To Study the Effect of Heavy Metal Ions on the Enzyme Activity of Free and Immobilized Biomaterial by Spectrophotometry

A

Thesis submitted

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in

Chemistry

Under the Supervision of

Dr. Susheel Mittal

Senior Professor

Submitted By

Manisha Pabbi

301002005

School Of Chemistry and Biochemistry

Thapar University, Patiala

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Manisha

Date: 13, July, 2012 **Manisha Pabbi**

Place: Patiala

Candidate's Declaration

I, hereby declare that the work being presented in the thesis entitled **"To study the effect of heavy metal on the enzymatic activity of free and immobilized biomaterial by spectrophotometry",** in partial fulfillment of the requirements for the award of the degree of Masters in Chemistry, School of Chemistry and Biochemistry, Thapar University, Patiala, is my own work during the period of Jan 2012 to July 2012, under the supervision of Dr. Susheel Mittal, Senior Professor, School of Chemistry and Biochemistry, Thapar University, Patiala. I have not submitted the matter embodied in this thesis for the award of any other degree.

Patiala

Date: 13, July, 2012

Manisha

Manisha Pabbi

301002005

This is to certify that the above statement made by the candidate is correct and true to the best of our knowledge.

Susheel Mittal 13/7/12

Dr. Susheel Mittal

Senior Professor

School of Chemistry & Biochemistry

Satnam Singh 13/7/12

Dr. Satnam Singh

Associate Professor and Head

School of Chemistry & Biochemistry

Certificate

This is to certify that the thesis entitled **"To study the effect of heavy metal on the enzymatic activity of free and immobilized biomaterial by spectrophotometry"**, being submitted by Ms. Manisha Pabbi in partial fulfillment of the requirements for the award of degree of Master of Science in the School of Chemistry and Biochemistry, Thapar University, Patiala, is a bonafide work carried out under the supervision of Dr. Susheel Mittal and that no part of this thesis has been submitted for the award of any other degree.

Susheel Mittal 13/7/12

Dr. Susheel Mittal

Senior Professor

School of Chemistry & Biochemistry

Satnam Singh 13/7/12

Dr. Satnam Singh

Associate Professor and Head

School of Chemistry & Biochemistry

Dr. S.K Mohapatra

Dean, Academic Affairs

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1 INTRODUCTION

Biosensors are the analytical devices which incorporate a biomolecule to provide recognition specificity for a particular analyte and transduction technology to deduce and quantitate the binding taking place between analyte and detector molecule. Many biosensors have been developed for a wide range of analytes in various formats, from dipsticks to large laboratory flow- injection analysis systems, and for wide range of application, from single chemical quantifications to biological effect measurements. Biosensors are playing vital role in areas such as the medical field, food analysis, defence and environmental applications. Detection of heavy metals is gaining interest now a day. Heavy metal ions are ubiquitous in nature. Living organisms require small doses of some essential heavy metals, including cobalt, copper, iron, manganese, molybdenum, vanadium, strontium and zinc.

Importance of heavy metals in biological system- Iron is present in hemoglobin, manganese act as cofactor in some enzymes and also acts as oxygen evolving complex in photosynthetic plants, molybdenum plays a vital role in nitrogen fixation, zinc also acts as cofactor in some enzyme such as alkaline phosphatase etc. Other heavy metals such as mercury, plutonium, and lead are toxic metals and have no known vital or beneficial effect on organisms. Their accumulation in environment offers severe heath problems and potential risk to ecology, so it becomes obvious need to determine these ions very rapidly and at trace level.

Heavy metals are typically analyzed by traditional methods, including inductively coupled plasma atomic electron spectrometry (ICP/AES), inductively coupled plasma mass spectrometry (ICP/MS), atomic absorption spectroscopy (AAS) or wet chemical methods. Traditional methods have detection limits ranging from parts per trillion to parts per billion, and relatively accurate analysis but consume significant time and money, particularly when large numbers of samples require testing. And also these methods require very sophisticated equipment and cannot be used for field monitoring. Therefore, there is a need for simple and potable detection method. Biosensors provide several advantages over traditional methods such as real time analysis, low cost, and low detection limit.

Two fundamental operating principle of a biosensor are “biological recognition” and “sensing.” Therefore, a biosensor can be generally defined as a device that consists of two basic components connected in series:

1. A biological recognition system, also known as bioreceptor
2. A transducer

Biological recognition includes cofactors, enzymes, nucleic acid (DNA), antibodies, organelles, tissues, microorganisms, cells from higher organisms, which have been used in the fabrication of biosensors. Bioreceptors are the key to specificity for biosensor technologies. Specificity and sensitivity are the unique abilities of bioreceptor. A transducer converts the recognition event into measurable signal. The transducer can take many forms depending upon the parameters being measured- electrochemical, optical, mass and thermal changes.

The main purpose of the recognition system is to provide the sensor with a high degree of selectivity for the analyte to be measured. The interaction of the analyte with the bioreceptor is designed to produce an effect measured by transducer which converts the information into measureable effect such as an electrical signal.

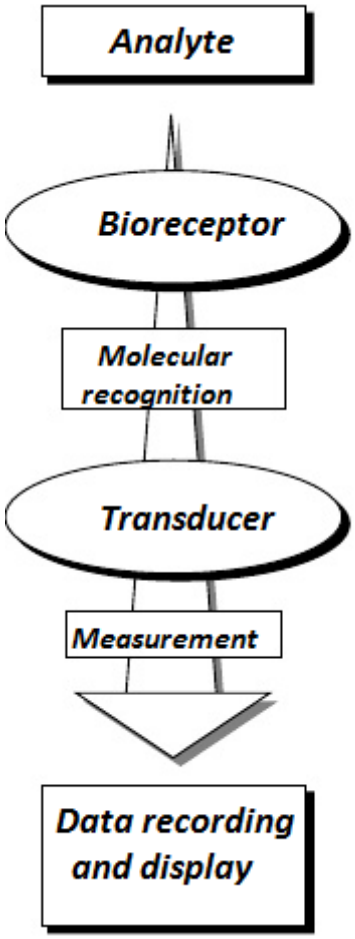

Fig: Systematic diagram of biosensing principle

In order to construct a successful biosensor, a number of conditions must be fulfilled:

1. The biocatalyst must be highly specific for the purpose of the analysis, be stable under normal storage conditions and show a low variation between assays.
2. Selection and design of a transducer that translates binding reaction into measurable signal.
3. The response should be accurate, precise, reproducible and linear over the concentration range of interest, without dilution or concentration.
4. For rapid measurements of analytes it is desirable that the biosensor can provide real-time analysis.
5. The complete biosensor should be cheap, small, and portable.

Biosensors are characterized by eight parameters. These are:

1. *Sensitivity* is the response of the sensor to per unit change in analyte concentration.
2. *Selectivity* is the ability of the sensor to respond only to the target analyte. Lack of response to other interfering chemicals is the desired feature.
3. *Range* is the concentration range over which the sensitivity of the sensor is desirable.
4. *Response time* is the time required for the sensor to respond.
5. *Reproducibility* is the accuracy with which the sensor output can be obtained.
6. *Detection limit* is the lowest concentration of the analyte upto which there is a measurable response.
7. *Life time* is the time period over which the sensor can be used without significant deterioration in performance characteristics.
8. *Stability* characterizes the change in its baseline or sensitivity over a fixed period of time.

1.1 Bioreceptors

1.1.1 Antibody receptors

Biorecognition elements based on antibody-antigen reaction are included in this group. Antibody based biosensors are also known as immunosensors. Antibodies are complex molecules having recognition site which are specific molecular structure. This structure will allow binding of a particular antigen. The binding of antigen- antibody is similar to lock and key mechanism; which means an antigen-specific antibody fits its unique antigen in a specific manner. Due to this property it is possible to detect an antibody that can recognize and bind to only one of a huge variety of molecular shapes. This unique property is key to synthesize immunosensors. Antibodies are usually immobilized on the surface of transducer by covalent attachment. The surface of the transducer must be functionalized with a amino, carbonyl or any other group so that the antibody can form a covalent attachment by conjugation of groups. The specific binding property of antibody to specific antigen allows detecting an analyte of interest that is present in small quantity in the pool of chemical substances. The property of antigen-antibody interaction is the strength or affinity; means the complex formed by

antigen-antibody interaction should have a reasonable life span which can be detected by a suitable transducer.

Immunosensors provide faster and in-field measurements using optical or acoustic transducer. Many immunosensors have been developed for determination of medical diagnostic molecules such as hormones (e.g. Steroids), clinical disease markers, drugs (therapeutic and abused), and food matrices and in the detection of pesticides and heavy metals in environmental samples.

