effect of copper, cadmium and zinc was studied on glutamate dehydrogenase (GLDH) in order to optimize the amount of GLDH required for immobilization on PPy-PVS/ITO electrode along with *Bacillus sp.* MD 028 biomass such that the response recorded by the *Bacillus*-GLDH/PPy-PVS/ITO bioelectrode in the presence of heavy metals is only due to the inhibition of the whole cell urease activity of *Bacillus sp.* MD 028 and any GLDH inhibition what so ever caused by heavy metal ions does not effect the biosensor response. In the earlier experimets of the present study the urease bioassay had revealed that the maximum inhibition of the whole cell urease activity of *Bacillus sp.* MD 028 was caused by copper, cadmium and zinc at the concentrations of 1.5 mgl^{-1}, 30 mgl^{-1} and 30 mgl^{-1} respectively. Therefore, the effect of these metal ion concentrations for the respective metal ions was studied on GLDH. Different amounts of GLDH (0.05-0.3 mg) were covalently immobilized using EDC-NHS on 1 cm^2 working area of PPy-PVS/ITO electrode and sqaure wave voltammetry was recorded after 300 secs in phosphate buffered saline (50 mM, 0.9% NaCl) containing 5 mM $[Fe(CN)_6]^{3-/4-}$) in the presence of 30 µl nicotinamide adenine dinucleotide (NADH, 0.5 mM), 70 µl α-Keto glutarate (2.5 mM) and 5 mM NH_4Cl. NH_4Cl was used as the source of NH_4^+ ions instead of urease and urea since the inhibition of only GLDH had to be monitored. Fig 35 shows that a maximum of 50 %, 20 % and 15 % GLDH inhibition was caused by copper, cadmium and zinc metals at their respective metal ion concentrations of 1.5 mg l^{-1}, 30 mg l^{-1} and 30 mg l^{-1} when 0.05 mg of GLDH was immobilized on the PPy-PVS/ITO electrode. The percentage inhibition decreased with increase in the amount of immobilized GLDH and no loss in the GLDH activity was observed when 0.25 mg of GLDH was immobilized on the 1 cm^2 PPy-PVS/ITO electrode. Also it was noted that the further increase in the amount GLDH did not improve the current response therefore 0.25 mg of the GLDH was found to be optimum for the preparation of bioelectrode. Therefore the *Bacillus*-GLDH/PPy-PVS/ITO bioelectrodes were prepared by covalently immobilizing 1 mg of lyophilized biomass of *Bacillus* sp. MD028 and 0.25 mg of GLDH on the surface of PPy-PVS/ITO electrodes.

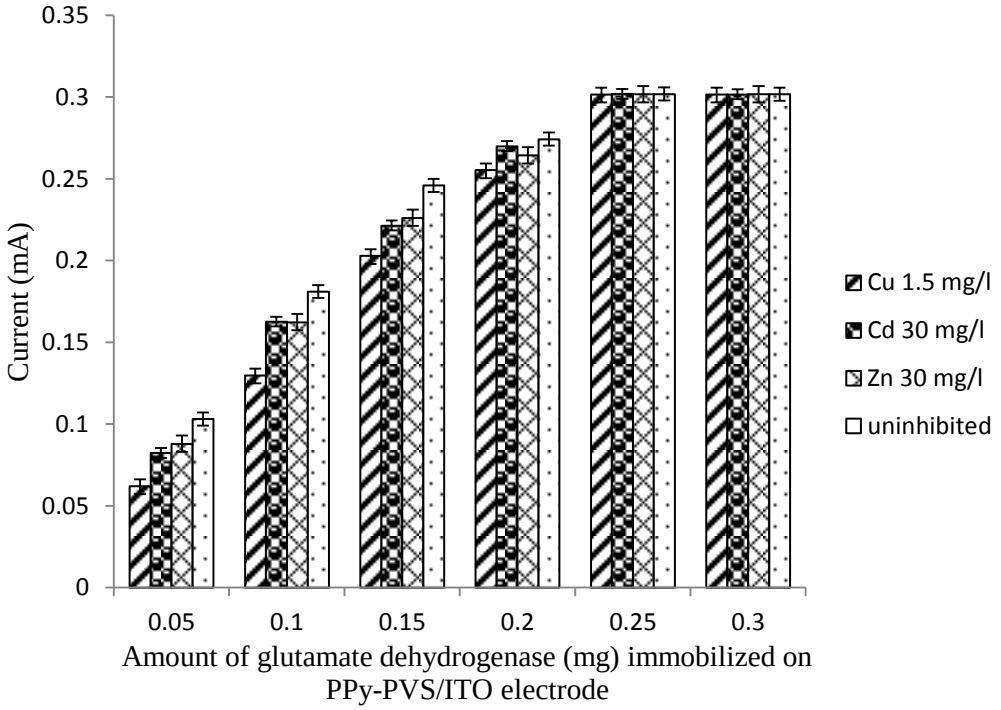

Fig 35 Amperometric response of glutamate dehydrogenase immobilized on PPy-PVS/ITO electrode at different concentrations in the presence of copper, zinc and cadmium

4.3.4 Cyclic Voltammetric characterization of PPy-PVS/ITO and *Bacillus*-GLDH/PPy-PVS/ITO bioelectrodes

The electrochemical behavior of PPy-PVS/ITO electrode after covalent immobilization of 1 mg lyophilized bacterial cells and 0.25 mg glutamate dehydrogenase were studied by cyclic voltammetry (CV). Fig 36 shows the cyclic voltammograms for (a) PPy-PVS/ITO electrode (b) and *Bacillus*-GLDH/PPy-PVS/ITO recorded in phosphate buffer saline (PBS, 50 mM, pH, 7.0, 0.9% NaCl) containing 5mM $[Fe(CN)6]^{3-/4-}$ in the potential range −0.6 to 1.3 V at 20 mV/s rate. The oxidation peaks of both PPy-PVS/ITO and *Bacillus*/PPy-PVS/ITO were observed at about 0.6 V with peak current values of 150 µA and 53 µA respectively. This decrease in peak current observed for bacterial biomass/Ppy-PVS/ITO indicated the proper immobilization of the bacterial biomass on the surface of the conducting polymer (PPy-PVS) that partially blocks transport of electrons between the reaction solution and electrode surface.

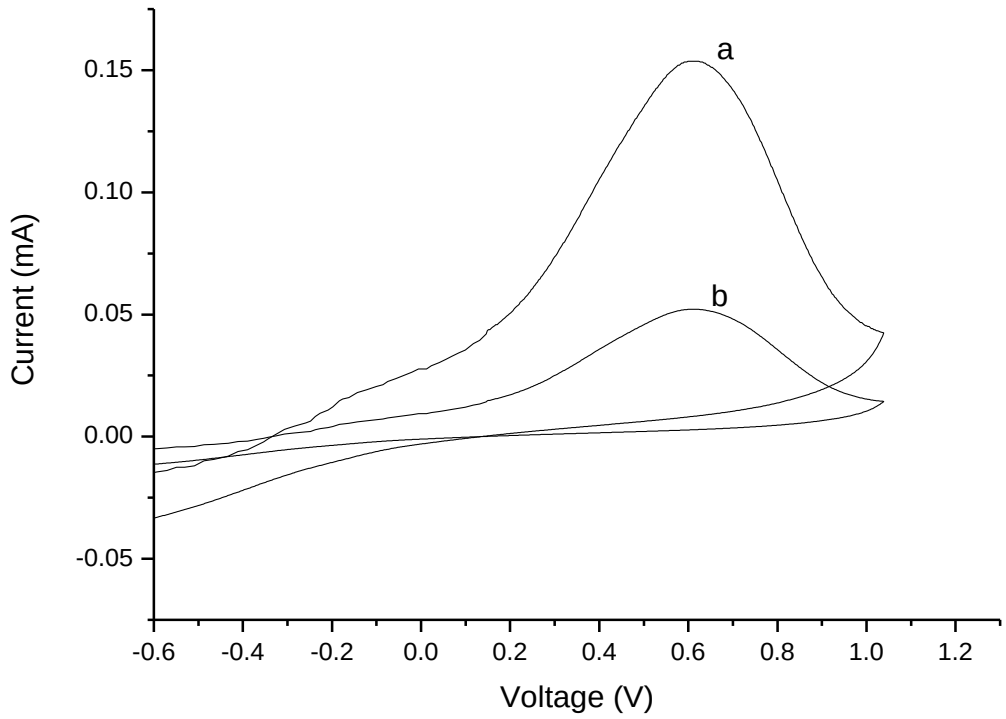

Fig 36 Cyclic voltammograms (a) PPy-PVS/ITO, (b) *Bacillus*-GLDH/PPy-PVS/ITO in phosphate buffered saline (PBS, 50mM, pH, 7.0, 0.9% NaCl) containing 5mM [Fe $(CN)_6]^{3-/4-}$ in the potential range −0.6 to 1.3 V

4.3.5 Effect of pH on the *Bacillus*-GLDH/PPy-PVS/ITO bioelectrodes

The effect of pH in the range of 2-9 on *Bacillus*-GLDH/PPy-PVS/ITO bioelectrodes was carried out using square wave voltammetry recorded in phosphate buffer saline (PBS, 50 mM, 0.9% NaCl) containing 5mM $[Fe(CN)_6]^{3-/4-}$ and in the potential range, -0.6 to 1.3 V at scan rate of 20mV/s (Fig 37 & 38). Slight shift in the peak potential towards the negative potential was observed with increase in pH. The peak current values were found to decrease from acidic to alkaline pH, with maximum peak current of 149 µA observed for pH 2 and minimum current of 11 µA observed for pH 9. Possibility of using extreme pH values for the measurement of urease activity was ruled out as the acidic pH conditions are highly unfavourable for the catalytic activity of urease and high alkaline pH of 9 drastically lowers the electroactivity of PPy-PVS/ITO electrode. pH 7 was chosen for further studies as it exhibited optimal current value and optimum urease activity. This suggests that *Bacillus*-

GLDH/PPy-PVS/ITO bioelectrodes showed maximum activity at pH 7 at which urease retains its natural structure and PPy-PVS film shows reasonably good conductivity.

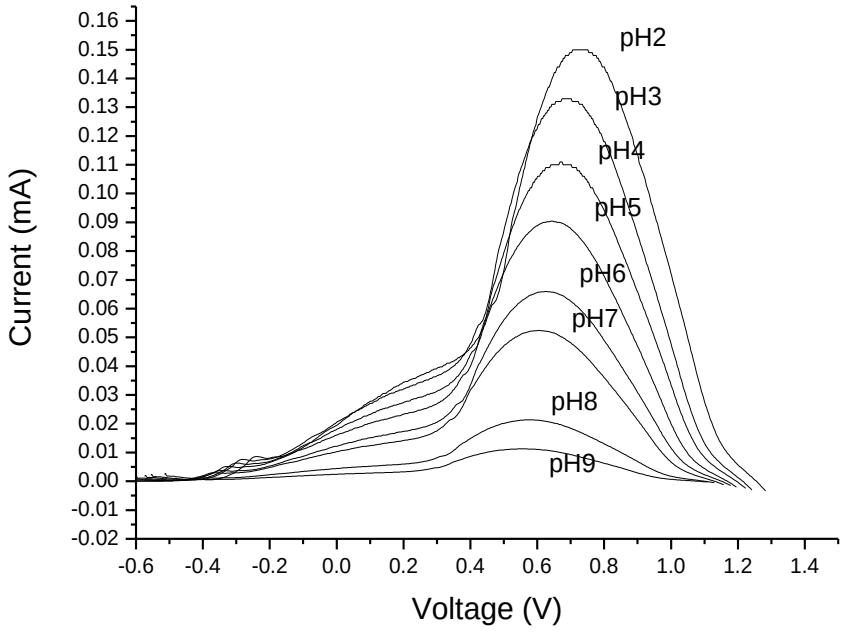

Fig 37 Square wave voltammograms of the *Bacillus*-GLDH/PPy-PVS/ITO bioelectrodes in phosphate buffered saline (PBS, 50 mM) of different pH (2-9), saline (0.9% NaCl) containing 5mM $[Fe(CN)_6]^{3-/4-}$ in the potential range -0.6 to 1.3 V.

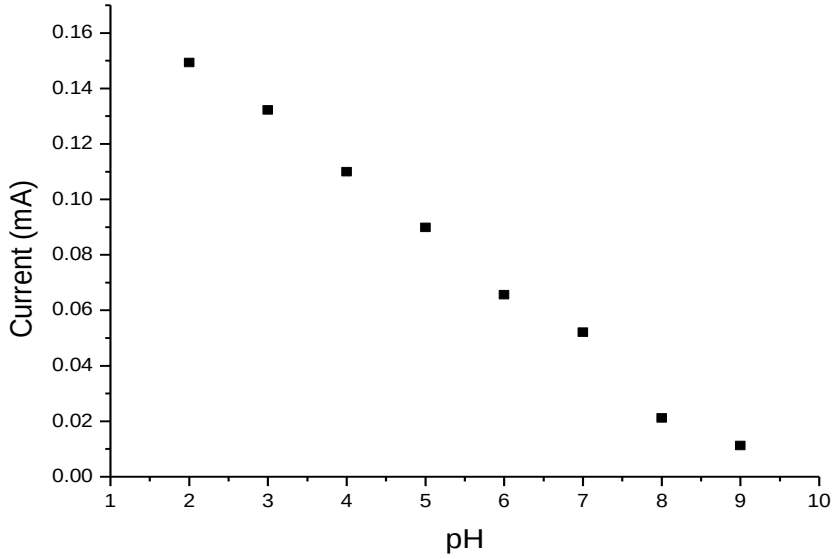

Fig 38 Peak current values of *Bacillus*-GLDH/PPy-PVS/ITO bioelectrode obtained using Square wave voltammetry in phosphate buffered saline (PBS, 50 mM) of different pH (2-9), saline (0.9% NaCl) containing 5mM $[Fe(CN)_6]^{3-/4-}$ at peak potential of 0.6V.

4.3.6 Electrochemical response studies of *Bacillus*-GLDH/PPy-PVS/ITO bioelectrode

Electrochemical response study of *Bacillus*-GLDH/PPy-PVS/ITO bioelectrode towards urea (5 mM) was carried out as a function of time in the presence of 30 µL of nicotinamide adenine dinucleotide (NADH, 3.7 mg/dL) and 70 µL of α-Keto glutamate (α-KG, 47.5 mg/dL) using square wave voltammetry in PBS solution (50 mM PBS (pH 7, 0.9 % NaCl) containing 5mM $[Fe(CN)_6]^{3-/4-}$). It was observed that magnitude of current obtained for the *Bacillus*-GLDH/PPy-PVS/ITO bioelectrode increased on addition of urea. This increase in the current value was consistant and gradual till the steady state current was achieved at about 210 seconds. The obtained response time of 210 seconds was reasonably good with respect to the whole cell biosensors and can be attributed to the efficient electrochemical detection method (Fig 39).

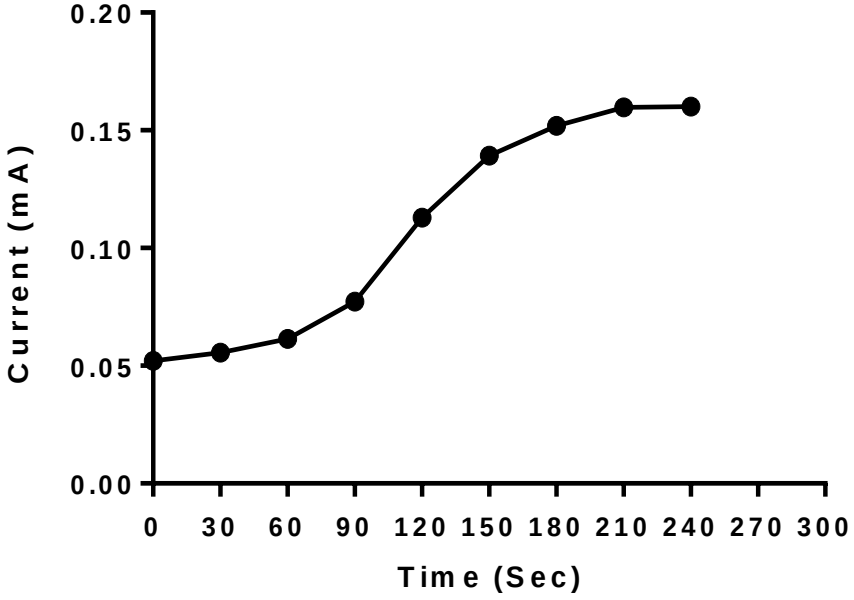

Fig 39 Amperometric response of *Bacillus*/PPy-PVS/ITO bioelectrode towards urea (5 mM) as a function of time in phosphate buffered saline (PBS) (50 mM PBS (pH 7, 0.9%NaCl) containing 5mM $[Fe(CN)_6]^{3-/4-}$).

