Microbial Biosensors for Some Heavy Metal Ions

A

Thesis submitted in

fulfillment of the requirement of the degree of

Doctor of Philosophy

Submitted by

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Under the Supervision of

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July, 2013

Certificate

Certified that the thesis entitled "**Microbial Biosensors for Some Heavy Metal Ions**" which is submitted by Mr. Jasminder Singh in fulfillment of the requirement for the award of the Degree of Doctor of Philosophy in the School of Chemistry and Biochemsitry, Thapar University, Patiala, is a record of candidate's own independent and original research work carried out by him 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 "**Microbial Biosensor for Some Heavy Metal Ions**" in fulfillment of the requirement for the award of the Degree of Doctor of Philosophy, School of Chemistry and Biochemsitry , Thapar University, Patiala, is an authentic record of my own work carried out under the supervision of Dr. Susheel Mittal, Senior Professor, School of Chemistry & Biochemistry, Thapar University, Patiala, India 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.

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Acknowledgement

There is always a sense of gratitude which one expresses to others for their help and support, but a statement of thanks will not reflect my deep sense of gratitude and obligation to all those who helped and supported me in one way or another in completion of thesis. I would like to take this opportunity to thank many people who have helped, encouraged and stood beside me during this this study.

I take this opportunity to thank my esteemed supervisor Dr. Susheel Mittal, Senior Professor, School of Chemistry & Biochemistry, Thapar University, Patiala for having faith in me and entrusting me with this opportunity to work under his guidance and supervision, providing all kinds of support and inspiration, wise counsel, constant encouragement, valuable suggestions and comments, expertise and fruitful advice throughout this exploration and preparation of the thesis. It's a great privilege to experience a sustained enthusiastic, involved interest, constructive criticism, tremendous enthusiasm and careful supervision from his side throughout the period of my work.

I express my gratitude to Director, Thapar University, Patiala and Dr. Satnam Singh, Head, School of Chemsitry and Biochemistry, for all the facilities which have been immensely helpful for the completion of my work.

I express my regards to Dr. Dinesh Goyal and Dr. Ranjana Prakash (PhD committee member- Subject expert) and Dr. Ashok Kumar SK (VIT, Vellore) for suggestions and taking interest in the progress of work along with laboratory facilities.

I extend my thanks to all the faculty members of School of Chemistry and Biochemsitry, Thapar University, Patiala for providing necessary guidance during my research work. I am thankful to Mr. Chandar Thakur and other non-teaching staff, School of Chemistry and Biochemsitry for the constant official help and cooperation.

I owe a debt of gratitude and heartfelt thanks to my sisters Mrs. Anju Bala, Mrs. Karamjeet Kaur Sidhu, Ms. Nidhi Gupta, Mrs. Taranpreet Kaur and Dr. Satnam Singh Aulakh (Big B) for their kind concern, critical observations in designing and planning experiments and above all their support, which always helped me endure hard times and to accomplish all my goals.

I extend my thanks to my seniors Dr. Santosh Pathak, Dr. Nirankar Singh and Mr. Pawan Kumar for their timely advice and support, which helped me throughout my endeavor.

I take this opportunity to thank Mrs. Shipra, Ms. Maitari Bhutani, Ms. Harpreet Kaur, Ms. Nayanjot Gill, Ms. Gurvinder Kaur Waraich, Ms. Kiran Sandhu Mr. Sumit Jaiswal, Mr. Ankush Kanwar, Mr. Subrat Mohapatra, Mr. Anirudh Sharma, Ms. Manisha Pabbi, Mr. Sanjeev Kumar, Mr. Kunal Garg and Ms. Rashmi Sharma, for their discussions and support. I owe a word of thanks to all research scholars at the School of Chemistry and Biochemistry for providing constant support, encouragement and a good working atmosphere.

I express my indebted gratitude to Mrs. Preeti Saluja Verma, Ms. Narinder Kaur, Mr. Hemant Sharma, Mr. Sunil Verma, and Mr. Sunil Thakur for helping me with various aspects of research.