1.1.2 Enzyme receptors

Biorecognition element is enzyme and analyte is detected by measuring the formation of product of a reaction with enzyme. Enzymes are the proteins which accelerate the rate of reaction for a particular product without being consumed in the reaction. Enzymes are suitable for use as a bioreceptor in the formation of biosensors it functions as continuous monitors of analyte concentration. The majority of enzyme based biosensors reported detect a particular analyte by converting it into detectable product by an immobilized enzyme. The product thus formed should have either electroactivity (e.g. redox active) or optical property (e.g. fluorescence, absorbance) which can be detected by a suitable transducer. Enzyme sensors can be enzyme electrodes (enzyme is immobilized on the surface of transducer), enzyme thermistors (where the heat evolved by enzyme catalyzed reaction of the analyte is measured), inhibited enzyme sensors (where a specific enzyme is inhibited thus altering the electrochemical or optical signal), enzyme optrodes (in which the optical signal originates from the enzyme itself rather than from a product of enzymatic reaction). The main principle behind the enzyme based biosensor is the high specificity and high sensitivity of enzyme-substrate interaction as the basis for a detection and measurement system. Amperometric based enzyme biosensors are widely used for various application. There are three generation related to the synthesis of amperometric enzyme based biosensors. The first generation of enzyme sensors were mediator free enzyme electrodes, which consist of immobilized enzyme close to the surface of ion-selective electrode such as Clark- type oxygen electrode. It involves either consumption or production of specific ion which could be monitored. Drawback of this technique was its limitation in the analysis of real samples.

In second generation, mediators were used to transfer electrons from enzyme after it reduces or oxidises the substrate to electrode. The first mediator based biosensor was glucose biosensor in which enzyme glucose oxidase was used as bioreceptor and ferrocenes as mediator. In third generation, modified electrode biosensors were developed. In this the surface of electrode is modified by the addition of molecules which allow the direct oxidation or reduction of enzyme at electrode (no mediators). Amperometric enzyme electrode involves oxidoreductase; Potentiometric and ion selective electrode with an immobilized enzyme are also used for preparation of enzyme electrode.

1.1.3 Whole cell receptors

The simple unicellular living systems like algae or bacteria which include enzymes, nucleic acid, ions etc can act as bioreceptor and biosensor which have these bioreceptors are called whole cell biosensors. The stability of biological cell is an important factor in the biosensor. The cells of organisms like algae and bacteria can be easily stabilized than complex cells of higher organisms. The advantage of using whole cell biosensors over enzymes based biosensors is that whole cells have versatility (cells respond to wide range of substrate), low cost of preparation, enzymes present in vivo have optimum activity as enzymes lose their activity when isolated in vitro. However, the presence of several enzymes usually lowers the substrate specificity.

Whole cell biosensors have been synthesized to determine target analyte in the field of environment, medicine, defence and food processing. Depending upon the type of transducer various types of whole cell biosensors have been developed such as (1) electrochemical whole cell biosensors (2) optical whole cell biosensors, (3) others based on baroxymeter, infrared analyser. Electrochemical biosensors includes amperometric, potentiometric, conductometric, voltammetric based biosensors.

Amperometric biosensors involve the measurement of current by oxidation or reduction of species at a fixed potential. It is a widely used technique and has been exploited for environmental application like determination of pesticides, heavy metals, organic pollutants etc. Other amperometric biosensors based on (1) monitoring of cell respiration include biosensors for surfactants using surfactant degrading bacteria, (2) in

the field of health and fermentation application- the detection of analytes such as glucose in the diagnosis of diabetes and quality control of fermentation and food, (3) determination of some compounds which are costly and time consuming by traditional methods. A whole cell amperometric herbicide biosensor based on magnetic retention of *Chlorella pyrenoidsa* cells functionalised with magnetic nanoparticles on the surface of screen printed electrode have been fabricated [1]. Another amperometric biosensor based on Saccharomyces cerevisiae immobilized in the gelation was developed for selective determination of thiamine in vitamin tablets [2].

Cyclic voltammetry (CV) is another common technique used. It offers a rapid location of redox potential of electroactive species. Biosensors for the detection of Cu^{2+} ion based on *Circinella sp.* as a bioreceptor on modified carbon paste electrode was developed [3]. Whole cell of recombinant *E.coli* was immobilized on the screen printed carbon electrode for detection of methyl parathion [4].

Potentiometric whole cell biosensor detects the amount of analyte by measuring the potential difference between the working electrode and reference electrode. It consists of an ion- selective electrode, gas sensing probe coated with immobilized microbe layer. Conductometric biosensors are attractive, fast and sensitive response to analytes. Microbe catalysed reaction involves a change in the conductivity of reaction solution due to change in ionic species which can be determined by conductometric biosensors.

Optical biosensor involves measurement of change of any optical characteristics for example change in optical absorption or emission. It include different types of spectroscopies such as IR and UV visible (optical absorption) and X-ray fluorescence (emission) to measure different spectrochemical properties of target species and fibre optics (optodes) which transmit light on the basis of the principle of total internal reflection. Fibre optics is very popular recently and can be synthesized: (1) immobilising sensing dye or fluorophore on the tip of fibre in membrane, (2) cladding can be stripped from the fibre and fluorophore can be immobilised on the surface of core. The basis of detection of optical biosensors can be fluorophore and chromophore based reaction or bioluminescence/ chemiluminescence based reaction.

1.1.4 Nucleic acid Bioreceptors

Nucleic acid biosensors involve DNA or RNA as biorecognition element which are based on highly specific hybridization of complementary strands of DNA or RNA molecules. DNA molecule is double stranded helix having four repeating nucleotides- adenine (A), guanine (G), cytosine (C) and thymine (T) which are held together by hydrogen bonds. Hydrogen bonding occurs between specific complimentary bases: A-T and C-G. In RNA nucleotide base thymine is replaced by uracil. The hydrogen bonds can be broken either by heat or high pH, which results in the formation of single stranded DNA (ssDNA). Reannealing between the ssDNAs is called hybridization. The stability of hybridization depends on the nucleotide sequences of both strands. A perfect match of sequence of nucleotides produces very stable dsDNA where as one or more bases mismatching imparts instability. The same principle applies to RNA. DNA biosensors are usually in the form of electrodes, chips and crystals. Both solution and solid support hybridization can occur. Solid phase hybridization is the longest established and most often used. DNA biosensors are used in the analysis of analyte present in environment, food, and for detection of bacteria and other pathogenic microorganisms.

1.1.5 Other bioreceptors

Many tissues of higher organisms, plants and cell organelles are also used as bioreceptorss; as they are closed system and can be used for long period of time. Rizzuto et al developed a biosensor based on mitochondria for signalling Ca^{+2} ions [5]. A rat basophilic leukemia mast cell based biosensor was also developed for numerous application including pharmaceutical screening, environmental monitoring, clinical diagnosis [6]. Plant tissue based chemiluminescence flow biosensor for glycolic acid was developed; spinach tissue acts as molecular recognition element [7].

1.2. TRANSDUCERS

1.2.1 Electrochemical transducers

Electrochemical transducers can be of various types for sensor applications such as potentiometry, voltammetry, amperometry and conductometry.

i. **Potentiometric transducers:** Potentiometric devices measure the accumulation of charge potential at the working electrode compared to the reference electrode in an electrochemical cell when zero or no significant current flows between them. Potentiometry provides information about the ion activity in an electrochemical reaction. The basic principle behind potentiometric measurement is the development of charge related to the analyte activity 'a' in the sample through Nernst equation:

$$\mathbf{E = E^0 \pm (RT/\ nF)\ ln\ a}$$

E^0 is standard electrode potential of a (mol/L), R is the gas constant, F is the faraday constant, T is the temperature (K), n is the number of charge on the ion, the signal + and – are for cation and anion respectively. The direct determination of the analyte concentration with the Nernst equation is referred to as direct potentiometry. The lowest detection limit for potentiometric devices are achieved with ion selective membrane (ISE) and FET based transducers. With potentiometric sensors, the recognition process is a selective binding event which transduces analyte ionic activity into a potential output.

In ion selective electrodes, the phenomenon of generation of response is the potential drop across the membrane. An ion is exchanged between two phases: membrane/ solution, which depends on the activity of target ion in these phases. Ion selective membranes are composed of plasticisers (influences extraction properties of membrane), polymeric matrix- PVC, polyurethane, cellulose (maintaining the liquid membrane), lipophilic salt and ionophore (determine selectivity of membrane). The bioreceptor can be either immobilised on the outer membrane or entrapped in the membrane.

The FET is a type of transistor that uses electric field to control the conductivity of a channel between two electrodes i.e. source and drain. FETs are adapted to be miniature transducer for detection and measurement of potentiometric signals produced by a potentiometric sensor process on the gate of FET. Control of conductivity is achieved by varying electric field potential, relative to source and drain electrode, at a third electrode known as gate. Depending on the configuration and doping of the semiconducting material, the presence of a sufficient positive or negative potential at the gate electrode would either attract charge carriers or repel charge carriers in the

conduction channel. This would either fill or empty the depletion region of charge carriers and thus form or deform the effective electrical dimensions of the conducting channel. This controls the conductance between the source and drain electrodes.