Linear Range and Detection Limit of urea using *Bacillus*-GLDH/PPy-PVS/ITO bioelectrode

The response of the *Bacillus*-GLDH/PPy-PVS/ITO bioelectrode towards urea was studied in the concentration range of 1-32 mM using square wave voltammetry (Fig 40 & 41).

Increase in the peak current values was observed with increasing urea concentrations. It was observed that the response of the bioelectrode exhibited good linearity in the urea concentration range (1.5-18 mM) and the current varies according to the following linear equation $y = 0.015 x + 0.0629$ with the correlation coefficient of (r^2= 0.9892) (Fig 42). At higher concentrations of urea (> 18 mM) the current response was found to level off. Increase in the current for higher concentrations of urea (18-32 mM) was observed to be slow possibly due to saturation of the enzyme active sites. The detection limit of *Bacillus*-GLDH/PPy-PVS/ITO bioelectrode is estimated to be about 1.5 mM. The sensitivity of the *Bacillus*-GLDH/PPy-PVS/ITO bioelectrode calculated from the slope of curve was found to be 15 µA/(mM cm^{-2}).

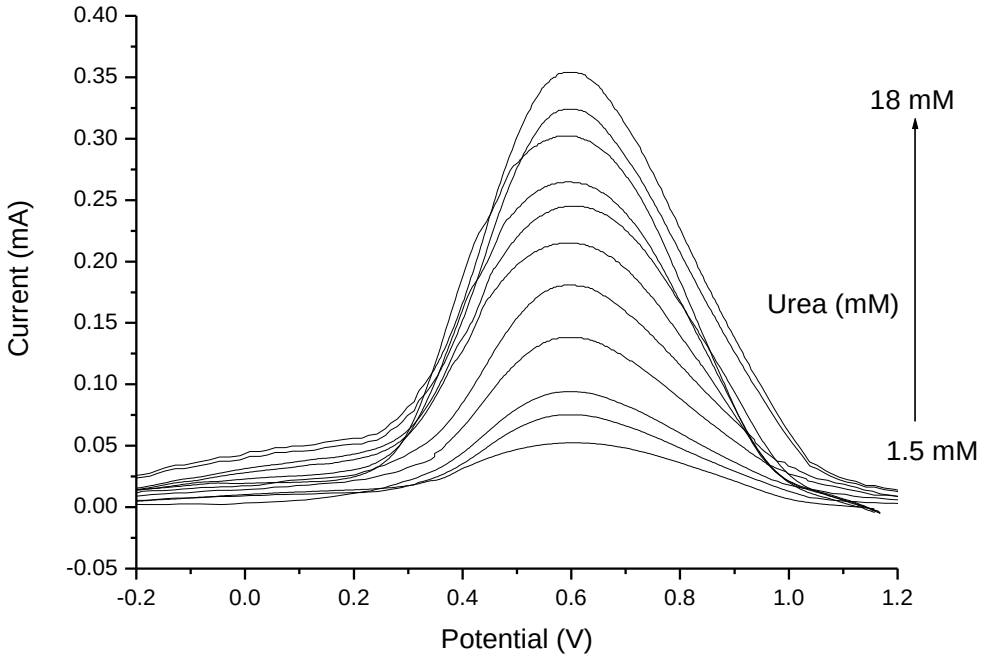

Fig 40 Electrochemical response of *Bacillus*-GLDH/PPy-PVS/ITO bioelectrode as a function of urea concentration (1.5-18 mM)

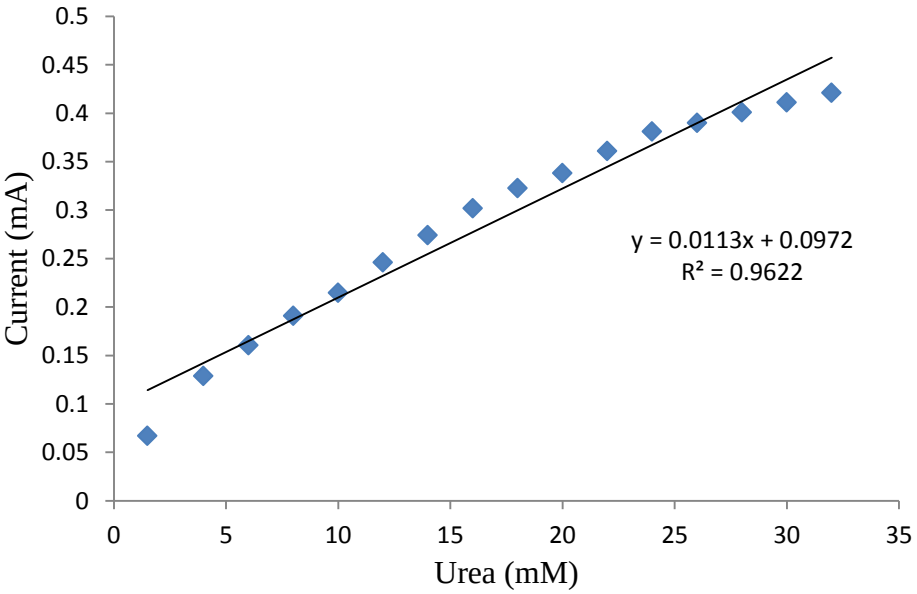

Fig 41 Amperometric response of *Bacillus*-GLDH/PPy-PVS/ITO bioelectrode towards urea (1.5-32 mM) using square wave voltammetry in phosphate buffered saline (PBS) (50 mM PBS (pH 7, 0.9%NaCl) containing 5mM $[Fe(CN)_6]^{3-/4-}$).

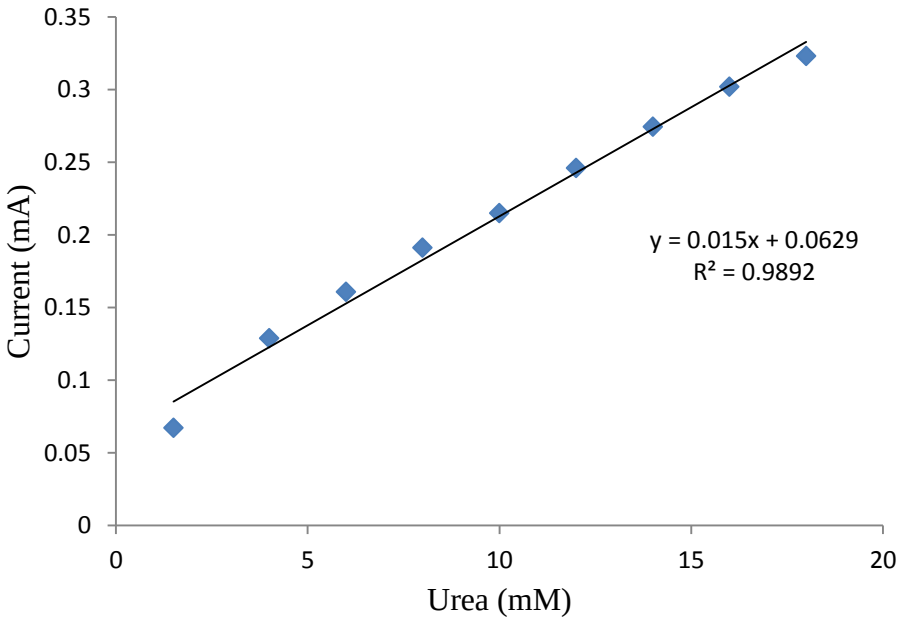

Fig 42 Amperometric response of *Bacillus*-GLDH/PPy-PVS/ITO bioelectrode towards urea (1.5-18 mM) using square wave voltammetry in phosphate buffered saline (PBS) solution (50 mM PBS (pH 7, 0.9% NaCl) containing 5mM $[Fe(CN)_6]^{3-/4-}$).

The value of the Michaelis–Menten constant (*K*m) was estimated to show the effect of covalent immobilization on the bacterial biomass and its response towards urea. Using Lineweaver–Burke plot (1/*I* versus 1/[S]) Fig 43 , *K*m value was found to be 9.35 mM for the *Bacillus*-GLDH/PPy-PVS/ITO bioelectrode which was only slightly higher than the Km for unimmobilized *Bacillus* sp. MD 028 (km= 8.13) (Fig 14) proving the efficiency of the covalent immobilization.

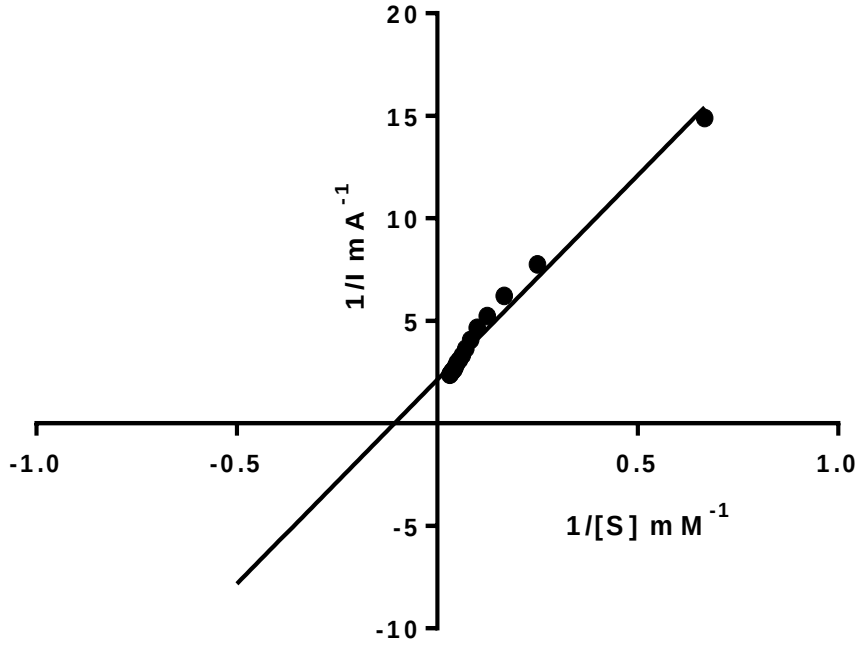

Fig 43 Lineweaver-Burke plot of electrochemical response of whole cell urease activity of *Bacillus* sp. MD028 at different concentrations of urea.

4.3.7 Electrochemical detection of copper using *Bacillus*-GLDH/PPy-PVS/ITO bioelectrode

Inhibition of the whole cell urease activity of *Bacillus sp*. MD 028 in the presence of copper ion was studied using square wave voltammetry technique recorded on Autolab Potentiostat/Galvanostat (Eco Chemie, Netherlands). The obtained square wave voltammograms were correlated to the corresponding copper ion concentration in the solution. The response of the *Bacillus*-GLDH/PPy-PVS/ITO bioelectrode towards the Cu^{2+} ion concentration was linear in the range of 0.008-1 mgl^{-1} (Fig 44). The linear relationship established between Cu^{2+} ion and decrease in urease activity followed y = -0.3682x+0.4328

with correlation coefficient of $r^2 = 0.9809$ and detection limit of 0.008 ppm (8 ppb). The detection limit of the electrochemical system (8 ppb) was marginally lower than the detection limit found by spectrophotometric system (10 ppb) possibly due to the efficiency of the amperometric sensing assembly.

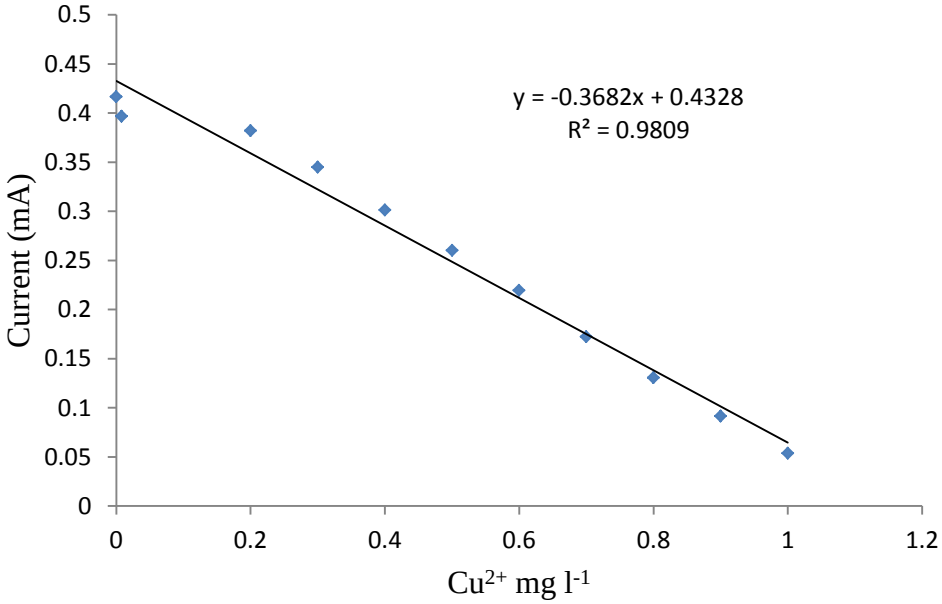

Fig 44 Amperometric response of the *Bacillus*-GLDH/PPy-PVS/ITO bioelectrode towards the Cu^{2+} ion concentration in the range of 0.008-1 mgl^{-1}

4.3.8 Electrochemical detection of cadmium using *Bacillus*-GLDH/PPy-PVS/ITO bioelectrode

Cadmium ions caused much less urease inhibition as compared to copper and hence a much wider linear range of inhibition was obtained for cadmium i.e., 1-30 mg l^{-1} as compared to copper. Results of both spectrophotometric and electrochemical methods of detection were in good agreement with each other. The detection limit of cadmium obtained using electrochemical method was found to be 1 mg l^{-1} which was same as that obtained from spectrophotometric method. Although the electrochemical method showed improved sensitivity and lower detection limit than the spectrophotometric method for copper but no such difference between the two methods was observed for cadmium. This could be explained by lesser urease inhibition caused by cadmium and hence cadmium did not cause

any significant urease inhibition below 1 mg l^{-1} detectable by the electrochemical assembly. Decrease in the current with increase in Cd^{2+} ion concentration followed $y = -0.0079x + 0.3989$ with the correlation coefficient of $r^2 = 0.9979$. The maximum inhibition percentage (I%) of whole cell urease activity of *Bacillus sp*. MD028 observed for was about 60% at a Cd^{2+} ion concentration of 30 mg l^{-1} (Fig 45).