I express my gratitude to my mentor Dr. Narinder Singh for development of a scientific aptitude, courage to go ahead and consistent support in the fields of science. All his support and guidance has brought me this stage

I am especially indebted to all students who worked with me and people, who taught me various lessons of life and helped me in polishing my skills of both personal and professional arena.

I feel a deep sense of gratitude for my grandfather, my parents for their blessings and belief in me and siblings (Rajwinder Kaur, Sukhminder Singh, Jatinderpal Singh, Avtar Singh) who have always showered unconditional love on me, encouraged and supported me in every aspect. They formed a part of my vision and taught me the good things that really matter in life.

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

Jasminder Singh

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Abstract

Whole cell microbes having phosphatase activity were identified for the preparation of biosensor using Pikovskaya media for bacterial culture and Beneck's media for algal culture from the rhizospheric soil. Identified microbial colonies were tested for their phosphatase solubilizing capacity. Microbes having maximum solubilizing capacity were cultured and re-cultured using BG-11 inorganic media and were starved with respect to phosphate by suspending the media in phosphate free BG-11 media, before being used for immobilization on to the electrode surface.

Starved microbes were immobilized on different electrode surfaces using bovine serum albumin (BSA) and were cross-linked with glutaraldehyde vapours for 30 minutes. The immobilized electrode was characterized for maximum output current by changing its cell density and optimum working environment by variation in pH of the solution. Immobilized electrode was also characterized for its composition, permeability and viability of the microbes in the membrane matrix with various techniques like scanning electron micrograph (SEM), cyclic voltammetry (CV) and spectrofluoremetry.

Whole cell based amperometric biosensor was fabricated using an identified phosphatase solubilizing unicellular microalgae *Chlorella* sp. as the biocatalyst. The microbial culture was entrapped in a polymeric membrane of BSA directly interfaced to the surface of a platinum electrode for the detection of bioavailable heavy metal ions like zinc, copper, cadmium, cobalt and nickel. . The fabricated biosensor had a life time of 7 days and was found sensitive to a lower concentration level of 10^{-9} M of nickel ions, 10^{-10} M of cadmium and cobalt ions, 10^{-11} M of copper and zinc ions. The electrode system was workable in a detection range of 10^{-12} M to 10^{-6} M. The amperometric biosensor responds to heavy metal ions with a relative selectivity in the order: $Zn^{2+} > Cu^{2+} > Cd^{2+} > Co^{2+} > Ni^{2+}$.

A whole cell based biosensor was also prepared by immobilizing *Chlorella* sp. microbes over glassy carbon electrode with optimised composition of cell density. The electrode responds linearly in concentration range of 10^{-14} M to 10^{-6} M for zinc, copper, cadmium, cobalt and nickel ions. Relative selectivity of the metal ions can be ordered in following

sequence: $Zn^{2+} >> Cu^{2+} \approx Cd^{2+} > Co^{2+} > Ni^{2+}$. The biosensor had a life time of 14 days, after which, its analytical properties did not remain stable. Zinc dominates the inhibition of microbial activity over all other heavy metal ions selected for the study. With modified glassy carbon electrode cadmium and copper do not interfere with each other but these are inhibited in presence of zinc ions only.

Prepared a whole cell based biosensor by immobilizing *Chlorella* sp. microbes having phosphatase activity over glassy carbon electrode. The electrode responds linearly in concentration range of 10^{-14} M to 10^{-6} M and lower detection limit of 10^{-14} M for mercury and showed its rare selectivity for mercury over other potential interferents like silver, alkali metals, alkaline earth metals and transition metals. The membrane electrode system had an expected life of 14 days.

The biosensor was also tested for determination of mercury in a continuous flow system. A new assembly for online voltammetric determination was designed and used for the measurement of mercury ions which showed an excellent selectivity, sensitivity and reproducibility in a range of flow rate of 0.5 mL/min to 1.5 mL/min.

The prepared biosensors were tested for their analytical applications in the determination of different heavy metal ions in real time samples collected from the laboratory drain and also those prepared artificially. The obtained results were compared with those obtained from atomic absorption spectrometer. Results of real time samples analyzed using flow system were compared with those by batch method and also with Atomic Absorption Spectroscopy (AAS).