There are different types of FET based biosensors: (1) ion selective FET (ISFET) which respond to ion in solution; (2) enzyme FET (ENFET) in which immobilised enzymes are used to measure enzyme substrate or species that are coupled to enzymatic reactions; (3) immunochemical FET (IMFET) which generates charge separation via antibody- antigen interaction; (4) suspended gate FETs, the operation of which is based on changes in the work function and dipole orientation that results from the interaction of the sensing element with various gases.

ii. **Conductometric transducers:** conductometric devices measure the ability of an analyte or a medium to conduct an electric current between electrodes or reference nodes. In most cases conductometric devices have been strongly associated with enzymes where ionic strength of a solution between two electrodes changes as a result of an enzymatic reaction. The variable ionic background and the requirement to measure small conductivity changes in media of high ionic strength limit the applicability of such enzyme based conductometric devices for biosensing. Another approach is to directly monitor the changes in the conductance of an electrode as a result of immobilisation of e.g. enzymes, antibody on to the electrode surface. The conductance is sensitive to temperature, faradic processes, double layer charging and concentration polarization so different methods with internal control must be used. The primary advantage of this technique is that it is inexpensive, reproducible, and disposable sensors. The main disadvantage is that ionic species produced must significantly change the total ionic strength to obtain a reliable measurement. This requirement increases the detection limit to unacceptable levels and results in the potential interferences from variability in the ionic strength of the sample.

iii. **Amperometric transducers:** Amperometric biosensors typically rely on a system that converts electrochemically non- active species into product that can be oxidized or reduced at a working electrode. These biosensors are quite sensitive and more suited for mass production than potentiometric. Amperometric biosensors measure the concentration dependent current through an electrochemical electrode coated with

biologically active material i.e. the current produced is linearly proportional to the concentration of electroactive product. The biologically active material usually an enzyme; oxidizes or reduces the analyte or the enzyme's substrate. The reaction is electrochemically connected to the electrode either directly or through mediator molecule or by the oxidation or reduction of co-reactant molecules or product molecules. The example would be an oxygen consuming enzyme coupled to Clark electrode. The main advantage of using amperometer as transducer is the low cost, disposable electrode are often use, the high degree of reproducibility, on site analysis is also possible. Limitation of this system is that a large over potential is required for the oxidation of analyte which may lead to oxidation of interfering compounds as well which have potential interferences to the response generating false current values.

Another type of biosensor which employ controlled potential technique i.e. chronoamperometry involves detection of chemical compounds. A square wave potential is applied to the working electrode and a steady state current is measured as a function of time.

iv. **Voltammetric transducers:** Voltammetry belongs to a category of electro-analytical method, through which information about an analyte is obtained by varying potential and then measuring the resulting current. There are various form of voltammetry: linear sweep, polarography, differential pulse, square wave, cyclic voltammetry and more. Cyclic voltammetry is one of most widely used form and it is useful to obtain information about the redox potential and electrochemical reaction rates of analyte solution. The voltage is measured between the reference electrode and the working electrode while the current is measured between the working electrode and counter electrode. The obtained measurements are plotted as current vs voltage, known as voltammogram. A number of enzyme based voltammetric sensors are available. An example is glucose sensor that is widely used in clinical laboratories for the determination of glucose in blood serums. Several other sensors are also available that are based upon voltammetric measurement; the analytes include sucrose, lactose, ethanol and L- lactate.

1.2.2 Optical transducers

Optical technique that could potentially be used in biosensors are UV- Visible spectroscopy, Fluorescence, Luminescence (bioluminescence, chemiluminescence) and Optical fibres. UV–Visible spectroscopy works on Beer- Lambert law. It is observed

that the electronic transitions of organic and biomolecules occur in ultraviolet and visible region. The major modes of excitation are:

- $\sigma \rightarrow \sigma^*$ and $\pi \rightarrow \pi^*$ i.e. from bonding to anti-bonding molecular orbital's
- $n \rightarrow \sigma^*$ and $n \rightarrow \pi^*$ i.e. from non-bonding to anti-bonding molecular orbital's

The measured absorbance at a particular wavelength is proportional to the concentration of a particular analyte; so UV- Visible spectroscopy has major application in quantitative analysis. Fluorescence spectroscopy is one of the most sensitive spectroscopic techniques and its sensitivity makes it uniquely suited for detection of very low concentration of bioanalytes. The principle of working involves the excitation of electrons in a molecule and causing them to emit light. It primarily concerned with electronic and vibrational states. The emitted fluorescent radiation can be measured in a similar way to UV- Visible radiation and subjected to Beer- Lambert law. Another optical technique is based on luminescence. Luminescence can be either chemiluminescence or bioluminescence. Chemiluminescence is the production of light by chemical reaction. During the reaction, one or more of the intermediates are formed in an electronically excited state, thus emitting light by means of fluorescence. Bioluminescence on the other hand generally involves multiple enzyme- catalysed reactions and enzyme co-factors with oxygen such as NADH. These reactions have been described as cold light, implying that the production of light from a biochemical reaction gives off no light. Bioluminescence and chemiluminescence molecules are important as they have picomolar range detection capabilities.

Optical fibres are most recently used transducers and have made possible to miniaturized optical sensors. Optical fibres can be used to direct light at a particular wavelength to a remote cell at the end of a fibre where a calorimetric reaction takes place. The magnitude of reflected radiation at this wavelength can be used as an analytic measurement. Optical fibres behave as waveguides for light and light waves are propagated along the fibre by total internal reflection.

Optical sensors offer various advantages such as speed and reproducibility of the measurement. Optical sensors can react on any change in the measurement properties literally with speed of light. Also non- contact nature of optical measurement avoids all systematic errors that come with tactile techniques. Optical transducers have been used for microbial based and few enzyme based biosensor.

1.2.3 Mass- sensitive transducers

Piezoelectric crystals act as mass sensors i.e. these transducers have been used where the biorecognition reaction causes change in mass. The utility of the piezoelectric crystal as a mass sensor arises from the linear relationship between the change in mass at the crystal surface and the change in its oscillating frequency. The vibration of piezoelectric crystals produces an oscillating electric field in which the resonant frequency of the crystal depends on its chemical nature, size, shape and mass. By placing the crystal in an oscillating circuit, the frequency can be measured as a function of the mass. Piezoelectric crystals also referred as quartz crystal microbalance (QCM). These transducers have been coupled with enzymes and antibodies to detect analytes.

1.3. Applications of Biosensors

The increase in the number of analytes to monitor present in environment, food and also in the field of health and others requires a technique which should be rapid, sensitive, reliable and less expensive. Conventional methods provide high accuracy but are time consuming and offer off- site analysis. Due to the development of biosensor technology better quantitative analysis of analyte is possible as it provides fast and sensitive measurement with lower cost and on- site analysis. Various biosensors have been developed for application including environment and bioprocess control, quality of food, agriculture, military and medical application. In the food industry, the detection of contaminants, verification of product's content, monitoring of raw materials conversion and product freshness are areas of biosensor application. Optic coated with antibodies are commonly used to detect pathogens and food toxins. Novel biosensors using suitable biological elements have been developed for medical application for the measurement of metabolites such as glucose monitoring in diabetes patients, detection of pathogens, artificial pancreas, protein engineering, and drug discovery.

Biosensors can also be used as environmental quality monitoring tools in the assessment of biological /ecological quality or for the chemical monitoring of both inorganic and organic pollutants. Common environmental analytes which are monitored are biological oxygen demand (BOD), atmospheric acidity, and river water pH, detergent, herbicides, heavy metal and fertilizer concentrations. In industrial process

control, biosensors have been developed for improving and controlling the products. Real-time monitoring of carbon sources, dissolved oxygen and carbon dioxide, and products of metabolism in fermentation are measured by biosensors. Biosensors can be also be used as defence tool for detection of hazardous materials such as germs or chemical warfare. The detection of illicit drugs and explosives, with airport security purposes, is also a major area of research in biosensors.

In spite of the past and current large amount of research in biosensor development, there is still a challenge to create improved and more reliable devices.

2 Literature Survey

Toxic nature of heavy metals adversely affects ecology and living organisms, so there is a need to determine heavy metals at trace levels. Conventional methods such as atomic absorption spectrometry, X- ray absorption spectroscopy suffer disadvantage of high cost, need for trained personnel and laboratory bound tests. Biosensors are now being utilized for monitoring heavy metal concentrations. The biological base makes biosensors ideal for toxicological measurement of heavy metal. Various types of biosensors have been reported in literature depending upon the type of biorecognition element such as enzyme, antibody-antigen, protein and whole cell. In this section main emphasis will be on whole cell based biosensors.

Whole cell biosensors involves cells of bacteria, yeast or algae with intimate contact with different types of transducers such as electrochemical (potentiometric, amperometric, voltammetric, conductometric), optical etc. The area of whole cell biosensor looks very promising for a multitude of uses. The response characteristics of cell based sensors are comparable to those exhibited by enzyme biosensors. The microbial strains are cheaper than isolated enzymes and the enzyme activity is often enhanced in microbial cells owing to optimal environment provided by cells. The inhibitory action of heavy metal to the enzyme activity form basis of its detection by cell based biosensor.