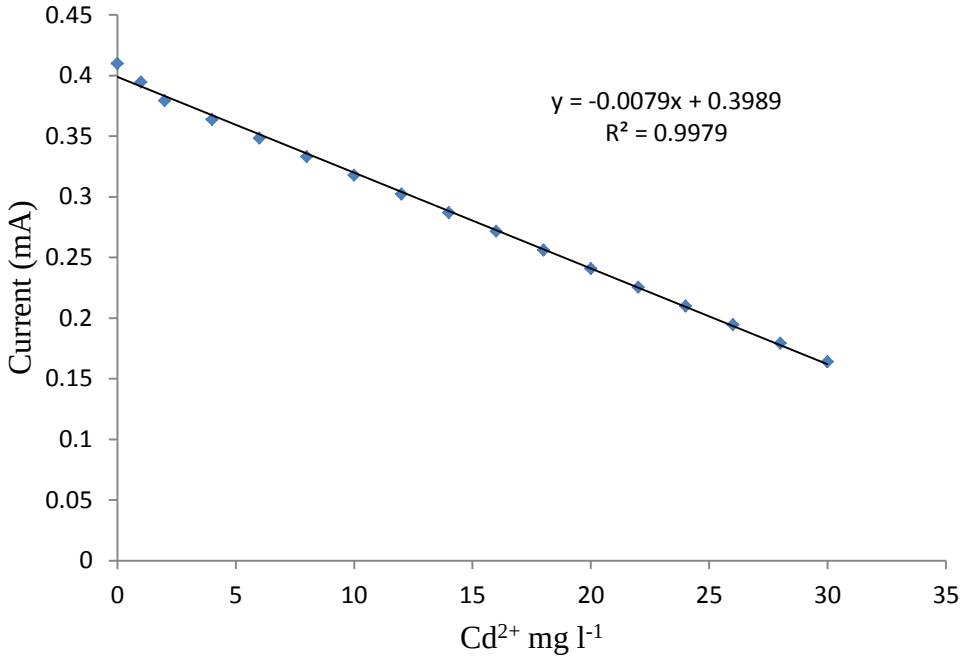

Fig 45 Amperometric response of the *Bacillus*-GLDH/PPy-PVS/ITO bioelectrode towards the Cd^{2+} ion concentration in the range of 1-30 mgl^{-1}

4.3.9 Electrochemical detection of zinc using *Bacillus*-GLDH/PPy-PVS/ITO bioelectrode

The degree of urease inhibition caused by zinc was comparable to that of cadmium and hence effect of zinc was also studied at a wide concentration range of 1 - 40 mg l^{-1} which was same as of cadmium attributing to its less toxic nature. The relationship between current and Zn^{2+} ion concentration was inverse and linear and followed the equation $y = -0.0066x + 0.3936$ with the correlation coefficient of $r^2 = 0.9932$ (Fig 46). The maximum inhibition percentage (I%) of 55 % was observed at Zn^{2+} ion concentration of 32 mgl^{-1} beyond which no decrease in the peak current was observed on increasing the zinc ion concentration. Detection limit of 0.7 mg l^{-1} similar to the spectrophotometric assay was obtained indicating good agreement between bioassay and biosensor measurements.

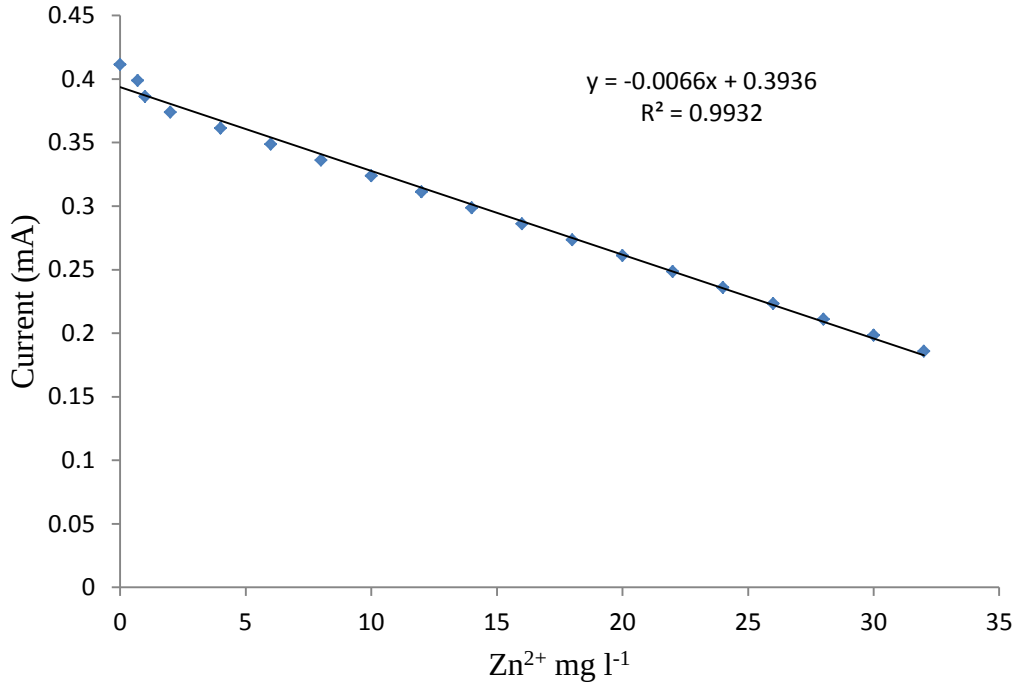

Fig 46 Amperometric response of the *Bacillus*-GLDH/PPy-PVS/ITO bioelectrode towards the Zn^{2+} ion concentration in the range of 1-30 mgl^{-1}

4.3.10 Shelf life of *Bacillus*-GLDH/PPy-PVS/ITO bioelectrode

The shelf-life of *Bacillus*/PPy-PVS/ITO bioelectrode was monitored by measuring the spectrophotometric response using phenol hypochlorite method (Natranjan 1995) with respect to time at regular interval of 2 week. It may be remarked that the response of the *Bacillus*/PPy-PVS/ITO bioelectrode recorded after 4 weeks exhibited no loss in the urease activity. The bioelectrode was observed to retain around 98% urease activity even after 12 weeks when stored under dry conditions at 4 °C. The urease activity decreased to 95% in about 24 weeks (Fig 47).

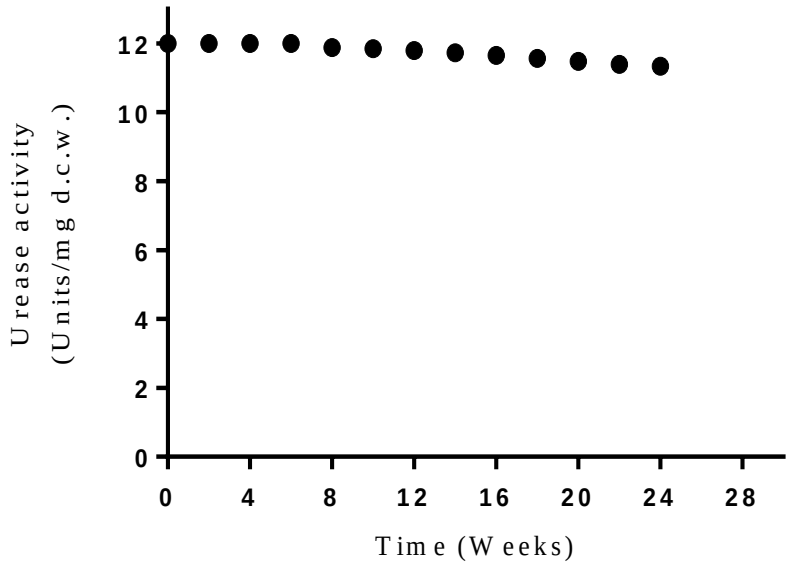

Fig 47 Urease activity of *Bacillus*-GLDH/PPy-PVS/ITO bioelectrode as a function of time tested over a period of 24 weeks at regular interval of 2 weeks in the presence of 20 mM urea.

4.3.11 Zinc selective potentiometric electrode based on *Pseudomonas striata* immobilized in PVC

The zinc selective PVC electrode was prepared by immobilizing lyophilized biomass of *Pseudomonas striata* in polyvinyl chloride matrix. Polyvinyl chloride (PVC) is highly hydrophobic and impermeable to any ions. It was plasticized (softened) by addition of a similarly hydrophobic solvent, e.g., DBP (Dibutyl pthalate), o-NPOE (ortho nitrophenyl octyl ether). So, far the membrane is just a flexible piece of plastic, which acts as a near perfect barrier to ions. To make it ion-selective, lyophilized biomass of *Pseudomonas striata* was added as a ligand and its selectivity towards heavy metals ions was tested.

Optimization of the membrane composition

The sensitivity and selectivity of an electrode are significantly affected by the nature of the plasticizer, the composition of ionophore, internal solution (Mi *et al*., 1999; Sokalaski *et al*., 1997; Sokalaski *et al*., 1999), etc. So, for optimization of the membrane, effect of the composition on the response characteristics of the electrode like slope of the calibration curve, measurement range and detection limit were studied (Table 20). The electrode with

the ratio PVC:DBP:bacterial cells:KTCIPB = 33%:60%:5%:2%, exhibited the best response with a slope of 26.2mV/decade. It was found that DBP is a more effective solvent medium than o-NPOE in preparing Zn^{+2} ion selective electrode. Amount of the ion carrier (Bacterial cells) affected the sensitivity. Sensitivity of the electrode increased with increasing ionophore content until a value of 5% (w/w) was reached. A further increase in the percentage of the ionophore resulted in decrease of the slope of the electrode. This may be due to the reason that equilibration of the ionophore with the metal ions is maximum at this concentration. Addition of potassium salt of tetrakis(4-chloro-phenyl) borate (KTClPB) is known to increase the sensitivity of the membrane as it reduces the anionic interference. It was observed that the addition of this lipophilic cation improved the working electrode sensitivity (Linear range: 10^{-1} to 10^{-5} M, Slope: 26.2mV/decade) and detection limit 5×10^{-5}M.

Table 20 Optimization of membrane ingredients

Sl. No.	PVC (wt%)	Plasticizers (wt%)	Ligand (wt%)	KTCIPB (wt%)	Slope (mV/decade)	Detection limit (M)
1	33	65 (DBP)	2	-	15	1×10^{-4}
2	33	64 (DBP)	3	-	18	1×10^{-4}
3	33	63 (DBP)	4	-	20	5×10^{-4}
4	33	62 (DBP)	5	-	22	1×10^{-5}
5	33	61 (DBP)	6	-	21	6×10^{-4}
6	33	60 (DBP)	7	-	21	5×10^{-4}
7	33	62(2-NPOE)	5	-	19	1×10^{-4}
8	33	61 (DBP)	5	1	25	1×10^{-5}
9	33	60 (DBP)	5	2	26.2	5×10^{-5}
10	33	59 (DBP)	5	3	21	1×10^{-4}

Effect of pH

Effect of pH was studied in the range of 2-12 using $1x10^{-2}$, $1x10^{-3}$ and $1x10^{-4}$ M Zn^{+2} concentrations. pH studies were done on membranes using 3 % and 5 % ionophore concentration. pH was adjusted by the addition of 0.1 N NaOH or HNO_3 as required. It was

found that the electrode response was optimum at a very narrow pH range of 3-4 (Fig 48). At pH above and below this range a sharp decrease in the emf (electromotive force) value was observed

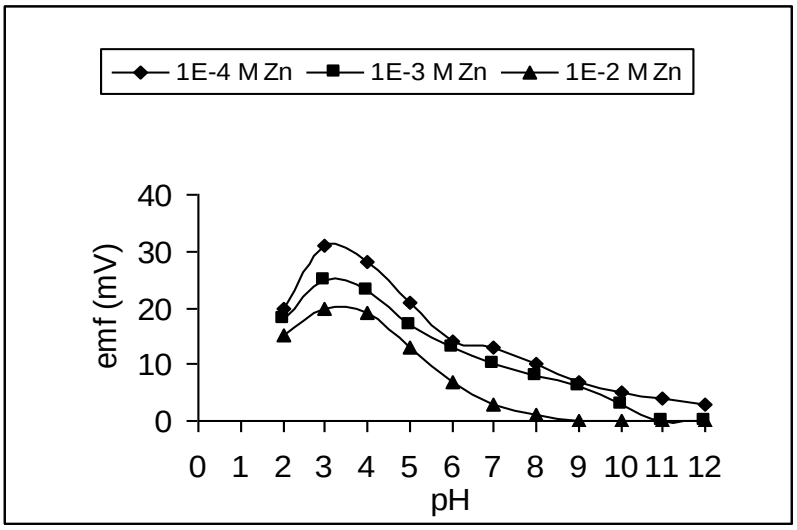

Fig 48 Effect of pH on potentiometric response of membrane at three different Zinc concentrations

Calibration curve, response time shelf life and detection limit

The electrode showed a linear response towards Zn^{+2} over a wide concentration range of 10^{-5} to 10^{-1} M. The calibration curve had a near Nernstian slope of 26.2mV/decade with a detection limit of $5x10^{-5}$ M which was obtained from the intersection of two straight-line portions of the curve (Fig 49). The slow decrease in the emf beyond 10^{-5} M may be due to the release of Zn^{2+} ions from membrane in to the solution. No particular emf trend was observed for Cd^{2+} and Cu^{2+} ions. The response time was measured by recording emf of the electrode as a function of time, when it is immersed in the solution to be studied. The estimated time to get stable potential was 6 s. Although always kept at 4-5ºC, the response of the electrodes stored in dry was much better than that of the electrode stored in 0.1 M Zn^{2+} solution. The soaked electrodes showed a 20%, 70% and 100% sensitivity decrease after 2, 4 and 6 days of storage, respectively (Fig 50). The longer life span of the electrodes which were stored under dry conditions could be attributed to the prevention of the oxidation of the cysteine residues present in the metalloproteins (Gonzalez-Bellavista *et al*., 2009).

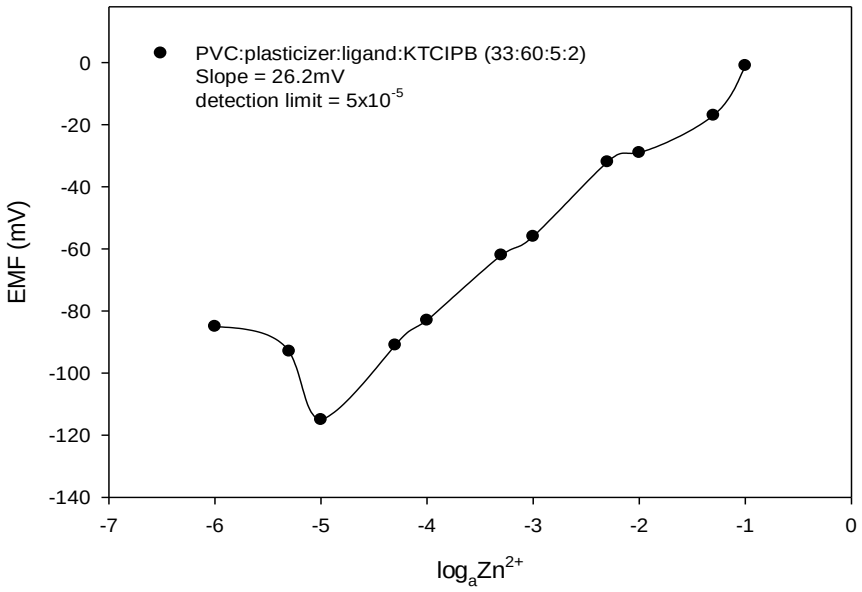

Fig 49 Response of PVC immobilized lyophilized biomass of *Pseudomonas striata* ISE (Ion selective electrode) towards zinc ions

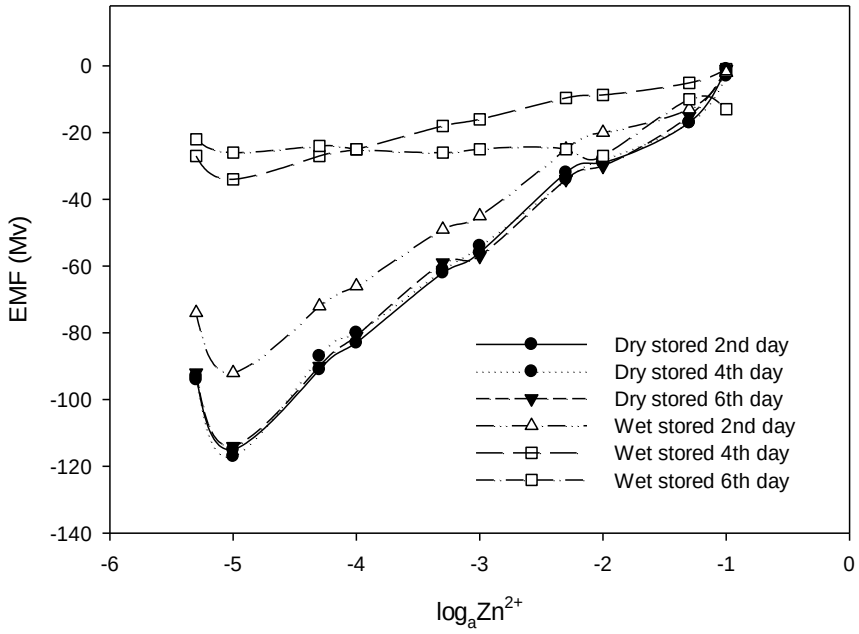

Fig. 50 Potentiometric response of *Pseudomonas striata* based ISE (Ion selective electrode) electrode stored in different conditions over the period of 6 days

Potentiometric selectivity Coefficient

One of the main features of any ion-selective electrode is its response to the primary ion in presence of other ions. Ion selective electrodes are rarely ion specific. The ability of an ion

selective electrode to distinguish between different ions in the same solution is expressed as the selectivity coefficient $-\log K^{pot}_{Ag+,B}$. The selectivity coefficient is not always constant and depends on several factors including the concentrations of both ions, the total ionic strength of the solution and the temperature. All electrodes are sensitive to some or other ions to some extent. For many applications these interferences are insignificant and can often be ignored. In some extreme cases, however, the electrode is far more sensitive to the interfering ions than the primary ions and can be used if the interfering ions are present only in trace quantities or completely absent. In our case, some interferences between Zn^{2+} and other metal ions could also be envisaged due to the well-known order of affinity of heavy metal ions for thiolates (Hg(II) >>Cu(I) $\approx$ Ag(I)>>Cd(II) > Pb(II) > Zn(II)) which is very close to that of Metallothionein. The observed values of the selectivity coefficients are presented in Table 21. For alkali and alkaline earth metal ions, the $-\log K^{pot}_{Ag+,B}$ values were of the order of 4 while the values for transition metal ions were in the range of 2-3 except for Pb^{2+} ions. Also the membrane electrode did not show any serious interference from Cu^{2+} ions. It is important to note that the selectivity coefficients for the Zn^{2+} electrode, with reference to most of the alkali and alkaline-earth metal ions, are quite small. This means that this membrane electrode will be highly efficient for determination of trace amounts of zinc in the presence of a large excess of alkali and alkaline-earth metal ions. The zinc response is seriously interfered with by small amounts of Hg^{2+} and Ag^{+} ions; so these two ions must be removed before the analysis of zinc from the samples.