1. Introduction

Biosensor is a device that uses biochemical reactions mediated by isolated enzymes, immunosystems, tissues, organelles or whole cells to detect chemical compounds usually by electrical, thermal or optical signals or the analytical device which responds to analyte in an appropriate sample and intercepts its concentration as an electrical or physical signal by a suitable combination of biological recognition material and transducer (Higgins et al, 1987). As a result of the recent advancement in technology and science, biosensors have established themselves as major components in generating analytical information regarding various human endeavours like medicine, living standards, industries, environmental analysis etc. (Zhylyak et al, 1995). All these efforts of advanced human societies are reliant on estimation, monitoring and control of various chemical species (Higgins et al, 1987). Major concern of human species is the monitoring and protection of environment, which is very important aspect of environmental control (Karube et al, 1995). Sustainable water management for human race requires determination and estimation of toxic matters (Wong et al, 1997) like heavy metals and pesticides etc., which pose a major threat to various water bodies of environment. These heavy metals and pesticides pose a serious threat as they show bio magnification. So, their rapid determination at trace levels becomes the need of the time.

Major part of chemical intelligence about these pollutants is obtained in sophisticated analytical laboratories with various traditional methods like flame photometry, atomic absorption spectrometry, inductive coupled plasma-mass spectrometry (ICP-MS) (Burlingame et al, 1996), anodic stripping voltammetry etc. (Turner, 1989). However, these facilities require either tedious sample preparation, long measurement time (Evtugyn et al, 1998) or in some centralized facilities and required capital and skilled personnel to handle and operate them (Karube et al, 1995; Preininger et al, 1996). Under various circumstances like time and finances these facilities become inadequate and unapproachable. So, a reliable, easy approachable, cost effective, sensitive, rapid in response and easy to use method is required for their determination. Biosensors present a perfect alternative for such estimation as they are easy to prepare, handle, cost effective, sensitive, and also offer a potential for continuous online analysis of various water bodies (Corbisier et al, 1996; Fennouh et al,

1998). Biosensor also provides an advantage over traditional methods as it gives real depiction of bioavailable content (Paton et al, 1995; McGrath et al, 1999) of heavy metal ions or pesticides, rather than total content as in all other methods (Gayet et al, 1993).

Biosensors have come up a long way since the discovery of 1st biosensor in 1956 by Prof. Leyland D Clark, also known as Father of Biosensors. Shapes, sizes and systems employed in making of biosensors have changed a great deal as compared with the first biosensor of Prof. Clark. Although, there is great deal of advancement in methods and techniques of collecting analytical information using biosensors, yet overall construction of biosensor has remained the same, i.e., close proximity of biological components with some transducers (Karube et al, 1995; Whitaker, 1972), which change the response generated by biological component due to some metabolic activities causing formation of product from substrate to some electrochemical or some physical change, which is further processed and refined using an amplifier and displayed on a digital display.

1.1 CLASSIFICATION OF BIOSENSORS

Biosensors can be classified:

a) On the basis of biological recognition element

b) On the basis of transducer

c) On the basis of analyte determined

1.1.1 Classification of biosensors on the basis of biological recognition element

Biological recognition element is identified keeping target analyte in mind, so that selectivity can be achieved without compromising with the sensitivity of the system. Biological element undergoes a biocatalytic or binding process by taking up the desired target analyte and produces signal, which is fed to transducer. Biosensors can be categorized into following types depending on biological recognition element used:

(a) Enzymatic assay based biosensors: Biosensors in which enzymatic assay is used as biocatalyst to produce metabolic changes by changing a substrate to product. This is one of the ancient techniques to prepare biosensors, having utmost sensitivity and selectivity as enzymes are very much selective in their action. Even the very first biosensor prepared by

Prof. Clark was made using glucose oxidase enzyme (Dhand et al, 2007; Wu et al, 2013; Lee et al, 2013; Wang et al, 2013).

(b) DNA based biosensors: DNA strand is being used as recognition element, expression of various sites of DNA and correspondingly increase or decrease in the activity of DNA strand is studied (Arora et al, 2007; Prabhakar et al; 2011).

(c) Proteins based biosensors: Various proteins are used as recognition element (Kim et al, 2013; Su et al, 2013).