2.1 Conductometric Biosensors

Chouteau et al has described a conductometric whole cell biosensor based on *Chlorella vulgaris* for the determination of Cd^{2+} ion in aquatic habitat by utilizing the alkaline phosphatase (APA) activity. The algae was immobilised inside bovine serum albumin cross linked with glutaraldehyde. The inhibition of *C.vulgaris* microalgae APA activities in the presence of Cd^{2+} was measured with detection limit of 1ppb [8]. Similarly Guedri et al and Durrieu et al described *Chlorella vulgaris* based biosensor for the detection of Cd^{2+} and Zn^{2+} with detection limit at ppb levels [9,10]. A conductometric biosensor using immobilised *Chlorella vulgaris* used as a bienzymatic biosensor utilizing activities of APA and acetylcholinesterase (AChE) was developed. AChE detected organophosphorous and carbamates and heavy metals (Zn^{2+}, Cd^{2+}) with a detection limit of 10 ppb [11]. A conductometric based biosensor based on *Chlorella*

vulgaris was also described by Claude et al and Berezhetskyy et al for detection of heavy metal such as Zn^{2+}, Cu^{2+}, Cd^{2+}, Ni^{2+}, Pb^{2+} [12,13]. For the detection of heavy metals biosensors have been developed based on sulphur- oxidizing bacteria (SOB). Hassan et al developed biosensor for the detection of Cd^{2+}, Zn^{2+}, Hg^{2+}, Pb^{2+} and CN^- based on *Acidithiobacillus caldus* a sulphur oxidizing bacteria. Toxicity was monitored by measuring changes in electrical conductivity and pH change. The sensor detects CN^- in range of 100- 1000 ppb and 50 ppb for the metals [14]. Similarly, Oh et al fabricated SOB based biosensor for the detection of Cr^{6+} with detection limit of 5 ppb [15].

2.2 Potentiometric Biosensors

An NH_4^+ selective electrode in conjugation with potentiometer based biosensor was developed for monitoring Ni^{2+} using *Bacillus Sphaerius* NTCC 5100 yielding urease enzyme as bioreceptor. The detection range reported for Ni^{2+} was 0.002-0.04 ppb [16]. Potentiometric biosensor for detection of Zn^{2+} ion was reported by Datta et al, *Pseudomonas Striata* was used as biosensing element and cells were entrapped in the polyvinyl chloride matrix [17].

2.3 Capacitance Biosensors

Genetically engineered microorganism (GEM) based biosensors are also exploited for the determination of heavy metals. A capacitance biosensor based on recombinant cyanobacterium *Synechococcus* PCC 7942 detected Hg^{2+}, Cu^{2+}, Zn^{2+}, and Cd^{2+} with detection of 10^{-15} M. Metallothionein from cyanobacterium and merR regulatory protein from transposon Tn501 allowed the detection of metals [18]. Another biosensor based on phytochelatin fused to protein domain of *Escherichia Coli* was developed for the detection of Pb^{2+}, Hg^{2+}, Cu^{2+}, Zn^{2+}, and Cd^{2+} with the detection range of 100 fM-10 mM [19].

2.4 Optical Biosensors

Various optical based biosensors have also been reported for the detection of heavy metals. The bacterial cells, *E.coli* and *Shigella sonnei* were used for the preparation of bioluminescence based biosensor for the detection of Cd^{2+}, Pb^{2+}, As^{5+} and Hg^+. The trends of sensitivity for the detection of heavy metals in case of *E.coli* was $As^{5+}>Hg^+>Pb^{2+}>Cd^{2+}$ and for *S.sonnei* it was $Hg^+>Cd^{2+}>Pb^{2+}>As^{5+}$ [20]. A live cell array biosensor for detection of Hg^{+2} was fabricated by immobilizing genetically

modified *E.coli* containing *lac Z* reporter gene fused with gene promoter *znt A* on the face of an optical imaging fiber. The detection limit obtained was 100nM [21]. Similarly another fluorescence based biosensor involving recombinant *E.coli* expressing a chimeric Cd binding peptide fused to green fluorescent protein was prepared for the detection of Cd^{2+} ions. The strains prepared were having CdBP4GFP, his6GFP and native GFP with detection limit of 0.5µM, 50µM and 0.5mM, respectively [22]. Roda et al developed luminescence based Hg^{2+} biosensor by utilizing transgenic *E.coli* with a detection limit of 1.67 $\times 10^{-13}$ M [23]. Similarly, Virta et al described a luminescence based mercury biosensor utilizing *luciferase* gene as a reporter under the control of mercury inducible *mer* promoter and *E. coli* as host. The lowest detection limit of mercury was 0.1 fM [24]. For the detection of uranium, biosensor was developed involving recombinant bacterium *Caulobacter crescentus* that produce UV- excitable green fluorescent protein in the presence of uranyl cation. The detection limit reported was 10 µM [25]. For the detection of Cd^{2+}, Pb^{2+} *Chlorella vulgaris* algal biosensor based on alkaline phosphatase activity was developed [26].

Genetically modified recombinant whole cell *Acinetobacter sp.* DF4 was prepared by Abd-El- Haleen et al for the detection of Zn^{2+}, Cd^{2+}, Fe^{2+}, Ni^{2+}, Co^{2+}, Cr^{6+} and Cu^{2+}. The detection limit reported was 10 mg/ L for Zn^{2+}, Cd^{2+}, Fe^{2+}, Cu^{2+}; 40 mg/L for Co^{2+}, Cr^{6+} and 30 mg/L for Ni^{2+} [27]. A rhizobium based luminescence (Rhizotox –C) was used as an indicator for Zn^{2+}, Cu^{2+} in soil solution from sewage sludge treated soils. The luminescence response of biosensor declined in the presence of Zn^{2+}, Cu^{2+} [28]. Three different type of *E.coli* based whole cell biosensors were constructed by fusing mercury inducible promoter P_{mer} and its regulatory gene *mer R* with reporter gene lux CDABE, *lac* ZYA and *gfp*. These biosensors responded to low levels of Hg^{2+} by producing light, β- galactosidase and green fluorescent light, respectively [29]. Babu et al described effect of heavy metal on electron transport on cyanobacterium *Spirulina platensis*. Heavy metals suppressed the intensity of fluorescence emitted from phycocyanin and also induce blue shift in the emission peak. Cr can cause 17%- 62% inhibitions from 25µm - 100µm and a 5nm shift of peak. Similarly, Ag can cause 51%- 66% inhibitions from 10µm - 15µm and a 7nm shift of peak [30].

Durrieu et al developed optical fiber based biosensor for determination of Cd^{2+} and Pb^{2+} that employ alkaline phosphatase present on the membrane of Chlorella vulgaris. The sensor detects Cd^{2+} and Pb^{2+} in range of 0.01- 1.0 mg/L [31]. Monk et al and

Marazuela et al described various types of optical fiber based biosensors in different reviews. They classified optical biosensors on the basis of biorecognition element used for sensing [32, 33].

2.5 Piezoelectric Biosensors

Inhibition of bacterial growth in the presence of copper ion was monitored based on the change in the frequency of an uncoated piezoelectric quartz crystal of a surface acoustic wave transducer upon contact with biomass. The inhibition effect of Cu^{2+} on the biomass was in the range of 18.0 – 25.0 ppm [34].

2.6 Amperometric Biosensors

Amperometric whole cell biosensors are very popular now a days. It provides a rapid and sensitive tool for the determination of heavy metal. A whole cell amperometric sensing system for Cu^{2+} using FIA was developed based on recombinant S.cerevisiae and an oxygen electrode with detection limit of 2.1mg/L. CUPI gene, serving as promoter was fused with reporter *lac Z* gene [35]. Similarly, Lehmann et al developed biosensor for the detection of Cu^{2+} using recombinant S.cerevisiae with detection limit of 0.5 – 2.0mM [36]. Another amperometric biosensor based on E.coli was developed for inhibitory effect of Hg^{2+}, Cu^{2+}, Zn^{2+} and Ni^{2+} with detection limits of 1.1µg/ml, 1µg/ml, 50µg/ml, and 50µg/ml respectively [37]. Verma et al developed an amperometric based whole cell *Bacillus sphaerius* biosensor using urease enzyme for the detection of Pb^{2+} in milk. The reported detection limit was 2.4×10^{-3} nM [38]. A whole cell based amperometric biosensor was fabricated using *Chlorella sp.* entrapped on the surface of platinum for the detection of heavy metal such as Zn^{2+}, Cu^{2+}, Cd^{2+}, Co^{2+}, Ni^{2+}. The order of selectivity was $Zn^{2+} > Cu^{2+} > Cd^{2+} > Co^{2+} > Ni^{2+}$. The detection range was 10^{-9}- 10^{-11}M [39].

2.7 Voltammetric Biosensors

Voltammetry such as cyclic voltammetry (CV) is another commonly used technique in electrochemical analysis. Alpat et al described a voltammetric biosensor based on *Circinella sp.* modified carbon paste electrode for the determination of Cu^{2+}. Measurement was done using CV and detection limit was 54nM [40]. Whole cell based biosensor was prepared by immobilizing *Chlorella sp.* microbes over glassy carbon electrode for detection of Hg^{2+} in the presence of Ag^{+} and the detection limit reported

10^{-14}M [41]. Zlatev et al proposed a modified stripping voltammetry by taking advantage of both bacterial adsorption on a mercury surface and metal fixation capacity on *A.ferrooxidans* for the detection of Cu^{2+}, Pb^{2+}, and Cd^{2+} [42]. Another biosorption based biosensor using *Tetraselmis chuii* microalgae was developed for the voltammetric measurement of Cu^{2+}. Cu^{2+} was accumulated on the algal circuit and voltammetric measurements were carried out by differential pulse cathodic stripping voltammetry. The detection limit of 4.6×10^{-10} M was obtained [43].