Table 21 Potentiometric selectivity coeffiecients ($K^{Pot}_{Zn^{2+},B}$) of interfering ions for *Pseudomonas striata* based ISE (Ion selective electrode).

Interfering ions (B)	$-\log K^{pot}_{Ag+,B}$	
	FIM	MPM
Co^{2+}	-2.2	-2.6
Mn^{2+}	-3.5	-2.8
Cd^{2+}	-4.2	-3.9
Ni^{2+}	-2.8	-2.1
Cu^{2+}	-3.9	-4.1
K^{+}	-3.5	-3.2
Fe^{3+}	-3.8	-2.9
Mg^{2+}	-4.3	-4.0
Pb^{2+}	+0.2	+0.5
Hg^{+}	+1.4	+0.9
Ag^{+}	+1.2	+1.0

Chapter 5
Discussion

Heavy metal toxicity poses a serious threat to the delicate balance of the ecosystem. The physiology of the all the living organisms at different strata of ecosystem is profoundly and adversely influenced by the elevated levels of heavy metals. Continuous and accurate monitoring of the heavy metals is an inevitable part of any environment pollution management system. Challenge lies in assessing the toxicity of metal ions in the environment which is related to the amount of bioavailable metal rather than total metal concentrations determined by classical methods like atomic absorption spectroscopy (AAS), inductively coupled plasma mass spectroscopy (ICP-MS) and anodic stripping voltammetry (ASV) (Corbisier *et al*., 1999). Bioavailability is however a loosely defined term as the toxic bioavailable concentration of different metals varies from one species to another (Williams *et al*., 2000). In the present study the whole cell enzyme activity of different bacterial strains has been used as parameter to assess the toxicity levels of zinc, cadmium and copper. The inhibitory effect of the heavy metals (Zn, Cd and Cu) was studied on the alkaline phosphatase activity of *Pseudomonas striata* procured from Division of Microbiology, IARI, New Delhi and urease activity of *Bacillus sp.* MD028 (FJ005050) isolated from the agricultural field of Thapar University, Patiala. The studies were conducted with un-immobilized and immobilized bacterial cells. The microbes were immobilized in two different matrices viz., polyvinyl chloride (PVC) membranes and electropolymerised films of polypyrrole-polyvinylsulphonate (Ppy-PVS). The work is a novel approach for bacterial cells immobilization in PVC and Ppy-PVS and their use in integration with suitable transducers systems to develop a biosensor. The immobilization matrices (polyvinylchloride and polypyrrole-polysulphonate) containing the microbes were integrated with suitable transducer systems (Digital potentiometer, (Equiptronics EQ602) and Autolab Potentiostat/Galvanostat (Eco Chemie, Netherlands) which recorded the chemical changes of enzyme reactions as electrical signals.

5.1 Screening, isolation and identification of microbes sensitive to Cu, Zn and Cd ions

Nutrient rich agricultural soil is an abundant source of phosphate solubilizing and urease producing microbes. Soil sample from the agricultural fields of Thapar University campus were collected for the isolation of alkaline phosphatase and urease producing bacteria. In view of the sensitivity of soil enzymes to heavy metals, the use of enzyme activity as a bioindicator to evaluate the degree of soil contamination by heavy metals was proposed (Dick and Tabatai, 1992; Nannipieri, 1995). This study presented the use of whole cell urease and alkaline phosphatase activity of the soil bacteria to monitor the toxicity of Cu, Zn and Cd.

5.1.1 Isolation of alkaline phosphatase producing bacteria

Alkaline phosphatase (APA) present in the cell membrane is commonly used as a tool for the detection of heavy metal ions (Rogers, 1995). In bacteria, alkaline phosphatase is located in the periplasmic space, external to the cell membrane. Since this space is much more subjected to environmental variation than the actual interior of the cell, alkaline phosphatase was used as an efficient environmental monitoring system (Durrieu and Tran-Minh, 2002, Chouteau *et al*., 2004, Chouteau *et al*., 2005). Even a trace amount of heavy metal ions would inhibit the activity of enzyme because enzyme has tendency to complex with heavy metal ions to favour the formation of a stable metal enzyme complex. The greater degree of inhibition by a metal ion species is due to greater stability of the metal enzyme complex. (Lakshmi *et al*., 1991). In the present study alkaline phosphatase activity of the whole cell bacteria was selected as a parameter to monitor the toxicity of the heavy metals. Hence, several alkaline phosphatase producing bacteria were isolated from the soil. Comparison between the alkaline phosphatase activities of the bacterial isolates from soil and *Pseudomonas striata* procured from IARI, New Delhi showed that the alkaline phosphatase activity of *P. striata* was higher than all the isolates. *Pseudomonas striata* showed the maximum activity of 3 U/mg (Dry cell weight) among all the isolates and was selected for further enzymatic assay. *Pseudomonas* spp., a soil microorganism, associated in the rhizosphere zones of crop fields are powerful phosphate solubilizers. Bacteria belonging to family Pseudomonadales, genus *Pseudomonas* and species *P. striata* and *P. liquifaciens* is an important Phophorus Solubilising Biofertilizer (Motsara *et al.*, 1995). The genus *Pseudomonas* was described by Migula (1894) and is one of the most diverse and ubiquitous bacterial genera whose species have been isolated worldwide from all kinds of

environments. The principal mechanism for mineral phosphate solubilization is the production of organic acids and acid phosphatases that play a major role in the mineralization of organic phosphorous in soil. (Rodríguez and Fraga, 2000). Alkaline phosphatase is one of the many phosphatases functioning in soil and is largely responsible for the mineralization of organic phosphate compounds in acid soils. Alkaline phosphatase (ALP, EC 3.1.3.1) is a nonspecific esterase that catalyzes hydrolysis of many monoesters of phosphoric acid. Low biocatalytic selectivity enables the development of various substrates for optical and electrochemical enzyme activity assays. A clinically recommended spectrophotometric method for ALP determination uses p-nitrophenylphosphate as chromogenic substrate. Several substrates for optical methods based on phosphorescence, chemiluminescence and fluorescence measurements have been developed (Koncki *et al*., 2006). ALP activity can be determined by measuring the hydrolysis rate of an artificial substrate under controlled reaction conditions. Such results are suitable for a quantitative estimation of the effects that ecological parameters may have on phosphatase activity (Orhanovic and Pavela-Vrancic, 2000). In the present study p-nitrophenylphosphate was used as the chromogenic substrate for monitoring the whole cell alkaline phosphatase activity of *Pseudomonas striata.* The Km value and Vmax values obtained for this substrate from lineweaver burke plot were 14.28 mM and 4.1 µmol. min^{-1}. High value of Km was obtained as the bioassay was performed using whole cells of *P. striata* which cause slower diffusion of the substrate through the cell membrane.

5.1.2 Isolation of Urease producing bacteria

Urease is a common biocatalytic receptor, recognizing selected heavy metals ions. Several urease-based biosensors reported in the literature are used for detection of these toxic ions (Ogo´nczyk *et al*., 2005). There are enzymatically modified optical (Andres and Narayanswamy, 1995, Tsai *et al*., 2003), conductometric (Zhylyak *et al*., 1995) and capacitance (Kukla *et al*., 1999, Starodub *et al*., 1999) devices which have been used for the detection of heavy metals and other environmental pollutanats. Potentiometric biosensors for detection of urease inhibitors are based on ammonium (Senillou *et al*., 1999) and pH-ISFETs (Volotovsky *et al*., 1997, Soldatkin *et al*., 2000), as well as on conventional pHelectrodes based on iridium oxide (Krawczynski *et al*., 2000) or polypyrrole (Kubota *et al*., 1998). In the present study urease was chosen as a parameter to detect the sensitivity of

the microbes towards heavy metal ions. Thus urease producing bacteria were isolated from the soil samples collected from agricultural fields of Thapar University Campus, Patiala (India) using urea enriched Nutrient Agar (Stanier *et al.*, 1987). Enrichment conditions for a given organism are those that enable it to compete successfully and outgrow other organisms that may be present in the inoculum. Thus various growth promoting factors like nature of carbon, energy and nitrogen sources, gaseous atmosphere, hydrogen acceptors, temperature, light, pH etc. can be imposed as per choice for isolation of selective organisms (Schlegel, 1993). Isolates produced urease activity in the range of 5.3-9.2 U/ml. Out of the10 urease producing isolates, 2 isolates were screened on the basis of their urease producing efficiency and characterized on the basis of morphology, biochemical test and molecular approaches.

5.1.3 Morphological and biochemical characterization of bacterial isolate

The selected bacterial isolates were efficient urease producers. The phenotypic and physiological properties of these isolates resemble with *Bacillus* species reported previously (Smith *et al*., 1952; Gordon *et al*., 1973). Both the isolates were Gram positive long rods A brownish and slimy extracellular substance could be seen around the colonies of the isolate P8 after 24 hours of growth. Salt tolerance, growth temperature range, growth pH range, and extracellular products are important taxonomic criteria which were used to differentiate species in the genus *Bacillus* (Claus and Berkeley, 1986). The bacterial isolates in this study were found to be alkalotolerants showing optimal growth between pH 7.0 and 9.0, but cannot grow above pH 9.5 unlike alkalophiles, which grow optimally above pH 10 (Sharp and Munster, 1986; Krulwich and Guffanti, 1989). Due to notable properties of these isolates to efficiently produce the urease enzyme under the wide range of pH and temperature, makes them the ideal candidate to be used as biorecognition elements in a biosensor construct.

5.1.4 Molecular identification based on the phylogenetic analysis

The development and increased availability of techniques in molecular biology have made it possible to obtain information regarding the diversity of bacterial cultures isolated from different habitats (Amarger *et al*., 1994). Polymerase chain reaction (PCR) can produce products to the more highly conserved 5S, 16S, and 23S ribosomal subunits which can

potentially differentiate species and also show intraspecific differences (Wakabayashi *et al*., 1999). So to further confirm the genetic variability in the isolates, 16S rDNA was amplified and sequenced. Phylogenetic analysis revealed both the isolates belonged to genera *Bacillus*. On the basis of the 16S rDNA sequence analysis the isolates P8 and P10 were identified as *Bacillus* sp. MD028 (FJ005050) and *Bacillus subtilis* strain MD008 (EU780733) respectively.

5.1.5 Optimization of parameters for maximum urease production

Temperature, pH, and substrate concentration affect the production and activity of all the enzymes and thus need to be optimized (Haltrich *et al*., 1993). The maximum urease activity was observed at 37º C by all isolates. Generally above 40-50ºC the enzyme activity decreased because denaturation of proteins occurs at these temperatures. Urease activity of all isolates was lower at 50 º C. The urease activity of almost all the isolates was maximum and comparable at pH 8 and pH 9. Other factors like speed of molecules, the activation energy of the catalytic reaction and the thermal stability of the enzyme and substrate are affected by temperature hence its effect on enzyme activity is very complex. At low temperatures the kinetic energy of the molecules is very low resulting in less frequent collisions with insufficient energy required for the reaction to occur. Thus, it can be said that the enzymes are deactivated at low temperatures. This energy barrier can be overcome by increasing the temperature. Increase in temperature enhances the kinetic energy and consequently the increase in enzyme activity is observed. The highest rate of enzyme activity is observed between 0-40°C and this increase is almost linear. The reaction rate decreases beyond 40°C because the increase in temperature disrupts the forces maintaining the shape of the molecule resulting in the denaturation of the enzyme. Enzymes are completely denatured above 65°C (Robinson *et al*., 2005). Neidhardt *et al*. (1990) emphasized on the necessity for placing cultures into balanced growth prior to collecting any data. Isolates exhibited abundant growth in the presence of urea indicating the coupling of urea hydrolysis and ATP generation (Jahns, 1996). Mobley and Hausinger (1989) also reported the use of urea as a source of energy by Bacillus cells, resulting in the production of ammonia and carbonate. Varying urea concentrations have a direct effect over urease activity. The urease production profile of the strain as a function of growth was examined in a complex nutrient medium. The production could be seen from the early exponential

phase onwards. It was very low during the early stages of exponential phases. The isolates showed a steady increase in urease production with the progression of growth from early exponential to early stationary phase. Different *Bacillus* species have been reported to be producing the maximum enzyme during the late exponential (Atalo and Gashe, 1993), post exponential (Ward, 1983; Manachini *et al*., 1998) and the stationary (Durham, 1987; Purva *et al*., 1998) phases of growth. The observations made with respect to optimization of growth conditions and process parameters that govern maximal production of urease by different bacterial isolates strengthen the potential of the organism as source of enzyme in a biosensor assembly.

5.2 Metal-microbe interaction studies to determine threshold concentration of metal ions

Bacteria have emerged extremely instrumental in monitoring positive and negative responses to different environmental conditions, such as the presence of nutrients or toxins, respectively. The ability to constantly sense their immediate environment makes bacteria ideal for determination of the bioavailability and/or toxicity of pollutants (Bjerketorp *et al*., 2006). Development of bacterial whole-cell biosensors in combination with high-throughput, low-cost instrumentation for the analysis of environmental samples has recently gathered attention of many researchers (Bjerketorp *et al*., 2006). Bacterial biosensors can be distinguished into two principle classes i.e., constitutive and inducible (Jansson, 2003, Gu *et al*., 2004). Constitutive reporter cells produce a constant measurable signal, and the general toxicity of a sample is estimated from the inhibition of this signal. Inducible reporter microorganisms are based on a reporter gene fused to an inducible promoter that is activated by a target compound or stress response (Jansson, 2003, Gu *et al*., 2004). In this study the alkaline phosphatase activity of *Pseudomonas striata* and urease activity of bacterial isolate P8 (*Bacillus sp.*) have been used as constitutive reporter cell systems to assess the heavy metal toxicity of the sample based on the inhibition of respective whole cell enzyme activities. Mechanism of the enzyme inhibition is based on interactions between heavy metal ions and thiol/methylthiol groups of cysteine/methionine presented in the active centre of the enzyme (Ogo´nczyk *et al*., 2005).