(d) Immunoassay or antibodies based biosensors: Immunoassay involves recognition between an antigen and its corresponding antibody. This recognition is very specific in general. The binding of analyte to an antibody depicts its concentration in the analytically important sample with an opportunity to quantify them. For such analytes process called coupling with heavy proteins like bovine serum albumin (BSA) is being used (Suri et al, 2009).

(e) Cell organelles based biosensors: Various cell organelles like mitochondria, plasmid etc. and their various actions and activities are used as recognition element for various analytical determinations.

(f) Whole cell based biosensors: Various whole cells like bacteria, fungi, algae, etc. are used as recognition elements for various estimations and measurements in analytical samples (Sharma et al, 2011).

Although, purified enzymes offer great deal about sensitivity and selectivity and whole cells lack selectivity in most of analyte determinations, yet they are better alternate to rest of biological recognition elements as they provide following advantages:

i. Can be handled easily.

ii. Can be easily cultured, cells proliferate with ease in both anaerobic and aerobic conditions.

iii. Cost effective.

iv. Enzyme system used for the study will work best within the system because it will get its optimum working environment of pH and temperature in the cell.

v. Wider range of analytes can be analysed as whole cell provide wider range of enzymes.

vi. Various metabolic functions such as respiratory and fermentation etc. involving more than one enzyme systems can be used for multi analyte determinations.

vii. Can be miniaturized with ease allowing potential rapid multi target analysis.

1.1.2 Classification on the basis of transducer employed

Biological recognition element is in close proximity with transducer. Transducer takes up the signal generated by product of a biocatalytic or binding process of biological recognition element with desired analyte, analyses it and changes to electrochemical or physical signal, which is further processed and displayed. Optical and electrochemical transducers are most widely used in biosensor preparations. Optical determination (Delfino, 2013; Citartan et al, 2013; Sciacca et al, 2013) is based on measurement of luminescent, fluorescent, colorimetric or other optical signals generated by interaction of biological recognition element with target analytes and correlates the observed signal with concentration of analytes.

The fluorescent sensing technique is based on measurement of fluorescence intensity, which is proportional to concentration of the target analyte. The shorter wavelength is used for excitation and emitted longer wavelength is analysed. Fluorescent material can be generated by the biological element or metabolic changes of biological element causes the surroundings to produce some fluorescent light emitting substance. Bioluminescent techniques rely on detecting the change in luminescence emitted by biological recognition element, which responds to target analyte in dose dependent manner. Colorimetric techniques involve conversion of a chromogenic substrate into a colourful compound by metabolic activity of biological recognition element. Colored product can be distinguished by naked eye or with a spectrophotometer.

Electrochemical transducers (Kestwal et al, 2008; Zhu et al, 2008; Yuce et al, 2010; Verma et al, 2011; Su et al, 2013; Gupta et al, 2013) can be divided into amperometry, potentiometry,

conductometry and voltammetry. In amperometric transducer (Xi et al, 2013; Zhu et al, 2013; Batra et al, 2013; Yagati et al, 2013) given voltage is applied and current signal is measured between working electrode and reference electrode and is related with concentration of desired compounds. Current signal is generated due to reduction or oxidation of an electro-active metabolic product generated by biological recognition element or intermediate from electro-inactive substrate on surface of working electrode. Conductometric transducer depends upon changes caused in conduction of the solution due to change in concentration of ionic species, due to their consumption or production in the appropriate analytical solution under investigation (Nasrabadi et al, 2009; Balan et al, 2012; kucherenko et al, 2012; Boisse et al, 2013).

Potentiometry (Konopinska et al, 2013; Zhang et al, 2013; Lawal et al, 2013) involves measurement of potential difference between the working electrode and the reference electrode and potential measured shows a concentration dependent behaviour. Voltammetry (Singh et al, 2012; Reza et al; 2013) is the most versatile technique in electrochemical analysis in which both current and potential are measured and recorded with respect to each other. The position of the peak is related to specific chemical species and peak current density is proportional to the concentration of corresponding species. Beside these optical and electrochemical transducers, various other properties like change in weight using piezoelectric (Guilbault et al, 1992) and change in heat energy using thermal transducers are also employed in biosensors preparation. Classification of biosensors can be summarised into following broad categories (Table 1.1) on the basis of transducer employed for various estimation and measurements:

Although optical transducers (Ganjali et al, 2009) are cost effective and selective in nature, yet electrochemical transducers are gaining importance, as no genetic engineering is required for getting the selectivity. An electrochemical species generated will give a selective response for estimation of target analyte. Miniaturization, multi analyte determination and ultra-sensitivity can be made possible with the use of electrochemical transducers.