3 Gap in the studies

A lot of work has been done in the field of biosensors based on enzyme using various techniques such as amperometry, voltammetry, conductometry, optical devices, and potentiometry. Comparatively less work is done on whole cell biosensors. Our present work is based on of whole cell biosensor using *Chlorella sp.* for the study of effect of heavy metal on the activity of alkaline phosphatase enzyme bound on the surface of algae. Whole cell offer many advantages over enzymatic bioreceptor which include low cost, easy to handle, optimum stability of enzyme which is not possible in case of purified enzymes. Although whole cells have low selectivity but they recognise a wide range of analytes.

We have carried out study of effect of heavy metal on both free and immobilized whole cells. The enzyme alkaline phosphatase acts on substrate p-nitrophenyl phosphate generating p-nitrophenol (PNP). The generation of PNP is studied using UV- Visible spectrophotometer. The spectrophotometer technique till date is used to observe the λ_{max} of PNP but not as a transducer in biosensor.

4 Experimental

4.1 Material

p-Nitrophenyl phosphate (PNPP), Tris-HCl buffer, $MgCl_2$, $CaCl_2$ were purchased from Sigma- Aldrich (India) and used without further purification. Sodium alginate was purchased from Loba Chemie (India). Hydrated salts like $Cd(NO_3)_2$, $Zn(NO_3)_2$, $Co(NO_3)_2$, $Ni(NO_3)_2$, $Cu(NO_3)_2$ and $Hg(NO_3)_2$ (analytical grade) were purchased from Sigma- Aldrich (India) and used as such.

4.2 Algae Culture

Algae *Chlorella sp.* was identified, cultured and sub-cultured in BG-11 media every three weeks. Algae was harvested by centrifugation and starved by suspending in phosphate free BG-11 media to induce maximum alkaline phosphatase activity.

4.3 Cell Immobilisation

Cells of algae were immobilized on Ca alginate beads. Grown algae cells (1mL) were suspended in 5% (w/v) solution of sodium alginate. The mixture was pumped drop-wise into 2% (w/v) $CaCl_2$ solution and beads were obtained.

4.4 Instrument

All experiments were performed using UV- Visible spectrophotometer (Analyticjena/ Specord 205/ Germany). Quartz cuvettes were used for measurements.

4.5 Spectrophotometric Measurements

The spectrophotometric study was performed by adding 10% *Chlorella sp.* (free/ immobilised) in a solution containing 1mM $MgCl_2$ (enzyme activator), Tris-HCl buffer (pH ~8.5). PNPP was added as a substrate. PNP was generated as product and absorption peak at 400 nm was observed.

4.6 Heavy metal testing

Stock solutions of metal salts (0.1M) were prepared by dissolving nitrate salts of Cd, Zn, Hg, Cu, Co and Ni. Fresh solutions were made by diluting the stock solution before the experiment as per requirement.

5 Result and Discussion

5.1 Absorbance Measurement with whole cell

Experiments were conducted to study the enzyme release activity of biomaterial by measuring absorbance at 400 nm (Figure 1) corresponding to absorption peak of PNP, the product of phosphatase activity on PNPP taken as substrate. 1 mL of *Chlorella sp.* biomaterial algae with cell count of 10 million cells per mL was incubated with 0.5 mL of $MgCl_2$ solution (1 mM) taken as enzyme activator, 0.5 mL of p- NPP taken as substrate and 8 mL Tris-HCl buffer of pH~8.5 shaken for 12 hours. The supernatant solution was separated after centrifugation for 5 min at 10000 rpm and measured it absorbance at 400 nm. This experiment was repeated a number of times to optimize different parameters like pH, biomaterial concentration, incubation time and concentration of substrate.

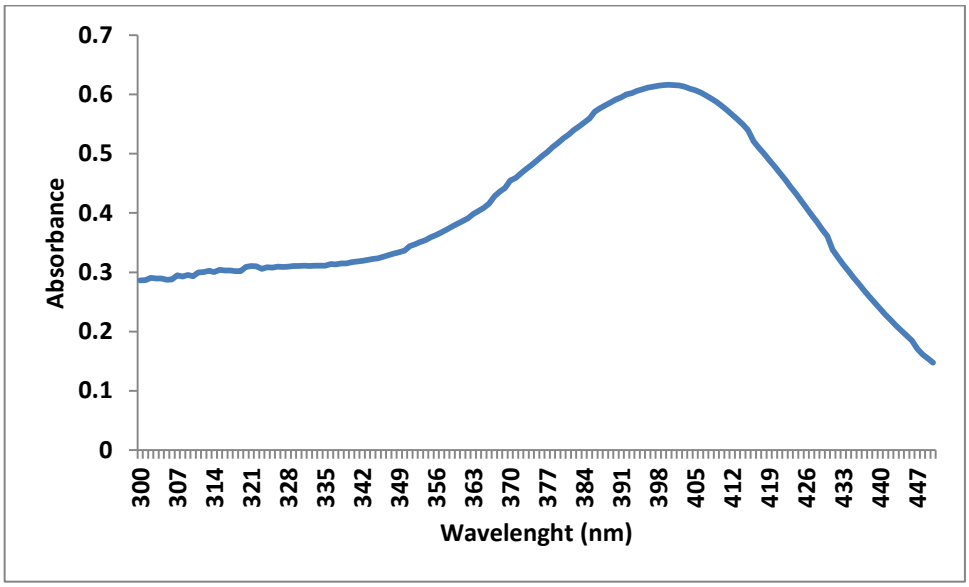

Figure 1. Absorbance vs wavelength plots showing the λ_{max} of PNP

5.1.1 Optimization of pH

Enzyme activity experiments were repeated by varying pH of the medium with Tris-HCl buffer. pH of the solutions were varied from 4 to 11 by adding hydrochloric acid and ammonium hydroxide, as per requirement. The results are shown in Figure 2.

Absorbance of the product remain stable in a pH range of 9- 11. At pH less than 9, the absorbance decreases sharply and results are not reproducible.

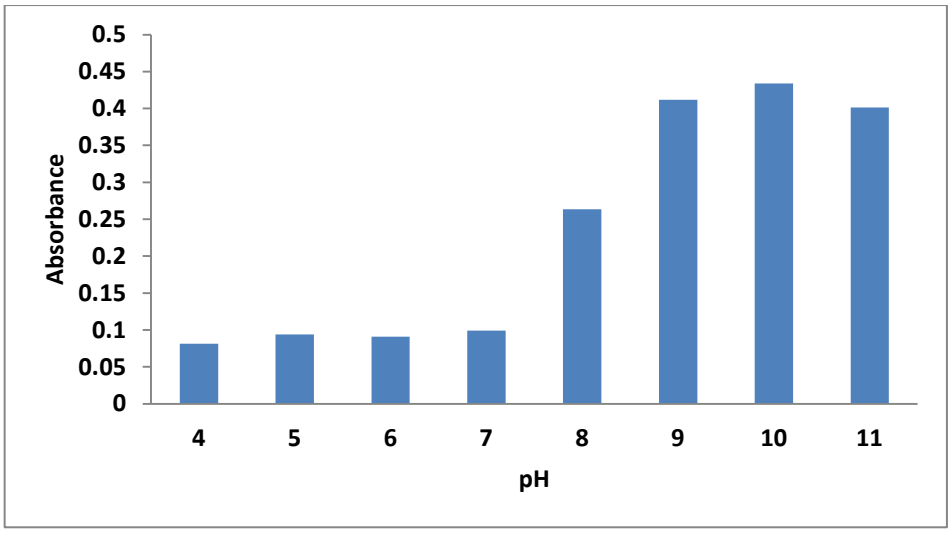

Figure 2. Absorbance vs pH plots of PNP in the presence of *Chlorella sp*. after 12 hours incubation

5.1.2 Optimization of biomaterial concentration

Different amounts of the biomaterial were taken from 0.1 mL to 1.5 mL and absorbance for each different amount of biomaterial was measured. Results are given in Figure 3 and it is seen that the absorbance increases with increase in amount of biomaterial upto 0.9 mL/ 10 mL of the mixture solution. Beyond this concentration and upto 1.3 mL/ 10 mL, the absorbance stays almost constant and decreases with further increase in the amount of biomaterial. Hence, 0.9 mL can be taken as optimized concentration of biomaterial.