5.2.1 Enzyme inhibition based bioassays for detecting heavy metals

Bioassays are the first step of an enzymatic screening system for chemical pollution in water (Durrieu *et al*., 2003). The present study reported a colorimetric bioassay using the alkaline phosphatase activity of *Pseudomonas striata* cells and urease activity of *Bacillus* sp. MD 028 to study the effect of zinc, copper and cadmium. Different inhibition trends were obtained for both the enzymes with zinc and copper being most inhibitive towards alkaline phosphatase and urease respectively. The results so obtained are in good agreement with those reported earlier. Out of the three metals studied zinc produced maximum inhibition of the alkaline phosphatase enzyme followed by cadmium and copper. Maximum alkaline phosphatase inhibition caused by zinc was 93.9% an effect that occurred at 1.7 mg l^{-1}. A low detection limit of 10 ppb was obtained. Similar toxic effects of zinc on the phosphatase activity have been reported earlier. Zinc has been reported to inhibit a variety of enzyme activities in soil, including acid and alkaline phosphatase (Juma and Tabatabai, 1977; Doelman and Haanstra, 1989). A significant negative correlation was found between acid phosphatase activity and the amount of Cu and Zn in polluted soils in Sweden (Tyler, 1974). Doelman and Haanstra (1989) reported that Zn was one of the heavy metals most toxic to phosphatase activity. Bioassays have been developed for the detection of *C. vulgaris* alkaline phosphatase activity and the influence of heavy metal ions (Durrieu *et al*., 2003). The microalgae Chlorella vulgaris has been used in different works to produce whole cell biosensors to monitor toxic pllutants in aquatic media (Durrieu and Tran-minh, 2002, Pandard *et al*., 1993). Guedri and Durrieu, 2008 reported the construction of whole cell algal biosensor using self assembled monolayers (SAM) of *Chlorella vulgaris* to detect heavy metals in the concentration range of 1 and 10 ppb. Toxic effects of cadmium on alkaline phosphatase activity have also been reported in the literature. Chouteau *et al*., 2005 studied the inhibition of *Chlorella vulgaris* microalgae alkaline phosphatase activities in presence of cadmium ions and obtained a very low detection limit of 1 ppb. The difference of these results from that reported in present study could be attributed to the difference in the sensitivities of the microorganisms used for the biosensor system.

5.3 Immobilization and process optimization for whole cell-electrode assembly and its application for detection of heavy metals

In the present study microbes were immobilized in two different matrices viz., polyvinyl chloride (PVC) membranes and electropolymerised films of polypyrrole-polyvinylsulphonate (Ppy-PVS) (Al-Hitti *et al*., 1984, Chaubey *et al*., 2000). Immobilization of whole cells improves the stability of the enzyme by retaining them in their natural surrounding and decreasing the cost for lengthy and expensive procedures for extraction and purification of the enzymes (D'Souza, 2001a; D'Souza, 1999). In this study the *Bacillus*-GLDH/PPy-PVS/ITO bioelectrodes were observed to have good stability and shelf life of 6 months. The bioelectrodes exhibited no loss in the urease activity for the first month and retained about 95% of the urease activity even after 6 months. Cell bound enzymes are more tolerant to environmental perturbations like pH, temperature, heavy metal poisoning denaturation and inactivation (D'Souza, 1989). Immobilized biomass can also act like self-proliferating biocatalyst within the matrix (D'Souza, 1989). One of the limitations of using whole cells in sensor is the low sensitivity and specificity because of permeability barrier and unwanted side reactions catalysed by other enzymes within the cell. These intereferences could be further enhanced by the immobilization matrix (D'Souza, 2001a,b). In the present study the limitation of the permeability barrier imposed by immobilization technique was over come by the use of covalent immobilization of the bacterial biomass. The lyophilized biomass of *Bacillus* sp. MD 028 was observed to retain 98-99% of the whole cell urease activity even after immobilization. Another strategy is to use recombinant DNA technology or controlled expression of the gene of interest for maximum yield of desired enzyme. The latter requires cultivation of microorganism in specific medium containing appropriate substances for controlled gene expression (Di Paolantonio and Rechnitz, 1982; Fleschin *et al*., 1998; Riedel *et al*., 1990). Thus microbial urease synthesis is repressed in presence of nitrogen rich compounds including ammonia and urea whereas derepressed under nitrogen starvation conditions (Harry and Robert, 1989). Similar results were obtained in the present study when the growth culture of *Bacillus* sp. MD 028 was harvested at late log phase and was observed to have maximum urease activity of 12 U/mg d.c.w. Choice of a suitable immobilization matrix is an important parameter for biosensor. Conducting polymers have emerged as an immobilization matrix that can also serve as a transducer (Contractor *et al*., 1994; Gerard *et*

al., 2002; Hoa *et al*., 1992; Sukeerthi and Contractor, 1998). They can be used for immobilization of pure enzyme (Hoa *et al*., 1992), aptamers (Liao *et al*., 2008), nanoparticles (Fredj *et al*., 2008) as well as microbial cells (Palmqvist *et al*., 1994).

5.3.1 PPy-PVS as the immobilization matrix

The electrical conductivity of polypyrrole can be modulated in the range of 10^{-3} to 10^{3} Ω/cm (Kros *et al*., 2005, Diaz and Bargon, 1986).Various forms of polypyrroles can be easily prepared by electrochemical techniques and oxidation of pyrrole in presence of desired dopant ions results in a doped film deposited at the surface of the electrode (Teasdale and Wallace, 1993, Sadik, 1995). Conducting polymer matrices have been reported to have improved environmental stability, biocompatibility, increased polymerization growth with higher compactness and conductivity when used with large polymeric anions such as *para*-tolutene sulfonate (pTS), polystyrene sulfonate (PSS), polyvinyl sulfonate (PVS) that helps in maintaining the charge neutrality during reduction process (Gaikwad *et al*., 2006). It has been suggested that size of dopant ions induces changes in molecular confirmation resulting in increased electrical conductivity (Kumar *et al*., 2001). Polypyrrole-polyvinyl sulfonate composite membrane has been shown to play important role as a 'charge controllable membrane' in which the fixed charges can be controlled electrochemically by an internal electrode (Shimidzu *et al*., 1988). In the present study the lyophilized bacterial biomass was immobilized on conducting polypyrrole-polyvinyl sulfonate (PPy-PVS) coated onto ITO glass plate electrode using entrapment and carbodiimide (EDC–NHS) coupling. Cyclic voltammetry and square wave voltammetry were used to characterize the bioelectrode. In the cyclic voltammograms the oxidation peaks of both PPy-PVS/ITO and *Bacillus*-GLDH/PPy-PVS/ITO were observed at about 0.6V with peak current values of 150 µA and 53 µA respectively. This decrease in peak current observed for *Bacillus*-GLDH/PPy-PVS/ITO indicated the proper immobilization of the bacterial biomass on the surface of the conducting polymer (PPy-PVS) that partially blocks transport of electrons between the reaction solution and electrode surface.

5.3.2 Advantages of amperometric system of urease detection

Enzymatic reactions have proved to be very promising tools to identify major pollutants such as heavy metals, enabling a very accurate toxicity identification evaluation (TIE)

based on their inhibition (Brack *et al*., 2000). Assays based on the inhibition of urease show a high selectivity for the sensitive and effect-based screening of heavy metals (Jung *et al*., 1995; Wittekindt *et al*., 1996; Brack *et al*., 2000). Most urease inhibition assays are based on the measurement of either pH changes (Shi *et al*., 1997; Kormos and Lengauer, 2000; Krawczyk *et al*., 2000) or ammonia production (Gil and Piedade, 1992; Soldatkin *et al*., 2000).The present study demonstrated the efficiency of the amperometric detection system for monitoring the urease activity. The *Bacillus*-GLDH/PPy-PVS/ITO bioelectrode used in the present study is a hybrid of two enzyme systems; urease and glutamate dehydrogenase (GLDH). Lyophilized biomass of the *Bacillus* sp. cells was used as a source of urease. The whole cell urease catalyzes the hydrolysis of urea to carbamine acid which is further hydrolysed to ammonia and carbon dioxide. GLDH catalyzed the reversible reaction between α -KG and NH_3 to NAD+ and linked oxidative deamination of l-glutamate in two steps. The first step involved a Schiff base intermediate being formed between NH3 and α-KG (Kaushik *et al*., 2009). The second step involved the Schiff base intermediate being protonated due to the transfer of the hydride ion from NADH which resulted in l-glutamate. NADH was utilized in the forward reaction of α -KG and free NH3 that were converted to l-glutamate via hydride transfer from NADH to glutamate. NAD+ was utilized in the reverse reaction, involving l-glutamate being converted to α-KG and free (NH_3) via oxidative deamination reaction (Kaushik *et al*., 2009). The electrons generated from the biochemical reactions are transferred to the PPy-PVS/ITO electrode through the Fe(III)/Fe(IV) couples that helped in amplifying the electrochemical signal resulted in increased sensitivity of the sensor. This mechanism of the urease and GLDH hybrid system was explained by Kaushik *et al*., 2009. Thus the above mechanism utilized all the free ammonia liberated due to the hydrolysis of urea and did not cause any unwanted pH change or consequent urease activity fluctutations in the electrochemical system which otherwise have been reported to be the major shortcoming of the urease inhibition tests based on measurement of ammonia evolution (Jung *et al*., 1995, Brack *et al*., 2000). Also, potentiometric sensors based on the determination of protons show the disadvantage of deactivating urease due to increase in pH value around the electrode when it is in use (Yoneyama *et al*., 2001). Again, strong buffers are usually required in these cases, making the sensor response dependent on buffer concentration (Yoneyama *et al*., 2001). Therefore, an amperometric detection system for monitoring urease activity will significantly

overcome the shortfalls listed above when using potentiomentry for urease activity measuring system.

5.3.3 Optimization of the cell density

The amount of biomass loading on electrode surface is a very important factor which determines the performance of the electrode. Both low and high biomass loading adversely affect the performance of the biosensor. If the biomass loading is high then the enzymatic reactions occur only at the border of the membranes preventing substrate molecules from diffusing inside and reacting with microbes situated near the sensitive areas. As a consequence, a low signal is observed. Moreover low biomass concentration also gives slight signal variations since only a few substrate molecules can react (Chouteau *et al*., 2004). It is interesting to note that for pure enzyme membranes the same conclusion has already been done (Mai Anh *et al*., 2002).

5.3.4 Effect of pH on the Bacillus/PPy-PVS/ITO bioelectrodes

Conducting polypyrrole (PPy) due to its operational compatibility at physiological pH and the ease of conductivity modulation (with the counter ions) has attracted attention of various researchers for application to biosensors (Arora *et al*., 2007). PPy-PVS electroactive in a wide pH range of 1-8. The peak current values were found to decrease from acidic to alkaline pH, with maximum peak current of 149 µA observed for pH 2 and minimum current of 11 µA observed for pH 9. Thus the possibility of using extreme pH values for the measurement of urease activity was ruled out as the acidic pH conditions are highly unfavourable for the catalytic activity of urease and high alkaline pH of 9 drastically lowers the electroactivity of PPy-PVS/ITO electrode hence making it unsuitable for the measurement of urease activity.

5.3.5 Detection of Urea using *Bacillus*-GLDH/PPy-PVS/ITO bioelectrodes

Good linearity towards the low urea concentration (1.5 mM-18 mM) was observed and thereafter the response became slightly sluggish due to saturation of enzyme active sites with urea. The observed sluggish increase in the current for solutions at higher concentration is likely to be due to restriction of the enzymatic reaction. At higher urea concentration, the original first order enzymatic reaction appears to have changed to 0th

order reaction at which the reaction rate becomes independent of substrate concentration (Cho and Huang, 1998).

Similar biosensor response towards urea was also reported by Kaushik *et al*., 2009. This whole cell urea biosensor had a high Km value of 9.6 mM as against 3-5 mM urea for purified urease from Jack beans suggests a low affinity of bacterial urease towards urea and therefore, thus the biosensor developed with this enzyme source would be suitable in higher range of urea concentration (Hirofumi *et al*., 1984). Thus a relatively high detection limit of 1.5 mM was obtained. High sensitivity value of 15 µA/(mM cm^{-2}) was obtained for the bioelctrode which can be attributed to the efficient immobilization strategy which resulted into unhindered transfer of electrons from electrolyte to the electrode surface and hence good sensor response was obtained. Arora *et al*., 2007 also reported higher sensitivity obtained for dCPPy-PVS/Pt electrodes prepared by direct immobilization due to the increased interaction of the oligonucleotides stationed near the PPy-PVS surface.

5.3.6 Detection of heavy metals using urea sensor

The effect of different metal ions on urease activity is related to the affinity toward the –SH groups present in the enzyme structure (Rodriguez *et al*., 2004). The results of the present study compare very well with findings of other authors. Krawczyk *et al*. (2000) developed a sensor with urease entrapped in PVC layer at the surface of a pH-sensitive iridium oxide electrode that allowed them to determine Hg(II) in the low µM range. The IC_{50} value calculated for this metal by these authors was 0.2µM (40 µg l^{-1}), while metals such as lead, cadmium or zinc produced the same effect at concentrations 500 fold higher. Volotovsky *et al*. (1997) obtained lower sensitivity for these metals with urease immobilised in Nafion on the surface of an ion sensitive field effect transistor that recorded pH changes (300 µg l^{-1} for Hg(II) and 317 µg l^{-1} for Cu(II)). Preininger and Wolfbeis (1996) developed a disposable cuvette test with integrated sensor layers for the determination of metal ions. The urease enzymatic reaction was followed by monitoring the colour of the layer, achieving IC50 values of 650 µg l^{-1} for Hg(II), 550 µg l^{-1} for Cu(II) and 210 mg l^{-1} for Pb(II). The discrepancy observed among authors in metals concentration required to produce 50% urease inhibition can be due to the amount of enzyme used in each case, since the higher urease activity in the assay, the less inhibition effect for the same metal concentration (Preininger and Wolfbeis, 1996). The effect of multiple metal ions in the

solution has been investigated by some authors. In the presence of multiple heavy metal ions a synergetic effect has been observed previously in such way that the total urease inhibition can be calculated by addition of the estimated inhibition values for individual cations (Krawczyk *et al*., 2000). The effect of various metals on urease activity produced an increase in the enzyme inhibition due to the addition of each metal effect. This effect allows the use of the developed assay for toxicity analysis produced by metal pollution (Rodriguez *et al*., 2004). The higher detection limits and IC_{50} values obtained in the present study could be attributed to the use of phosphate buffer as the presence of phosphate in the assay would bind the metals (Jung *et al*., 1995) or inactivate the enzyme (Krajewska and Zaborska, 1999). The other possible reason for high detection limit could be the use of whole cell as the source of enzyme which are comparatively more stable and resistant to the presence of heavy metals in the surrounding media than pure enzymes.

5.3.7 Comparison of bioassays and biosensors

Inhibition (or activation for short exposures) rates are higher using biosensors than bioassays. It can be explained by the different ratios 'number of alga cells/cadmium' in both cases. Indeed in membranes, low amounts of algae were immobilised compared to bioassays using free algae: for biosensors, the ratio algae/cadmium is lower than for bioassays. As inhibitions rates are inversely proportional to these ratios, as a result, biosensors give higher inhibition rates and they seem to be more sensitive to detect the APA modifications (Chouteau *et al*., 2004). In the present study similar results were obtained for urease bioassay (free cell) and biosensor (immobilized cells) as the biomass amount was kept same for both the methodologies. Therefore the detection limits and linear range obtained for zinc, copper and cadmium were quite similar for colorimetric urease bioassay and amperometric *Bacillus*-GLDH/PPy-PVS/ITO bioelectrode.

5.3.8 Potentiometric zinc selective biosensor based on *Pseudomonas striata* cells

The zinc selective PVC electrode was prepared by immobilizing lyophilized biomass of *Pseudomonas striata* in polyvinyl chloride matrix. Polyvinyl chloride (PVC) is highly hydrophobic and impermeable to any ions. It was plasticized (softened) by addition of a similarly hydrophobic solvent, e.g.,DBP (Dibutyl pthalate), o-NPOE (ortho nitrophenyl octyl ether). So, far the membrane is just a flexible piece of plastic, which acts as a near

perfect barrier to ions. To make it ion-selective, lyophilized biomass of *Pseudomonas striata* was added as a ligand and its selectivity towards heavy metals ions was tested. Many chemical ionophore based potentiometric biosensors have been reported for the detection of metal ions, but all these suffer from the drawback of low ion selectivity as the ionophores used are not ion specific. The use of bioligands as the ionophore for the construction of potentiometric biosensor is a novel concept. An Ag+-ion selective electrode using polysulfone matrix embedding metallothioneins as ionophores with the detection limit of about 10^{-5} M was reported by Gonzalez-Bellavista *et al*., 2009. Since construction of such biosensors required small amount of proteins they can be dry-stored and have long-lifetimes. In this study lyophilized cells of *Pseudomonas striata* were used as the source of alkaline phosphatase (Ligand) containing Zinc ligating sites. These Zinc ligating sites of the enzymes are exposed to the external solution and made available for binding to the metal ions due to the rupturing of the bacterial cell walls by Tetrahydrofuran (THF) (solvent used for the preparation of PVC membrane). This was confirmed by observing the THF cell suspension under the microscope.