Table 1.1: Classification of biosensors based on transducer employed

S.No	Type of Transducer	Measured property
1.	Electrochemical	• Potentiometric • Amperometric • Voltammetric • Conductivity
2.	Optical	• Florescence • Luminescence • Colorimetry
3.	Mass sensitive	• Piezoelectric
4.	Thermal	• Heat of Reaction • Heat of Adsorption

1.1.3 Classification on the basis of target analyte determined

Biosensors can also be classified on the basis of targeted analytes for estimation from the analytical samples like organic pollutants, various metal ions, pesticides, phenols, polluting gases etc. from the analytical samples.

(a) Biological oxygen demand (BOD) based biosensors: Biological oxygen demand is the oxygen required by aerobic microorganisms to breakdown of various organic pollutants present in water bodies for their food and energy. BOD values serve as an indicator of concentration of organic pollutants in various water bodies (Karube et al, 1977; Kim et al, 1999).

(b) Biosensors for pesticides determination: Pesticides have become an indispensable tool in field of agriculture for efficient control of weeds, insects and fungi, which have a detrimental effect on crop production. Pesticides can be further classified into herbicides, fungicides and insecticides depending upon the targeted species. Depending upon the mode of use and their solubility these pesticides cause the pollution to water bodies and soil by the modes of deposition and leaching. Effect of pesticides is studied as disrupter of various metabolic activities of biological recognition medium, causing decrease in the activity of the metabolic process (Kaur et al, 2008; Bhalla et al, 2012; Cesarino et al, 2012; Sgobbi et al,

2013; Oujji et al, 2013). This is measured by various transducers like optical, electrochemical etc.

(c) Biosensors for heavy metal determination: Heavy metals like pesticides are of great importance to human as well as plant cells. Various metal ions like copper, magnesium, zinc, etc., are of great importance to cells of both eukaryotic and prokaryotic origin, acting as co-factor in enzymes of various metabolic processes. But these metal ions have the ability to get accumulated in livestock and excess of any of these ions proves very fatal for the cells of both plant and animal origin. In human beings they cause diseases like alzheimer, willson's disease, parkinson's disease, kidney and liver failure etc. by disrupting the functioning of various enzymes and causing decrease in activity of that enzyme. As the activity of an enzyme is inhibited by heavy metal ions, which in turn caused the decrease in bio-catalysed reaction, which in turn measured by various transducers (Poursaberi et al, 2012; Narsaiah et al, 2012; Babu et al, 2012; Veselova et al, 2012; Ghica et al, 2013).

(d) Biosensors for phenols: Phenol is one of the most widely used chemical as it has been used in various industries like pesticide, pharmaceuticals, surfactant, plasticizers and explosive industries. Effluents of these industries give rise to phenols, which contaminate soil, air and water. Some pesticides are based on phenolic skeleton and their degradation by various activities and produces phenol as product. Various metabolic activities cause the oxidation of phenol to catechol and then quinones. Oxidation of phenol to quinone is concurrent with depletion in oxygen which can be monitored with Clark oxygen electrode or generation of quinone using electrode biased at a negative potential vs Ag/ AgCl reference electrode.

(e) Biosensors for monitoring polluting gases: Increasing concerns over estimation and monitoring of environmentally relevant gases like NO_x, SO_x, CO_2, O_2, methane and ozone has provided a need for reliable monitoring and estimation of air pollution. Various biosensors can be used for effective determination of various gases like CO_2 and O_2 in gas phase or in dissolved solution. Various other pollutants like cyanide, poly aromatic hydrocarbons have also been detected using biosensors.