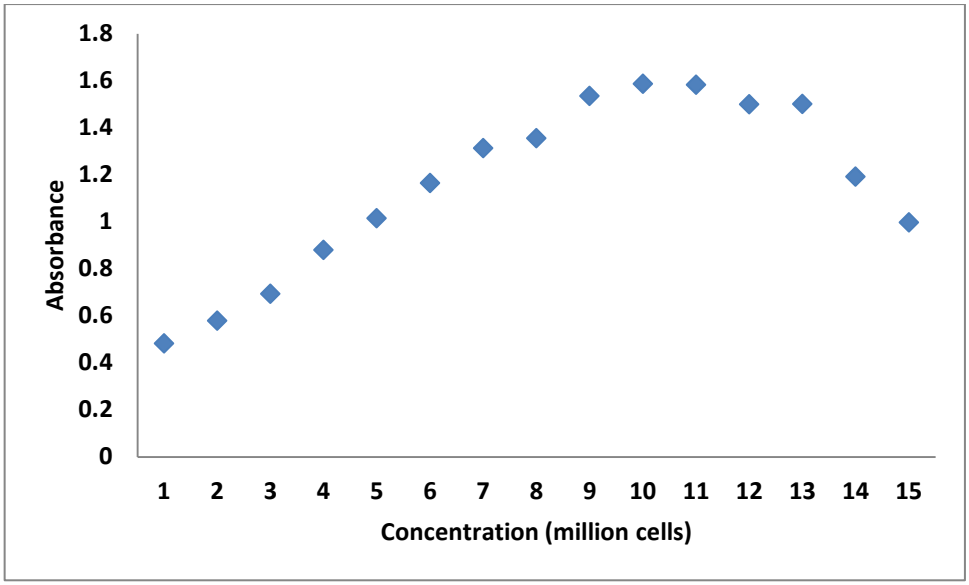

Figure 3. Absorbance vs concentration plots of PNP at different concentrations of *Chlorella sp.* after 12 hours incubation

5.1.3 Optimization of incubation time

Enzyme activity measurement experiments were carried out at different durations of incubation time of the mixture solution. Samples were incubated from 4 to 24 hours to know the most stable incubation time. Results are shown in Figure 4. It can be seen that after 12 hours of incubation there is no change in absorbance of the solution. All subsequent experiments were run with 12 hours incubation period.

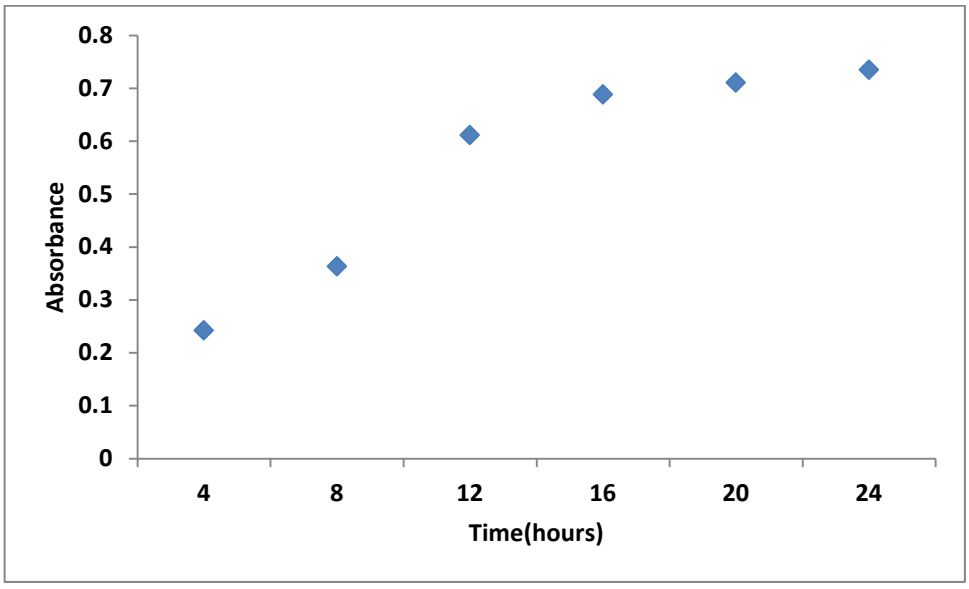

Figure 4. Absorbance vs time plots of PNP at optimized concentration of Chlorella sp. at different incubation periods

5.1.4 Optimization of substrate concentration

To understand the effect of different substrate concentrations on the enzyme release activity of the algae, experiments were conducted by taking different substrate concentration in the range of 0.2 mg/ 10 mL to 1.0 mg/ 10 mL. From the results in Figure 5, it can be seen that minimum concentration of 0.5 mg/ 10 mL is required to get stable absorbance.

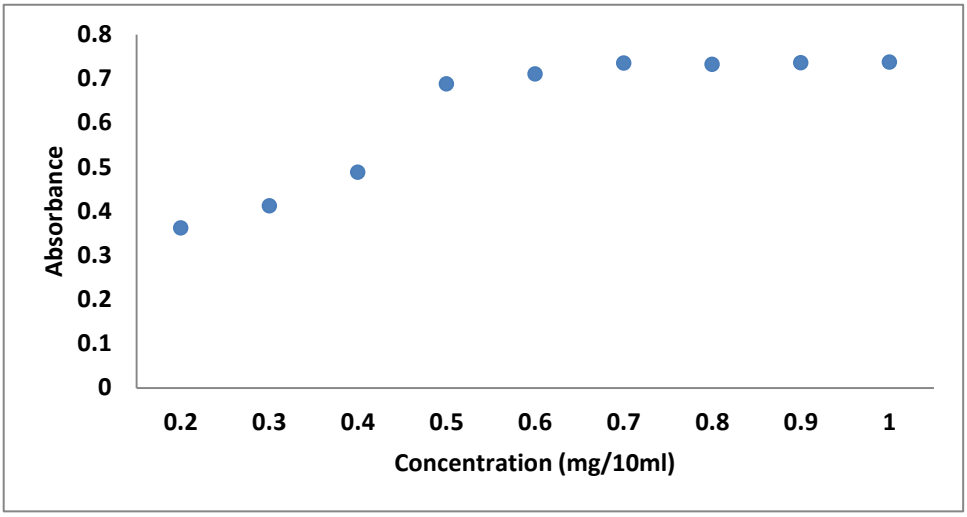

Figure 5. Absorbance vs concentration plots of PNP at optimized concentration of *Chlorella sp*. at different concentration of PNPP

5.2 Effect of heavy metal on the enzyme activity performance

It is well established that presence of heavy metals like transition elements- Zn, Co, Cd, Hg influence the enzyme activity of the biomaterial. But different metals affect the enzyme activity to different extent, probably due to difference in the stability of their respective complexes with the enzyme. Experiments were done in presence of different heavy metal ions like Co^{2+}, Ni^{2+}, Cu^{2+}, Cd^{2+}, Zn^{2+} and Hg^{2+}. Absorbances at 400 nm (corresponding to λmax of p- nitrophenol) were measured with each metal varying in the concentration range 10^{-4} to 10^{-1} M. Although trends of inhibition by different metal ions were not in regular pattern yet these can be arranged in the decreasing order of their toxicity towards the *Chlorella sp.* On the basis of measurements carried out with different metal ions at different given concentration of 10^{-3} M, 10^{-2} M, 10^{-1} M, these can

be arranged in decreasing orders of their toxicity as given in Figure 6, 7 and 8, respectively.

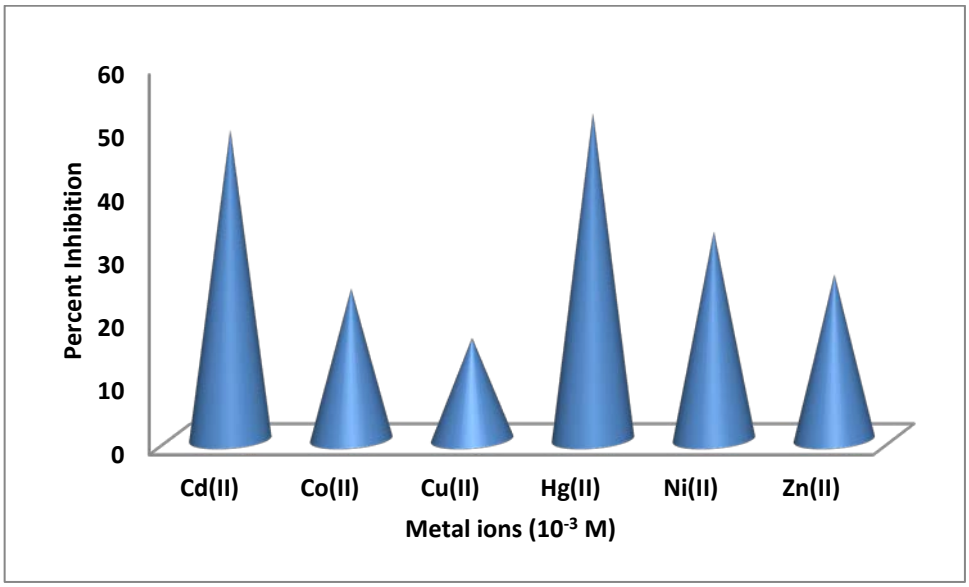

Figure 6. Extent of inhibition of enzyme activity by different heavy metals at a concentration of 10^{-3}M