Zinc binding sites in proteins are often distorted tetrahedral or trigonal bipyramidal geometry, made up of the sulfur of cysteine, the nitrogen of histidine or the oxygen of aspartate and glutamate, or a combination. Zinc in proteins can either participate directly in chemical catalysis or be important for maintaining protein structure and stability. In all catalytic sites, the zinc ion functions as a Lewis acid. An understanding of naturally occurring zinc-binding sites will aid in creating de novo zinc-binding proteins and in designing new metal sites in existing proteins for novel purposes such as to serve as metal ion biosensors. (McCall *et al*., 2000). Zinc, always occurring as a divalent cation Zn^{2+} in biological systems, is the second most abundant (common) transition metal following iron. Today more than 300 different zinc proteins are known. These include numerous essential enzymes which catalyze the metabolic conversions (synthases, polymerases, ligases, transferases) or degradation (hydrolases) of proteins, nucleic acids, lipids, porphyrin precursors and other important bioorganic compounds (Urbanova *et al*., 2008) .

Also, it was comfirmed that the activity of alkaline phosphatase which contains the zinc binding sites released due to cell rupture is not lost as was confirmed spectrophotometrically by performing the enzyme assay using para- nitrophenylphosphate (p-NPP) as the substrate at pH-8.3 and 37ºC (Barnes and Morris, 1957). The enzymatic

reaction leads to the conversion of the substrate (p-NPP) to a yellow colored compound para-nitrophenol (p-NP) whose optical density was measured at 420 nm using a UV-Vis spectrophotometer (Hitachi).

The sensitivity and selectivity of an electrode are significantly affected by the nature of the plasticizer, the composition of ionophore, internal solution (Mi *et al*., 1999; Sokalaski *et al*., 1997; Sokalaski *et al*., 1999), etc. In neutral carrier membranes, plasticizers that are compatible with the ionophore provide a smooth surface to the membrane and hence enhance the response characteristics (Cammann, 1979). The nature of the plasticizer influences the dielectric constant and the mobility of the ions in the membrane. These membrane solvents are seen to strongly influence the working concentration range and the slope of the sensor. It was observed that the electrode with DBP as plasticizer was found to give the best response in terms of the slope and the concentration range. The slopes in the case of the o-NPOE are sub- Nernstian. The potentiometric response of the sensor towards Zn (II) ions is found to be dependent on the concentration of the ionophore used. Different compositions (w/w%) of the ionophore were also tried to obtain the right composition of ionophore that gives the best response characteristics. The maximum sensitivity was observed for 5% (w/w) of the ionophore. On increasing the ionophore content, the slopes are affected; this may be related to the change in the water uptake capacity of the membrane (Kumar *et al*., 2006) and also may be due to the reason that equilibration of the ionophore with the metal ions is maximum at this concentration (Mittal *et al*., 2007).

The linearity observed in the case of Zn^{2+} (Fig 1) can be attributed to the Zinc ligating sites present on Zn-metalloenzymes. These Zn-ligating sites lying at the interface of the internal and test solution are exposed to the concentration gradient across the membrane which leads to the generation of potential difference measured in terms of electromotive force (emf). This further supports the non-linear trends observed for Cd and Cu, confirming the selective binding of zinc by the zinc metalloenzymes.

The results presented reveal that the potentials are independent of pH in a very narrow range of 3-4 and this range is taken as the working pH range of the Zn(II) sensor. Variation of potentials above and below these pH values can be related to hydrolysis of Zn(II) (at higher pH) and the competition of H+ with Zn(II) (at lower pH values).

5.3.9 Potentiometric selectivity Coefficient

One of the main features of any ion-selective electrode is its response to the primary ion in presence of other ions. Ion selective electrodes are rarely ion specific. The ability of an ion selective electrode to distinguish between different ions in the same solution is expressed as the selectivity coefficient $-\log K_{Ag+,B}^{pot}$. The selectivity coefficient is not always constant and depends on several factors including the concentrations of both ions, the total ionic strength of the solution and the temperature. All electrodes are sensitive to some or other ions to some extent. For many applications these interferences are insignificant and can often be ignored. In some extreme cases, however, the electrode is far more sensitive to the interfering ions than the primary ions and can be used if the interfering ions are present only in trace quantities or completely absent. In our case, some interferences between Zn^{2+} and other metal ions could also be envisaged due to the well-known order of affinity of heavy metal ions for thiolates (Hg(II) >>Cu(I) $\approx$ Ag(I)>>Cd(II) > Pb(II) > Zn(II)) which is very close to that of Metallothionein (Vasak, 1991). For alkali and alkaline earth metal ions, the $-\log K_{Ag+,B}^{pot}$ values are of the order of 4 while the values lie in the range of 3, 2 for transition metal ions, except for Pb^{2+} ions. Also the membrane electrode did not show any serious interference from Cu^{2+} ions. It is important to note that the selectivity coefficients for the Zn^{2+} electrode, with reference to most of the alkali and alkaline-earth metal ions, are quite small. This means that this membrane electrode will be highly efficient for determination of trace amounts of zinc in the presence of a large excess of alkali and alkaline-earth metal ions. The zinc response is seriously interfered with by small amounts of Hg^{2+} and Ag^{+} ions; so these two ions must be removed before the analysis of zinc from the samples. This is in good correspondence with literature data regarding electrodes of different nature (Siswanta *et al*., 1996, Wroblewski *et al*., 1995 and Chen *et al*., 2000) and with the higher affinity of this metal ion for the Cys residues if compared with that of the other metal ions studied (Pb^{2+}, Zn^{2+}, Cd^{2+} and Cu^{2+}).

Conclusions

1. Two urease producing strains were isolated from soil and identified as *Bacillus* sp. MD028 (FJ005050) and *Bacillus subtilis* strain MD008 (EU780733) using 16S rDNA sequencing. *Bacillus* sp. MD028 (FJ005050) showed good urease activity (12 U/mg (Dry cell weight)) as compared to *Bacillus subtilis* strain MD008 (EU780733) [9 U/mg (Dry cell weight)].

2. Among the various bacterial isolates screened for phosphatase activity, *Pseudomonas striata* procured from IARI, New Delhi showed the maximum alkaline phosphatase activity (3 U/mg (Dry Cell weight)).

3. The effect of three heavy metals viz., Cu, Cd and Zn on the alkaline phosphatase activity of free and immobilized *P. striata* cells showed an inhibition trend of Zn>Cd>Cu. The inhibitory response of heavy metals was linear in the concentration range of 0.01-1 mgl^{-1} for Zn, 0.1-4 mgl^{-1} for Cd and 1-30 mgl^{-1} for Cu.

4. The effect of three heavy metals viz., Cu, Cd and Zn on the urease activity of free and immobilized *Bacillus* sp. MD028 (FJ005050) cells showed an inhibition trend of Cu>Zn>Cd. The inhibitory response of the heavy metals was linear in the concentration range of 0.01-1 mgl^{-1} for Cu, 1-30 mgl^{-1} for Cd and 1-30 mgl^{-1} for Zn.

5. This work presented a novel and efficient strategy for immobilization of lyophilized cell mass of *P. striata* in poly vinyl chloride matrix which upon immobilization retained more than 85 % of the alkaline phosphatase activity which indicated that the protein structure was not damaged after immobilization. An equally efficient and novel strategy of immobilization was used for the immobilization of lyophilized biomass of *Bacillus* sp. MD028 (FJ005050) by covalently attaching the bacterial cells on the conducting polymer matrix of PPy-PVS/ITO electrodes. The *Bacillus* sp. MD028 cells retained approximately 98 % of the urease activity after immobilization. Both these immobilization techniques are novel and are being reported for the first time for the immobilization of bacterial cells.

6. The potentiometric bioelectrode fabricated by immobilizing *Pseudomonas striata* cells in PVC was found to be fairly selective and sensitive for the Zn ions. The present work is the first report on the use of bacterial cell as metal ligands in a

sensing device. The potentiometric response generated by the zinc selective electrode can be attributed to the selective binding of the zinc ions to the zinc ligating sites present in the bacterial biomass immobilized in the PVC membrane. This zinc bioelectrode exhibited a linearity range of 10^{-1} to 10^{-5} M for zinc with near nernstian slope of 26.2 mV per decade. The membrane electrode showed a sharp response time of 6-10 sec and detection limit of 5×10^{-5} M at 25±1ºC in the pH optima of 3-5. The interference was found to arise only from few transition metals such as Hg^{+2}, Ag^{+} and Pb^{+2}. For alkali and alkaline earth metal ions, the $-\log K^{pot}_{Ag+,B}$ values are quite small. Also the membrane electrode did not show any serious interference from Cu^{2+} ions.

7. The urease bioelectrode fabricated by immobilizing bacterial cell mass on PPy-PVS matrix supported on indium tin oxide coated glass had a response time of 210 s and showed linear response towards urea in the range of 1.5-18 mM. The urease bioelectrode was used for the detection of heavy metals ions and showed linearity in the concentration range of 0.008-1 mgl^{-1} for Cu and 1-30 ppm for Cd and 0.7-30 mgl^{-1} for Zn. This bioelectrode retained 95% of enzyme activity after 6 months at $4^{o}C$.

8. Results of the urease bioassay and urease bioelectrode were in good agreement with each other implicating the efficiency of the covalent immobilization which did not cause any interference to the permeability of substrate and metal ions. Therefore the extent of whole cell urease inhibition by heavy metal ions was same for urease bioassay and urease bioelectrode.

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Indian Journal of Science and Technology Vol.2 No. 7 (July 2009) ISSN: 0974- 6846

Sensor based on polyvinyl chloride immobilized *Pseudomonas striata* cells as metal-ionophore

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Abstract: PVC membrane containing lyophilized cell mass of *Pseudomonas striata* was prepared using dibutyl-pthalate as the plasticizer. Anionic interferences were excluded by use of potassium salt of tetrakis (4-chloro-phenyl) borate (KTCIPB). The electrode was found to be fairly selective and sensitive for the zinc ions. It exhibited a linearity range of 10^{-1} to 10^{-5} M with near nernstian slope of 26.2 mV per decade. The membrane electrode showed a sharp response time of 6-10 sec and detection limit of 5×10^{-5} M at 25±1ºC in the pH optima of 3-5. The interference was found to arise only from few transition metals such as Hg^{+2}, Ag^{+} and Pb^{+2}.

Keywords: Zn-metalloenzymes, Heavy metal determination, Potentiometric biosensor, Ionophore.

Introduction

The toxic nature of the heavy metals necessitates the need of their determination in biological materials, natural waters, soils and air even at trace levels. Bioaccumulation of the heavy metals has been reported to be higher in the upper trophic levels at concentrations surpassing those found in water supplies (Krawczyk *et al*., 2000). The conventional methods used for the determination of the heavy metals based on spectrophotometry, chromatography, mass spectrometry and various hyphenated techniques require sophisticated and expensive equipments, highly trained staf: besides they are usually time-consuming (Sherma & Zweig 1983; Dzyadevych *et al*., 2005). Also these conventional methods give the estimate of the total heavy metals present in the environment which is different from the bioavailable concentration that actually affects the living organisms. Thus, need arises for the fast and inexpensive methods to detect bioavailable heavy metals. Biosensors are useful analytical devices in this respect, and several configurations have been described in the past for heavy metal detection.

Wide spectrum of biological recognition elements and transducer systems has been used for the fabrication of biosensors (Bentley *et al*., 2001; Castillo *et al*., 2004; Amine *et al*., 2006). Of the different biorecepotors used for the fabrication of the heavy metal sensor, metalloenzymes/metalloproteins are potentially most promising because of their specificity for metal binding (McCall *et al*., 2000). Different metalloproteins/peptides have been used for developing heavy metal sensors (Cherian *et al*., 2003; Chow *et al*., 2005). The high selectivity of these metal binding molecules even in complex natural solutions like sea water or blood when combined with a suitable transducer has a great promise as an indicator system that may in the future replace the current techniques of measuring very low concentrations of metal ions (Kielland, 1937; Thompson *et al*., 1996). In the present work plasticized PVC membranes were used as a support matrix for the entrapment of lyophilized bacterial cells to fabricate a Zn^{2+} selective potentiometric electrode. *Pseudomonas striata* was selected because this strain produces sufficient amount of alkaline phosphatase which is Zn-metalloenzyme and has zinc ligating sites. In addition, there are many other Zn-metalloenzymes which are present in prokaryotic systems that might be responsible for the zinc selective nature of the electrode. Literature searches have ascertained sequences, zinc content and functional characteristics of the catalytic, cocatalytic and structural zinc sites for families of zinc enzymes. The X-ray structure analyses of 11 enzymes containing a single catalytic zinc atom identify their ligands. This metal forms complex with any of the nitrogen and oxygen ligands of histidine and glutamate residues with a binding frequency of His>>Glu (Vallee & Auld, 1993).

In the present work the zinc ligating property of *in-vivo* alkaline phosphatase and other Zn-metalloenzymes has been explored for the purpose of making a biosensor for Zn^{2+} ions. Lot of ionophore-based chemical sensors have been reported till date which make use of a large number of chemical metal ligands as the ionophore but lack selectivity. Al-Hitti *et al*. (1984) demonstrated the immobilization of GOD (Glucose oxidase) within plasticized polyvinylchloride membrane, which was then used for glucose determination. The methodology followed for the preparation of electrode is same as described by Mittal *et al*. (2007).

Materials and methods

Reagents

Reagents like dibutyl phthalate (DBP), o-nitrophenyloctyl ether (o-NPOE) were procured from Sigma-Aldrich. All other chemicals were of analytical reagent grade. Double distilled deionized water was used throughout the experiments.

Ligand preparation

Pseudomonas striata was cultured on nutrient agar plates for 17 h. The cells were harvested using Tris-HCl buffer pH-8.3. The cell suspension was centrifuged at 8000 rpm for 10 min to obtain a cell pellet which was lyophilized at -50ºC under vaccuum using a freeze dryer (Modulyod, ThermoElectron Corporation) to obtain dry cell mass.

Electrode preparation

Indian Journal of Science and Technology Vol.2 No. 7 (July 2009) ISSN: 0974- 6846

Membranes of ~ 0.2 mm thickness were obtained by pouring a solution of the membrane components of PVC 33%, bio-ligand (lyophilized bacterial cells) 1-7%, potassium salt of tetrakis(4-chloro-phenyl) borate (KTCIPB) 1-3% and dibutyl phthalate/ o-nitrophenyloctyl ether 59-65%, dissolved in 2-3 ml of tetrahydrofuran (THF). The viscous solution of the polymer thus obtained was poured in a glass ring of 30 mm diameter placed on a dust free pyrex glass plate. The solvent was allowed to evaporate slowly for about 24 hrs at room temperature. To obtain the membrane with similar characteristics, viscosity of the casting solution and rate of solvent evaporation were controlled so that the thickness and morphology of the membranes remained unchanged and the appearance of the film looked pale yellow in colour. The membranes were then removed from glass ring and circular pieces of 1.25 cm diameter were cut and mounted on the ground end of a pyrex glass tube with an adhesive and conditioned with a metal solution ($ZnSO_4/CuSO_4/CdSO_4$) (0.1 M) for 2 h.