Heavy metals are well known to human race for ages and are being used in various applications. Copper, nickel, iron, were the first of the metals to be used. Heavy metals like cobalt, iron, selenium, chromium, in particular, and to a lesser degree other elements molybdenum, zinc, are the so-called micro-elements (Tauriainen et al, 1998), also known as nourishing metals. Some of the heavy metal ions except cadmium and lead (Tauriainen et al, 1998) play a very important physiological role (Merian et al, 1991) in human body, e.g. iron is one of the major components for transportation of oxygen and fat in human body as a part of hemoglobin in human body, copper and cobalt act as co-factor, chromium and zinc are part of some important enzymes (Thompson et al, 1993). Like humans, these metal ions are of supreme importance to plants too. For example, manganese is an integral component of chlorophyll, which helps in photosynthesis. Various metal ions also act as activators e.g. magnesium is an activator in alkaline phosphatase. Uptake of heavy metal ions and their incorporation into metalloenzymes or utilization in enzyme activation occurs in all microbes.

Nitrogenase contains iron and manganese, cytochrome contains iron and superoxide dismutase contains iron, manganese, copper and zinc. A number of microbes are able to use some metals or metalloids as electron donors or acceptors in energy metabolism (Nies, 1999). Besides being in a healthy system, they are present in a quite precisely determined proportion with other heavy metals for proper working of the body. Any change in the proportion of these metal ions is a causing agent of various health issues in humans. Heavy metals occur naturally in soil and rocks. Weathering of rocks and soil break down and release them into air and water sources.

Beside these natural sources, various anthropogenic activities and various industries like mining, agriculture, casting, pharmaceutical, polishing, smelting, processing industries etc., burning of fossil fuels, burning of tobacco and even forest fires are major sources of heavy metal pollution. These metal ions taken in have a unique property of bioaccumulation, causing the metal to accumulate with in the body. Hence, even the non-lethal dosage also changes to a lethal one (Bontidean et al, 1998; Diels et al, 1999). These soil, air and water polluting heavy metal ions are washed away or carried away by flowing water and air current, which takes them to miles away from the source making point source to a diffuse source.

Leaded petrol introduced in 1920's caused a gradual and then a fast increase in lead content in the bones of the whole population, who came in contact with dust covering towns and cities. Body does not distinguish it from calcium, thus accumulating it where 96 % of bodily calcium is accumulated, i.e. in the bones. Leaded petrol was phased out with unleaded one, which quickly brought about normalization of lead content in city and town environment. Leaded petrol is still being used in piston moved jet engines. Metals like mercury, gold, arsenic etc. trigger autoimmunity, which causes the cells to identify cells of its own kind and starts acting against them by destroying them. Similarly mercury and aluminum also show bioaccumulation. Despite some noted improvements in worker safety and cleaner production, mining remains one of the most hazardous and environmentally damaging industries

Nowadays, most common heavy metal contamination involves: lead, mercury, cadmium, nickel, manganese, zinc, copper, etc. These metal ions work through free radical production mechanism in oxygen. The system remains same for both useful and non-useful heavy metal ions. These metal ions cause diseases like heart attacks, atherosclerosis, hemorrhages, diabetes, blindness, deafness, reduced growth, and other even more dramatic ones. Mercury acts as cytoplasmic poison, hardly removable by normal mechanisms. It has enormous affinity for the nervous system, where it accumulates. It is from there that the most dramatic disease symptoms are observed. Despite of having such rigorous effects of heavy metal pollution, heavy metals are still extensively used in various industries. A need to monitor and estimate exact amount of heavy metal ions in a given system or analytical sample has risen. In last two to three decades biosensors have come up by leap and bounds as a perfect alternate to chemical sensors for detection of heavy metal ions in various analytical and environmental samples.

Close inspection of literature reveals that there is hardly any rigorous comparison between chemical analysers and biosensor performance towards heavy metal determination. Later show great deal of superiority in detection limit and accuracy of measurement. Biosensors present following arguments in support of them being considered as superior tool for analytical estimation and monitoring of heavy metal ion in various samples of analytical and environmental importance from chemical or biological origin, food, industries and environment.

a) Biosensors measure bioavailable i.e., the amount which is freely available to cross the bioreceptor from the medium it is dipped in, at a given time rather than total quantities of analytes of importance.
b) Biosensors can be used in specially restricted environments.
c) Biosensors can be used for general toxicity measurement as first line screeners.
d) Biosensors can be used as alternate with reproducible, cost effective and sensitive tools for various analytical measurements
e) Biosensors can be miniaturized and used in multi target analysis in both in-situ and ex-situ conditions.