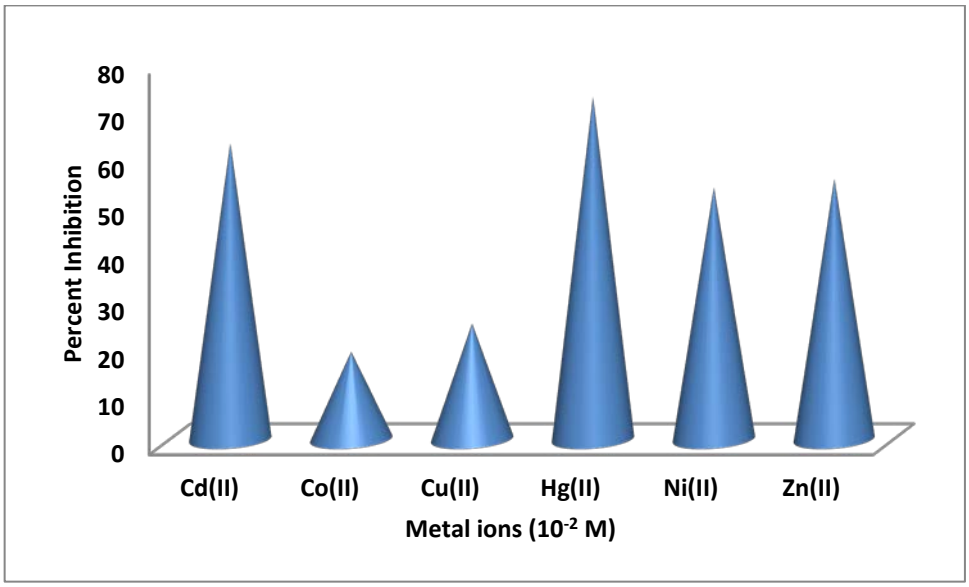

Figure 7. Extent of inhibition of enzyme activity by different heavy metals at a concentration of 10^{-2}M

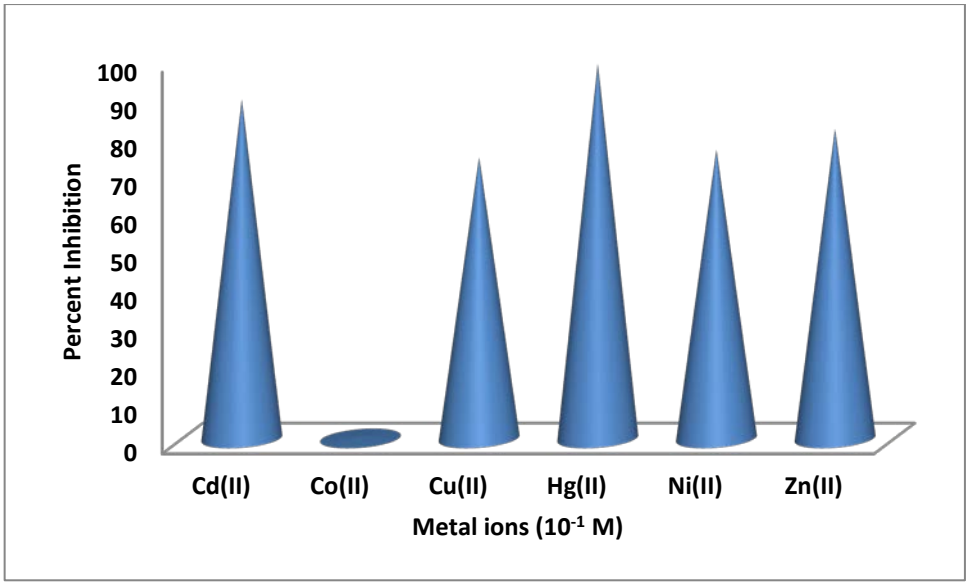

Figure 8. Extent of inhibition of enzyme activity by different heavy metals at a concentration of 10^{-1}M

Order of inhibition at 10^{-3}M: $Hg^{2+} > Cd^{2+} > Zn^{2+} > Ni^{2+} > Cu^{2+} > Co^{2+}$

Order of inhibition at 10^{-2}M: $Hg^{2+} > Cd^{2+} > Zn^{2+} > Ni^{2+} > Cu^{2+} > Co^{2+}$

Order of inhibition at 10^{-1}M: $Hg^{2+} > Cd^{2+} > Zn^{2+} > Ni^{2+} > Cu^{2+} > Co^{2+}$

Plots of absorbance measurement were drawn against concentration of each heavy metal in the range of 10^{-4}M to 10^{-1}M. Figure 9 shows trends of change in absorbance with increasing concentration of heavy metal ion.

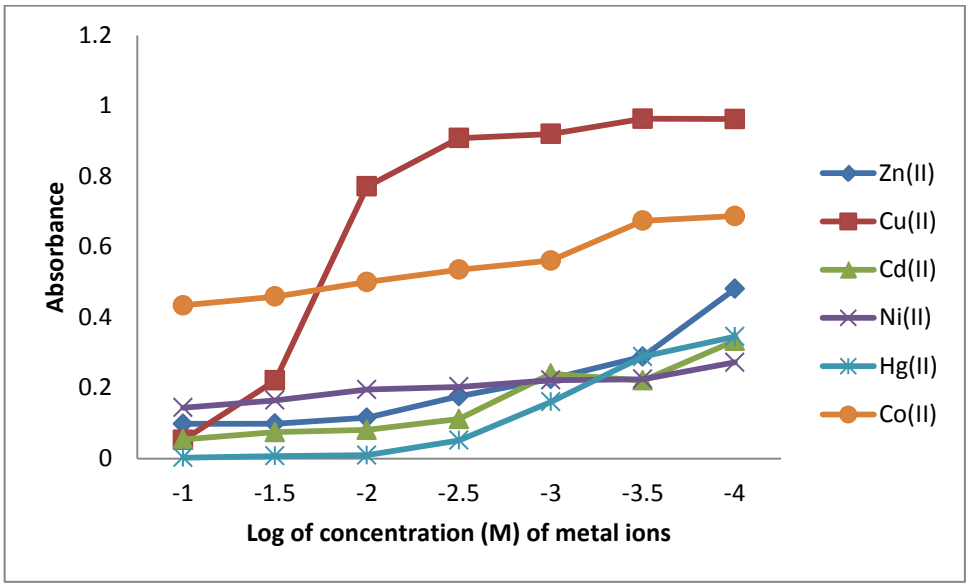

Figure 9. Absorbance vs concentration plots of different metals at optimized concentration of *Chlorella sp*. after 12 hours of incubation

For each metal ion absorbance of p-NP measured at λ_{max} 400 nm decreases with increase in concentration. But the trends are not linear. For metals like Cd, Hg and Zn, there is no further change in absorbance after the decrease from 10^{-4}M till 10^{-2}M. For Ni, there is very little change in absorbance after an initial decrease in presence of 10^{-4}M metal ion. Presence of heavy metals like Co and Cu are much different from rest of the metals. It is further observed that Cu^{2+} ions inhibit the enzyme activity to the minimum and there is very small change till 10^{-2}M concentration, beyond which the absorbance drops sharply and finally becomes almost negligible at 10^{-1}M. In the presence of Co^{2+} ions there is very less decrease in absorbance from 0.687 to 0.434 with change in concentration of metal salt from 10^{-4} to 10^{-1}M. It is easy to compare relative inhibition of a given metal ion with other metal ions.

5.3 Effect of heavy metal on enzyme activity in whole cell using Ca-alginate beads

Ca –alginate beads were generated by precipitation when sodium alginate mixed with biomaterial was added to a cold solution of $CaCl_2$. The Ca- alginate beads thus prepared contained biomaterial encapsulated in its core. It can be seen from Figure 10 that in general, absorbance is relatively less for a system with biomaterial present in free form as compared to that when entrapped in Ca- alginate beads.

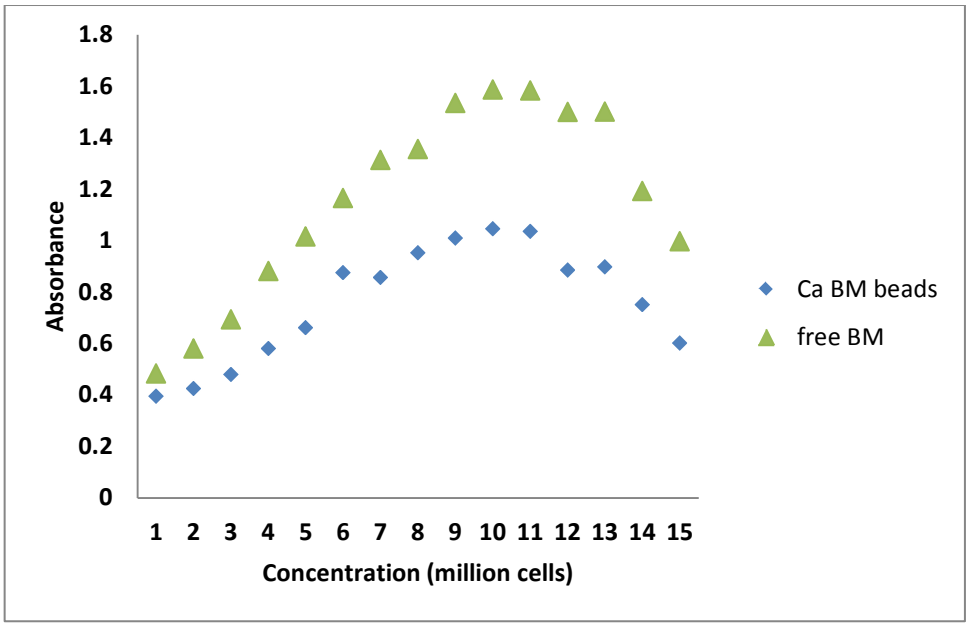

Figure 10. Comparison plots of Absorbance vs concentration of algal cells for p-NP at different concentrations of free BM and BM entrapped in Ca alginate beads (1-15%) after 12 hour incubation

Enzyme activity of entrapped whole cells was studied by measuring absorbance of the aqueous medium containing the heavy metals. This experiment was repeated with all the heavy metals at different concentration also. It can be seen from Figure 11 that the trends of inhibition in enzyme activity due to the presence of different heavy metals is similar to that when the biomaterial is present in free form except for Co where the trend of the inhibition is relatively to a lesser extent.