Table 1. Optimization of membrane ingredients

PVC (wt%)	*Plasticizers (wt%)*	*Ligand (wt%)*	*KTCIP B (wt%)*	*Slope (mV/decade)*	*Detection limit (M)*
33	*65 (DBP)*	*2*	*-*	*15*	1×10^{-4}
33	*64 (DBP)*	*3*	*-*	*18*	1×10^{-4}
33	*63 (DBP)*	*4*	*-*	*20*	5×10^{-4}
33	*62 (DBP)*	*5*	*-*	*22*	1×10^{-5}
33	*61 (DBP)*	*6*	*-*	*21*	6×10^{-4}
33	*60 (DBP)*	*7*	*-*	*21*	5×10^{-4}
33	*62(2-NPOE)*	*5*	*-*	*19*	1×10^{-4}
33	*61 (DBP)*	*5*	*1*	*25*	1×10^{-5}
33	*60 (DBP)*	*5*	*2*	*26.2*	5×10^{-5}
33	*59 (DBP)*	*5*	*3*	*21*	1×10^{-4}

EMF measurements

All the EMF measurements were carried out using the following cell assembly:

Ag/AgCl, KCl (sat.) ‖0.1 M $Zn^{+2}/Cd^{+2}/Cu^{+2}$ |membrane| $Zn^{+2}/Cd^{+2}/Cu^{+2}$ test solu‖ KCl (sat.), AgCl/Ag

Salt bridges containing KCl were used to provide electricity links between KCl and metal solutions on both sides of the membrane. A digital potentiometer having sensitivity of 0.1 mV (Equiptronics EQ602, India) was used for the potential measurements at 25±0.1ºC. Activities were calculated according to the Debye-Huckle equation (Kielland, 1937). Standard metal solutions were obtained by gradual dilution of 0.1 M metal stock solution and their potential measurements were performed.

The membranes were calibrated for the three metal ions viz, Zn Cd and Cu at a concentration range varying from 10^{-7} to 10^{-1} M. Percentage weight of ionophore was also optimized and effect of pH on the EMF response was studied.

Potentiometric selectivity coeffeicients

Selectivity coefficients were evaluated by the fixed interference method (FIM) (interfering ion concentration fixed at 1×10^{-3}M) and matched potential method (MPM), a specified amount of primary ions is added to a reference solution and the membrane potential is measured. In a separate experiment, interfering ions are successively added to an identical reference solution until the membrane potential matches with that one obtained before with the primary ions (Umezawa *et al.,* 1995).

Results and discussion

The membrane material is a plastic, polyvinyl chloride (PVC) that is highly hydrophobic and impermeable to any ions. It is plasticized (softened) by addition of a similarly hydrophobic solvent, e.g., DBP (Dibutyl pthalate), o-NPOE (ortho nitrophenyl octyl ether). The membrane is just a flexible piece of plastic, which acts as a near perfect barrier to ions. To make it ion-selective, a neutral ligand which is selective for the analyte and lipophilic in nature is added.

Optimization of the membrane composition

The sensitivity and selectivity of an electrode are significantly affected by the nature of the plasticizer, the composition of ionophore and internal solution (Mi *et al*., 1999; Sokalaski *et al*., 1997; Sokalaski *et al*., 1999). Hence, for optimization of the membrane, effect of the composition on the response characteristics of the electrode like slope of the calibration curve, measurement range and detection limit were studied (Table 1). The electrode with the ratio PVC:DBP:bacterial cells:KTCIPB = 33%:60%:5%:2%, exhibits the best response with a slope of 26.2mV/decade. It was found that DBP is a more effective solvent medium than o-NPOE in the preparing the Zn^{+2} ion selective electrode. Amount of the ion carrier (Bacterial cells) affects the sensitivity. Sensitivity of the electrode increases with increasing ionophore content until a value of 5% (w/w) is reached. A further increase in the percentage of the ionophore results in decrease of the slope of the electrode. This may be due to the reason that equilibration of the ionophore with the metal ions is maximum at this concentration. Addition of potassium salt of tetrakis (4-chloro-phenyl) borate (KTCIPB) is known to increase the sensitivity of the membrane as it reduces the anionic interference. It is observed that the addition of this lipophilic cation improved the working electrode sensitivity (Linear range: 10^{-1} to 10^{-5} M, Slope: 26.2mV/decade) and detection limit 5×10^{-5}M.

Table 2. Potentiometric selectivity coefficients ($K^{Pot}_{Zn^{2+},B}$) for interfering ions

Interfering ions (B)	$-\log K^{pot}_{Ag+,B}$	
	FIM	*MPM*
Co^{2+}	*-2.2*	*-2.6*
Mn^{2+}	*-3.1*	*-2.8*
Cd^{2+}	*-3.2*	*-2.9*
Ni^{2+}	*-2.8*	*-2.1*
Cu^{2+}	*-3.2*	*-3.3*
K^{+}	*-3.5*	*-3.2*
Fe^{3+}	*-3.1*	*-2.9*
Mg^{2+}	*-3.5*	*-3.1*
Pb^{2+}	*+0.2*	*+0.5*
Hg^{+}	*+1.4*	*+0.9*
Ag^{+}	*+1.2*	*+1.0*

Zinc ligating sites

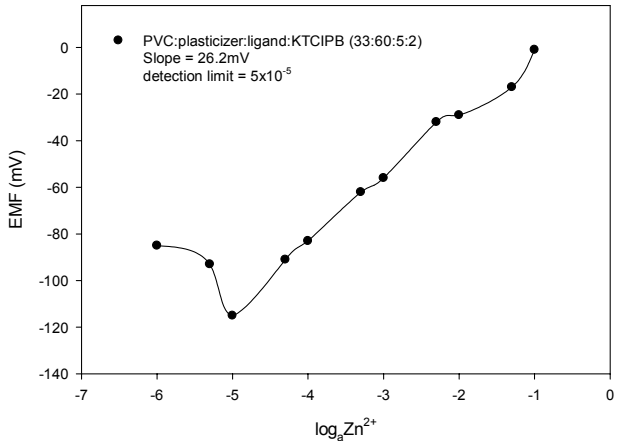
Tetrahydrofuran (THF) used as a solvent for the preparation of PVC

Indian Journal of Science and Technology Vol.2 No. 7 (July 2009) ISSN: 0974- 6846

membrane leads to the rupture of the bacterial cell walls as was confirmed by observing the THF cell suspension under the microscope, releasing the cell content and hence exposing the Zn-ligating sites of the enzyme. Also, it was observed that the activity of alkaline phosphatase released due to cell rupture is not lost as was confirmed spectrophotometrically by performing the enzyme assay using para- nitrophenylphosphate (p-NPP) as the substrate at pH-8.3 and 37ºC (Barnes & Morris, 1957).

The enzymatic reaction leads to the conversion of the substrate (p-NPP) to a yellow colored compound para-nitrophenol (p-NP) whose optical density was measured at 420 nm using a UV-Vis spectrophotometer (Hitachi).

Fig. 1. Response of Bio-ligand based ISE (Ion selective electrode) towards zinc ions



Calibration curve, response time shelf life and detection limit

The electrode shows a linear response towards Zn^{+2} over a wide concentration range of 10^{-5} to 10^{-1} M. The calibration curve has a near Nernstian slope of 26.2mV/decade with a detection limit of $5x10^{-5}$ M which was obtained from the intersection of two straight-line portions of the curve (Fig.1). The slow decrease in the emf beyond 10^{-5} M may be due to the release of Zn^{2+} ions from membrane in to the solution. No particular emf trend was observed for Cd^{2+} and Cu^{2+} ions (data not shown). The reason for the linearity observed in the case of Zn^{2+} can be attributed to the Zinc ligating sites present Zn-metalloenzymes. These Zn-ligating sites lying at the interface of the internal and test solution are exposed to the concentration gradient across the membrane which leads to the generation of potential difference measured in terms of electromotive force (emf). Many chemical ionophore based potentiometric biosensors have been reported for the detection of metal ions, but all these suffer from the drawback of low ion selectivity as the ionophores used are not ion specific. The use of bioligands as the ionophore for the construction of potentiometric biosensor is a novel concept. An Ag^{+}-ion selective electrode using polysulfone matrix embedding metallothioneins as ionophores with the detection limit of about 10^{-5} M was reported by Gonzalez-Bellavista *et al.*, 2009. Since construction of such biosensors required small amount of proteins they can be dry-stored and have long-lifetimes.

The response time is measured by recording emf of the electrode as a function of time, when it is immersed in the solution to be studied. The estimated time to get stable potential was 6 s. Although always kept at 4-5ºC, the response of the electrodes stored in dry is much better than that of the electrode stored in 0.1 M Zn^{2+} solution. The soaked electrodes showed a 20%, 70% and 100% sensitivity decrease after 2, 4 and 6 days of storage, respectively (data corresponding to 5 days of storage is shown in Fig. 2).

The longer life span of the electrodes which were stored under dry conditions could be attributed to the prevention of the oxidation of the cysteine residues present in the metalloproteins (Gonzalez-Bellavista *et al.*, 2009).

Effect of pH

pH was studied in the range of 2-12 using $1x10^{-2}$, $1x10^{-3}$ and $1x10^{-4}$ M Zn^{+2} concentration. pH studies were done on membranes with 3 % and 5 % ionophore concentration. pH was adjusted by the addition of 0.1 N NaOH or HNO_3 as required. It was found that the electrode response was optimum in a very narrow pH range of 3-4 (Fig.3). At pH above and below this range a sharp decrease in the emf value was observed

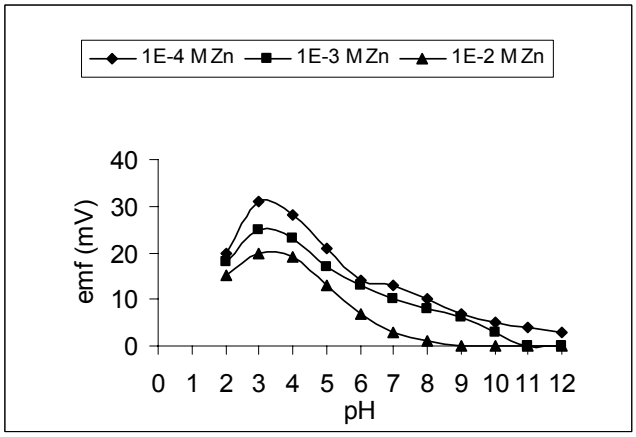
Potentiometric selectivity coefficient

One of the main features of any ion-selective electrode is its response to the primary ion in presence of other ions. Ion selective electrodes are rarely ion specific. The ability of an ion selective electrode to distinguish between different ions in the same solution is expressed as the selectivity coefficient $-\log K^{pot}_{Ag+,B}$. The selectivity coefficient is not always constant and depends on several factors including the concentrations of both ions, the total ionic strength of the solution and the temperature. All electrodes are sensitive to some or other ions to some extent. For many applications these interferences are insignificant and can often be ignored. In some extreme cases, however, the electrode is far more sensitive to the interfering ions than the primary ions and can be used if the interfering ions are present only in trace quantities or completely absent. In our case, some interferences between Zn^{2+} and other metal ions could also be envisaged due to the well-known order of affinity of heavy metal ions for thiolates (Hg(II) >>Cu(I) ≈ Ag(I)>>Cd(II) > Pb(II) > Zn(II)) which is very close to that of Metallothionein (Vasak, 1991). The observed values of the selectivity coefficients are presented in Table 2. For alkali and alkaline earth metal ions transition metal ions, the $-\log K^{pot}_{Ag+,B}$ values lie in the range of 3, 2 except for Pb^{2+} ions. Also the membrane electrode did not show any serious interference from Cu^{2+} ions. It is important to note that the selectivity coefficients for the Zn^{2+} electrode, with reference to most of the alkali and alkaline-earth metal ions, are quite small. This means that this membrane

Indian Journal of Science and Technology Vol.2 No. 7 (July 2009) ISSN: 0974- 6846

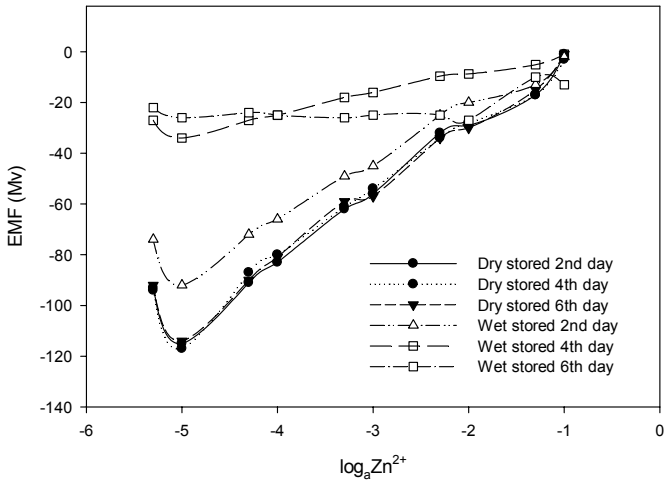
electrode will be highly efficient for determination of trace

Fig. 2. Potentiometric response of the electrode stored in different conditions over the period of 6 days



amounts of zinc in the presence of a large excess of alkali and alkaline-earth metal ions. The zinc response is seriously interfered with by small amounts of Hg^{2+} and Ag^{+} ions; so these two ions must be removed before the analysis of zinc from the samples. This is in good correspondence with literature data regarding electrodes of different nature (Wroblewski & Brzozka, 1995; Siswanta *et al*., 1996; Chen *et al*., 2000) and with the higher affinity of this metal ion for the Cys residues if compared with that of the other metal ions studied (Pb^{2+}, Zn^{2+}, Cd^{2+} and Cu^{2+}).

Fig. 3. Effect of pH on potentiometric response of membrane at three different zinc concentrations



Conclusions

The present study reports the use of *in vivo* Zn-metalloenzymes as a zinc ligand to fabricate a selective biosensor. Potentiometeric response of Zn-metalloenzymes present in the bacterial cell mass was quite specific for Zn^{2+} ions with a near Nernstian slope of 26.2mV/decade and a sharp response time of 6-10 sec. The optimum pH for the detection of zinc was 3. The electrode did not show any response towards Cd^{2+} and Cu^{2+}, which indicates towards the zinc selective nature of the electrode. The electrode was found to be quite selective for zinc ions except for the interference shown by Hg^{+2}, Ag^{+} and Pb^{+2}.

Acknowledgement

The first author is highly thankful to the Director, Thapar University for providing necessary infrastructure and facilities. This work was supported by a Senior Research fellowship (Ref. 9/677/(8)/2005-EMR-I) from CSIR, New Delhi.

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Potentiometric Zn^{2+} Biosensor Based on Bacterial Cells

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Abstract: *Pseudomonas striata* cell mass was immobilized in polyvinylchloride (PVC), a neutral carrier to prepare zinc selective membranes using Dibutyl-phthalate as the plasticizer. Membranes were prepared using 1, 2, 3, 5 and 7% of bacterial biomass, of which the highest response was obtained for 5%. Potentiometric response of the electrode was studied for Zn, Cd and Cu in the concentration range of 10^{-7} to 10^{-1} M. A linear trend between the electrode response and the varying metal concentrations was seen only for Zn^{2+} ions in the range of 10^{-4} to 10^{-1} M. Calibration slope of 22 mV/decade and detection limit of 5×10^{-4} M was obtained for zinc. Electrode showed a sharp response time of 6 sec and pH optima of 3. Thus, the PVC membrane containing the bacterial biomass was found to selectively bind the Zn^{2+} ions and generate the corresponding potential response at different zinc concentrations.

Key words: Zn-metalloenzymes, heavy metal determination, potentiometric biosensor, ionophore

INTRODUCTION

The determination of traces of toxic heavy metals in biological materials, natural waters, soil and air has become very important because the environment is highly vulnerable to this class of pollutants. Heavy metals are accumulated and stored in the living organisms; especially in the marine organisms a very high bioaccumulation of heavy metals can take place (Krawczyk *et al*., 2000). Currently, a huge array of analytical methods for the toxic agents' detection is used. These methods based on spectrophotometry, chromatography, mass spectrometry and various hyphenated techniques; require sophisticated and expensive equipments, highly trained staff and is usually time-consuming (Dzyadevych *et al*., 2005; Sherma and Zweig, 1983). Moreover, these methods can only detect the total amount of heavy metals and not the bioavailable concentrations accessible to the living organisms. Therefore, development of new and inexpensive methods for the detection of bioavailable heavy metals concentrations is highly desirable. Biosensors are useful analytical devices in this respect and several configurations have been described in the past for heavy metal detection. Wide spectrum of biological recognition elements and transducer systems has been used for the fabrication of biosensors (Castillo *et al*., 2004; Amine *et al*., 2006; Bentley *et al*., 2001). The use of metalloenzymes/ metalloproteins as the biological sensing element for heavy metals has several advantages over the other bioreceptors as these are more specific in regard of metal binding (McCall *et al*., 2000). There are certain reports on the use of metal binding proteins/peptides for the fabricaton of heavy metal biosensor (Cherian *et al*., 2003; Chow *et al*., 2005). These biomolecules besides detecting the bioavailable content also allow high selectivity in the recognition of analytes, such as metal ions, in complex natural solutions, e.g., seawater or blood. Combination of this property of biomolecules with

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a suitable transducer system has a great promise as an indicator system that may in the future replace the current techniques of measuring very low concentrations of metal ions (Thompson *et al*., 1996; Kielland, 1937). In the present study, plasticized PVC membranes were used as a support matrix for the entrapment of lyophilized bacterial cells to fabricate a Zn^{2+} selective potentiometric electrode. Al-Hitti *et al*. (1984) demonstrated the immobilization of GOD (Glucose oxidase) within plasticized polyvinylchloride membrane, which was then used for glucose determination. The methodology followed for the preparation of electrode is same as described by Mittal *et al*. (2007). Ligand is that component of the potentiometric sensor which makes the inert support matrix (which is polyvinylchloride in this study) ion-selective in nature. Lyophilized cell mass of *Pseudomonas striata* was chosen as a ligand in the membrane electrode. *Pseudomonas striata* was selected because this strain produces good amount of alkaline phosphatase which is Zn-metalloenzyme and has zinc ligating sites. In addition to this there are many other Zn-metalloenzymes which are present in prokaryotic systems that might be responsible for the zinc selective nature of the electrode. Literature searches have ascertained sequences, zinc content and functional characteristics of the catalytic, cocatalytic and structural zinc sites for families of zinc enzymes. The X-ray structure analyses of 11 enzymes containing a single catalytic zinc atom identify their ligands. This metal forms complexes with any of the nitrogen and oxygen ligands of histidine and glutamate residues with a binding frequency of His>>Glu (Vallee and Auld, 1993). In the present study, the zinc ligating property of *in vivo* alkaline phosphatase and other Zn-metalloenzymes has been explored for the purpose of making a biosensor for Zn^{2+} ions. Lot of ionophore-based chemical sensors have been reported till date which make use of a large number of chemical metal ligands as the ionophore but lack selectivity. This study is a novel attempt to explore the potential of the Zn-metalloenzyme as Zn-ionophore.

MATERIALS AND METHODS

The microbiological study was carried out in Department of Biotechnology and Environmental Studies and the potentiometric studies were done in School of Chemistry and Biochemistry, Thapar University, Patiala (Punjab) from February to April 2008.

Reagents

Reagents like dibutyl phthalate (DBP), o-nitrophenyloctyl ether (o-NPOE) were procured from Sigma-Aldrich. All other chemicals were of analytical reagent grade. Double distilled deionized water was used throughout the experiments.

Ligand Preparation

Pseudomonas striata was cultured on nutrient agar plates for 17 h. The cells were harvested using Tris-HCl buffer pH 8.3. The cell suspension was centrifuged at 8000 rpm for 10 min to obtain a cell pellet which was lyophilized at -50°C under vaccuum using a freeze dryer (Modulyod, ThermoElectron Corporation) to obtain dry cell mass.

Electrode Preparation

Methodology followed for the preparation of PVC membranes and potential measurements were same as described by Mittal *et al*. (2007). Membranes of ~ 0.2 mm thickness were obtained by pouring a solution of the membrane components of PVC 33%, bio-ligand (lyophilized bacterial cells) 1-7% and dibutyl phthalate/o-nitrophenyloctyl ether 63% dissolved in 2-3 mL of tetrahydrofuran (THF). The viscous solution of the polymer thus obtained was poured in a glass ring of 30 mm diameter placed on a dust free pyrex glass plate. The solvent was allowed to evaporate slowly for about 24 h at room

temperature. To obtain the membrane with similar characteristics, viscosity of the casting solution and rate of solvent evaporation were controlled so that the thickness and morphology of the membranes remained unchanged and the appearance of the film looked pale yellow in colour. The membranes were then removed from glass ring and circular pieces of 1.25 cm diameter were cut and mounted on the ground end of a pyrex glass tube with an adhesive and conditioned with a metal solution ($ZnSO_4$/$CuSO_4$/$CdSO_4$) (0.1 M) for 2 h.

EMF Measurements

All the EMF measurements were carried out using the following cell assembly:

Ag/AgCl, KCl (sat.) ‖0.1 M Zn^{2+}/Cd^{2+}/Cu^{2+} |membranel Zn^{2+}/Cd^{2+}/Cu^{2+} test solul| KCl (sat.), AgCl/Ag

Salt bridges containing KCl were used to provide electricity links between KCl and metal solutions on both sides of the membrane. A digital potentiometer having sensitivity of 0.1 mV (Equiptronics EQ602, India) was used for the potential measurements at 25±0.1°C. Activities were calculated according to the Debye-Huckle equation (Meier *et al*., 1980). Standard metal solutions were obtained by gradual dilution of 0.1 M metal stock solution and their potential measurements were performed.

The membranes were calibrated for the three metal ions viz., Zn Cd and Cu at a concentration range varying from 10^{-7} to 10^{-1} M. Percentage weight of ionophore was also optimized and effect of pH on the EMF response was studied.

RESULTS

Optimization of the Membrane Composition

For optimization of the membrane, effect of the composition on the response characteristics of the electrode like slope of the calibration curve, measurement range and detection limit were studied. The electrode with the ratio PVC:DBP:bacterial cells = 33:62:5%, exhibits the best response with a slope of 22 mV/decade. It was found that DBP is a more effective solvent medium than o-NPOE in preparing the Zn^{2+} ion selective electrode. Amount of the ion carrier/ligand (bacterial cells) affects the sensitivity of the electrode. Sensitivity of the electrode was found to increase with increasing ionophore content until a value of 5% (w/w) was reached. A further increase in the percentage of the ionophore results in decrease of the slope of the electrode.

Calibration Curve, Response Time and Detection Limit

The response time is measured by recording emf of the electrode as a function of time, when it is immersed in the solution to be studied. The estimated time to get stable potential was 6 sec. The electrode shows a linear response towards Zn^{2+} over a wide concentration range of 10^{-4} to 10^{-1} M. The calibration curve has a slope of 22 mV/decade with a detection limit of 5×10^{-4} M, which was obtained from the intersection of two straight-line portions of the curve (Fig. 1). A slow decrease in the EMF is observed beyond 5×10^{-5} M. No particular emf trend was observed for Cd^{2+} and Cu^{2+} ions.

Effect of pH

pH was studied in the range of 2-12 using 2×10^{-2}, 2×10^{-3} and 2×10^{-4} M Zn^{2+} concentration. pH studies were done on membranes with 3 and 5% ionophore concentration. pH was adjusted by the addition of 0.1 N NaOH or HCl as required. It was found that the electrode response was optimum in a very narrow pH range of 3-4 (Fig. 2).

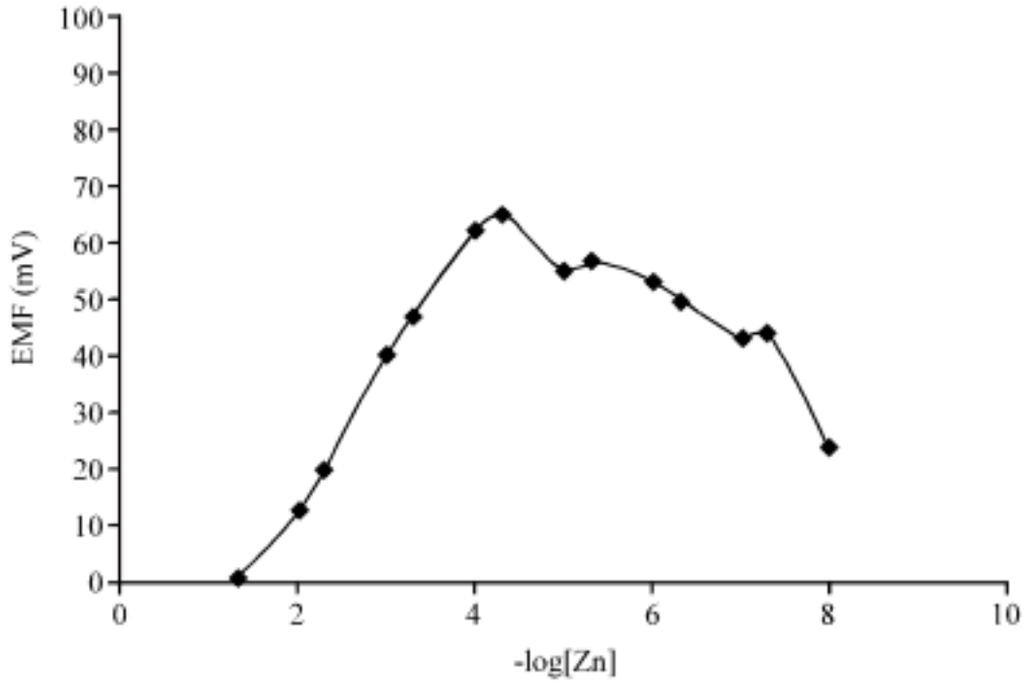

Fig. 1: Response of bioligand based ISE (Ion selective electrode) towards zinc ions

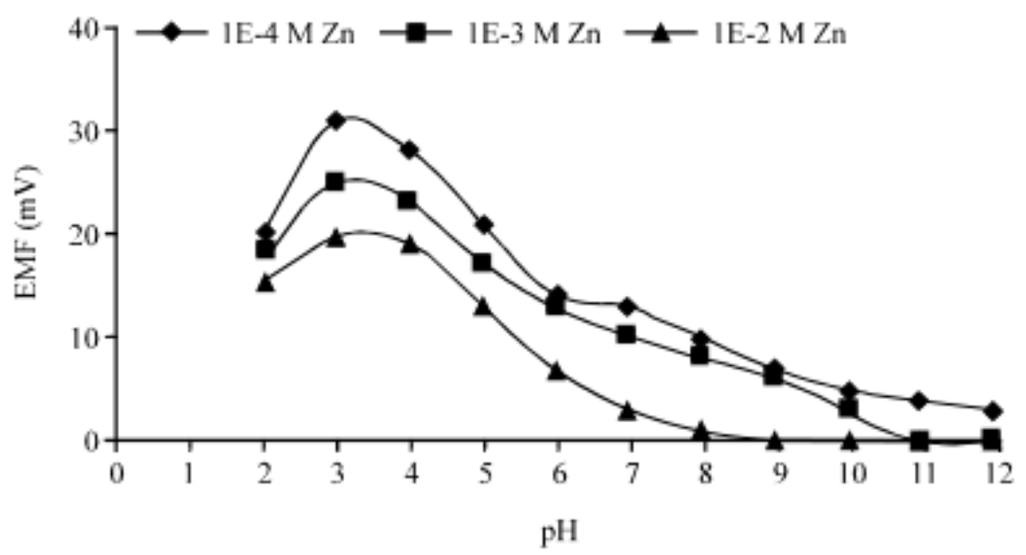

Fig. 2: Effect of pH on potentiometric response of membrane at three different zinc concentrations

DISCUSSION

The membrane material is a plastic, polyvinylchloride (PVC) that is highly hydrophobic and impermeable to any ions. It is plasticized (softened) by addition of a similarly hydrophobic solvent, e.g., DBP (Dibutyl pthalate), o-NPOE (ortho nitrophenyl octyl ether). So far, the membrane is just a flexible piece of plastic, which acts as a near perfect barrier to ions. To make it ion-selective, a neutral ligand which is selective for the analyte and lipophilic in nature is added. Many chemical ionophore based potentiometric biosensors have been reported for the detection of metal ions, but all these suffer from the drawback of low ion selectivity as the ionophores used are not ion specific. The use of bioligands as the ionophore for the construction of potentiometric biosensor is a novel concept. An Ag^{+} ion selective electrode using polysulfone matrix embedding metallothioneins as ionophores with the detection limit of about 10^{-5} M was reported by González-Bellavista *et al*. (2009). Since, construction of such biosensors required small amount of proteins they can be dry-stored and have long-lifetimes. In this study, lyophilized cells of *Pseudomonas striata* were used as the source of alkaline phosphatase (Ligand) containing zinc ligating sites. These zinc ligating sites of the enzymes are exposed to the external solution and made available for binding to the metal ions due to the rupturing of the bacterial cell walls by tetrahydrofuran (THF) (solvent used for the preparation of PVC membrane). This was confirmed by observing the THF cell suspension under the microscope.

Zinc binding sites in proteins are often distorted tetrahedral or trigonal bipyramidal geometry, made up of the sulfur of cysteine, the nitrogen of histidine or the oxygen of aspartate and glutamate, or a combination. Zinc in proteins can either participate directly in chemical catalysis or be important for maintaining protein structure and stability. In all catalytic sites, the zinc ion functions as a Lewis acid. An understanding of naturally occurring zinc-binding sites will aid in creating de novo zinc-binding proteins and in designing new metal sites in existing proteins for novel purposes such as to serve as metal ion biosensors (McCall *et al*., 2000). Zinc, always occurring as a divalent cation [zinc(II)] in biological systems is the second most abundant (common) transition metal following iron. Today more than 300 different zinc proteins are known. These include numerous essential enzymes which catalyze the metabolic conversions (synthasis, polymerasis, ligasis, transferasis) or degradation (hydrolasis) of proteins, nucleic acids, lipids, porphyrin precursors and other important bioorganic compounds (Urbanová *et al*., 2008).

Also, it was comfirmed that the activity of alkaline phosphatase which contains the zinc binding sites released due to cell rupture is not lost as was confirmed spectrophotometrically by performing the enzyme assay using para-nitrophenylphosphate (p-NPP) as the substrate at pH 8.3 and 37°C (Barnes and Morris, 1957). The enzymatic reaction leads to the conversion of the substrate (p-NPP) to a yellow colored compound para-nitrophenol (p-NP), whose optical density was measured at 420 nm using a UV-Vis spectrophotometer (Hitachi).

The sensitivity and selectivity of an electrode are significantly affected by the nature of the plasticizer, the composition of ionophore, internal solution (Mi *et al*., 1999; Sokalaski *et al*., 1997; Sokalaski *et al*., 1999), etc. In neutral carrier membranes, plasticizers that are compatible with the ionophore provide a smooth surface to the membrane and hence enhance the response characteristics (Cammann, 1979). The nature of the plasticizer influences the dielectric constant and the mobility of the ions in the membrane. These membrane solvents are seen to strongly influence the working concentration range and the slope of the sensor. It was observed that the electrode with DBP as plasticizer was found to give the best response in terms of the slope and the concentration range. The slopes in the case of the o-NPOE are sub-Nernstian. The potentiometric response of the sensor towards Zn (II) ions is found to be dependent on the concentration of the ionophore used. Different compositions (w/w%) of the ionophore were also tried to obtain the right composition of ionophore that gives the best response characteristics. The maximum sensitivity was observed for 5% (w/w) of the ionophore. On increasing the ionophore content, the slopes are affected; this may be related to the change in the water uptake capacity of the membrane (Kumar *et al*., 2006) and also may be due to the reason that equilibration of the ionophore with the metal ions is maximum at this concentration (Mittal *et al*., 2007).

The linearity observed in the case of Zn^{2+} (Fig. 1) can be attributed to the zinc ligating sites present on Zn-metalloenzymes. These Zn-ligating sites lying at the interface of the internal and test solution are exposed to the concentration gradient across the membrane which leads to the generation of potential difference measured in terms of electromotive force (EMF). This further supports the non-linear trends observed for Cd and Cu, confirming the selective binding of Zinc by the Zinc metalloenzymes.

The results presented as Fig. 2 reveal that the potentials are independent of pH in a very narrow range of 3-4 and this range is taken as the working pH range of the Zn(II) sensor. Variation of potentials above and below these pH values can be related to hydrolysis of Zn(II) (at higher pH) and the competition of H^+ with Zn(II) (at lower pH values).

CONCLUSION

The present study is a novel report on the use of *in vivo* Zn-metalloenzymes as a zinc ligand to fabricate a selective biosensor. Potentiometeric response of Zn-metalloenzymes present in the bacterial

cell mass was quite specific for Zn^{2+} ions with a slope of 22 mV/decade and a sharp response time of 6 sec. The optimum pH for the detection of zinc was 3. The electrode did not show any response towards Cd^{2+} and Cu^{2+}, which indicates towards the zinc selective nature of the electrode. Further work would include testing the selectivity of the electrode which is the ability of an ion electrode to distinguish between different ions in the same solution, to study the effect of different solvent media on the electrode response and to determine the target ion (Zn^{2+}) with probable interfering ions.

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