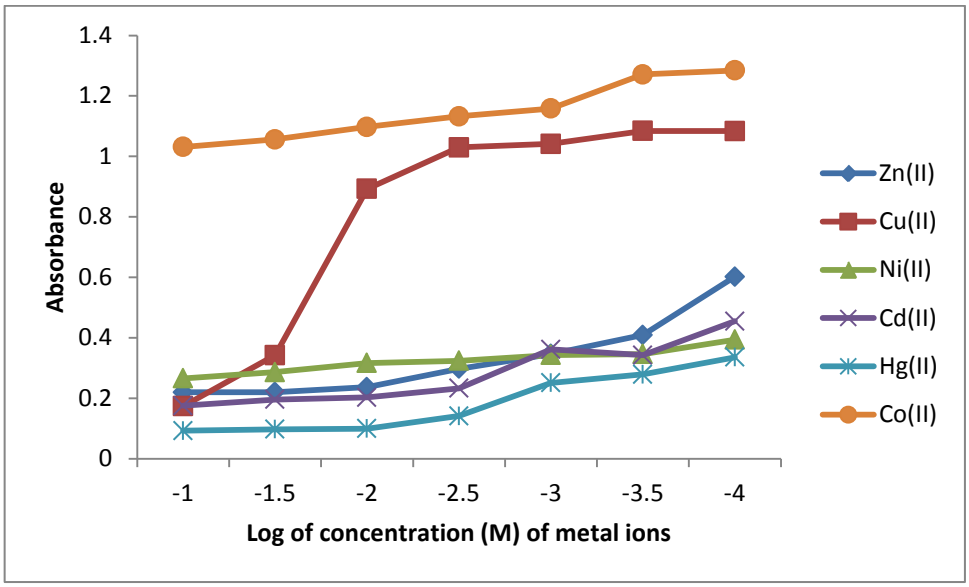

Figure 11. Absorbance vs concentration plots of different metals at optimized concentrations of biomaterial entrapped in Ca alginate beads (*Chlorella vulgaris*) after 12 hrs incubations

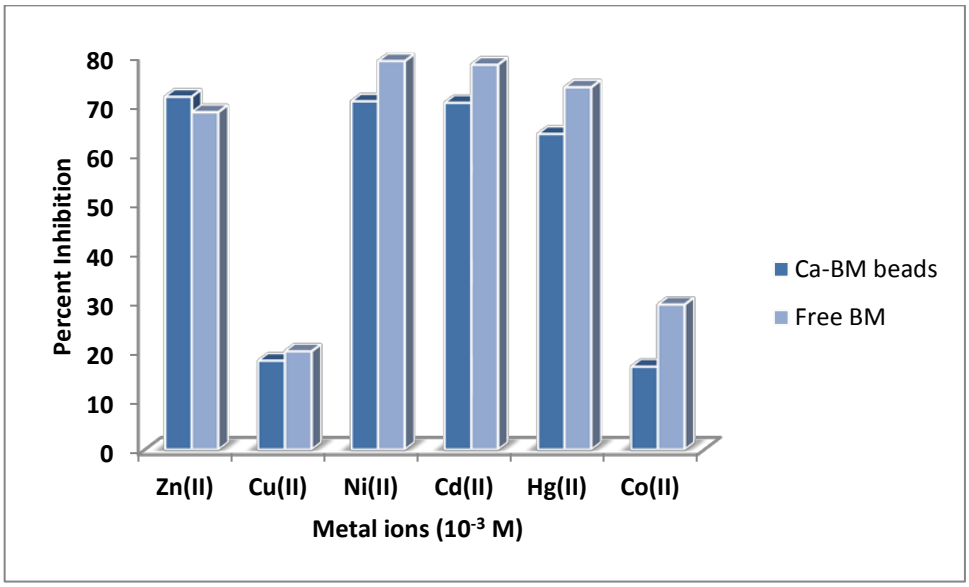

Figure 12. Comparison of percent inhibition of enzyme activity with different heavy metals (at a concentration of 10^{-3}M) on both free and encapsulated biomaterial

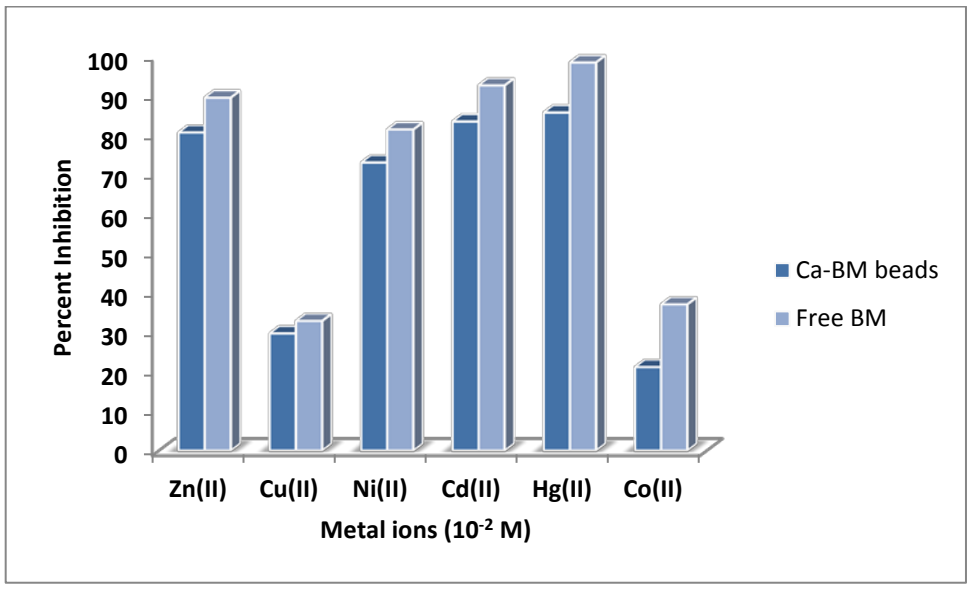

Figure 13. Comparison of percent inhibition of enzyme activity with different heavy metals (at a concentration of 10^{-2}M) on both free and encapsulated biomaterials

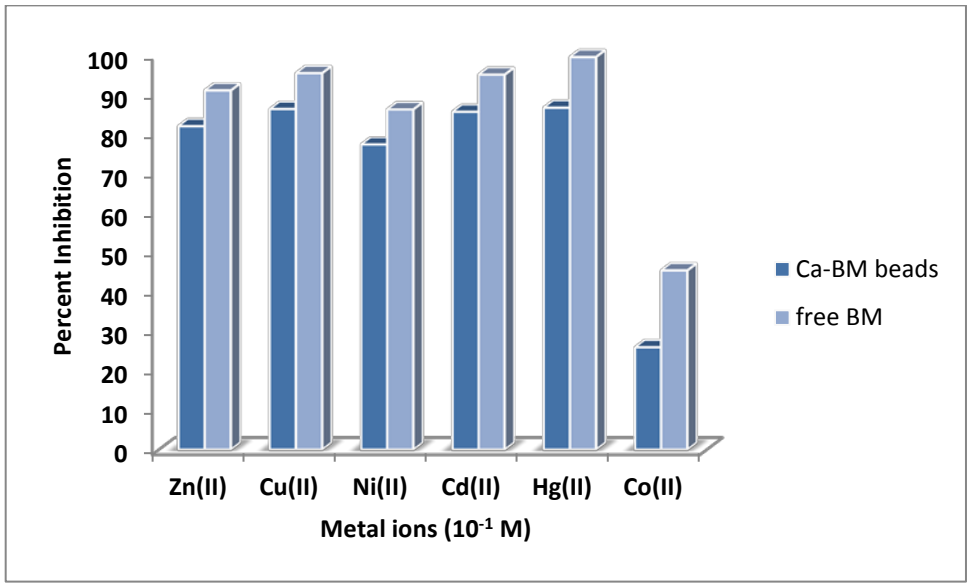

Figure 14. Comparison of percent inhibition of enzyme activity with different heavy metals (at a concentration of 10^{-1}M) on both free and encapsulated biomaterials

Figures 12-14 show inhibition of each metal ion on biomaterial activation when present as trapped Ca- alginate beads and in free form, respectively. For almost all cases the extent of absorbance is less for biomaterial as Ca-alginate bead than as free biomaterial.

6 Conclusions

1. Effect of heavy metals on the enzyme activity of whole cells (free and encapsulated biomaterial) can be studied by spectrophotometrically.
2. The parameters such as pH, concentration of biomaterial, substrate and incubation time need to be optimized to get desired results.
3. In presence of heavy metals the activity of enzymes present in the whole cells is decreased. But the extent of inhibition is different with different metal ions.
4. Inhibition of enzyme activity by different heavy metals have been classified as $Hg^{2+} > Cd^{2+} > Zn^{2+} > Ni^{2+} > Cu^{2+} > Co^{2+}$. The order is independent of concentration of the metal ions.

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