One of the main goals in designing new biosensor systems is to achieve greater sensitivity, which can be achieved with incorporation of one or more amplification steps with in the assay configuration. Although, there will be a limit of how far this can go because of inherent characteristics of the biological component interfaced on it. Such amplification steps are not that necessary in electrochemical detection. Hence, the signal generated by biological component of biosensor can be amplified to a greater extent than normal optical methods.

Other major consideration is being given to miniaturization in various biosensors, especially for investigation of in-vivo (Starodub et al, 1999; Kukla et al, 1999; Gupta et al, 2013) or continuous system. Needle electrodes, coated wire electrodes are the best working examples of miniaturization with a diameter reduced to 1-2 mm. Further reduction can be achieved by using ion selective field effective transistors (ISFET) (Schöning, et al, 2002; Khanna et al, 2009; Linkhor et al, 2010; Vieira et al, 2012), screen printed electrodes (SPE), microelectrodes etc. These electrodes are inexpensive, simple, reusable, and durable for a long period of time, invariably give a better sensitivity and response characteristics to normal and traditional methods and electrodes. Microelectrodes are receiving a great deal of attention in recent years as they offer a possibility of monitoring trace level of analytically important analytes. Electrochemical biosensors offer a great deal of future as an analytical device in clinical, health care and environment monitoring tool due to their greater sensitivity, reproducibility, portability (Starodub et al, 1999) and reliability.

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2. Review of Literature

Design and technology of biosensors has been a developing area in the quest of innovative approaches to analysis in the last two to three decades. It has come up as a potential field for ultra-trace exploration of various analytically important analytes in samples of analytically important environmental sources like air, water, soil, etc. (Frew et al, 1987; Karube et al, 1995). Biosensors have revolutionized analytical methodology by providing very powerful and considerably less expensive alternative to various older, well established laboratory techniques. Electrochemical biosensors hold a leading position among the systems presently available for sensitive and selective ultra-trace determination of various analytes of interest. In past two decades biosensors have evolved by leaps and bounds. The first biosensor reported by Dr. Leland C Clark in 1956 as an oxygen probe (Clark, 1956, Palchetti et al, 2010) expanded its range of analytes that could be measured. In 1962, in a Symposium in the New York Academy of Sciences he described how to make electrochemical sensors (pH, polarographic, potentiometric or conductometric) more intelligent by adding "enzyme transducers as membrane enclosed sandwiches". In the same paper Lyon and Clark coined the term "enzyme electrode" by trapping glucose oxidase enzyme entrapped in dialysis membrane (Clark et al, 1962). Clark's idea of biosensor was soon commercialised by "Yellow Springs Instrument Company" (Ohio) in 1975, producing glucose analyser based on hydrogen peroxide determination amperometrically.

A new semi-continuous catheter-based blood glucose analyser has recently (Clemens *et al* 1976) been introduced by VIA Medical (San Diego). In the same year, La Roche (Switzerland) introduced the Lactate Analyser LA 640 in which hexacyanoferrate was used to transport electrons between lactate dehydrogenase and an electrode. This worked as a beginning to present day lactate analysers for sports and clinical applications. In all these commercial endeavours researchers also came forward for analysis and determination of various analytes as alcohol (Alferov et al, 2011; Schlangen et al, 2013), solid waste (Corbiser et al, 1996; Nistor et al, 1999), heavy metal ions (Malitesta et al, 2005; Ghica et al, 2013), pesticides (Kim et al, 2013; Carneiro et al, 2013; Meng et al, 2013) and for various environmental analysis (Treccani et al, 2013; Zhang et al, 2013) using biosensors.

In last two decades use of biosensors for various determinations of analytical importance can be found in literature. For convenience of understanding the literature can be divided into two major categories.

(a) Biosensors on the basis of biomaterial employed for its preparation, which is further classified broadly:
   i. Whole cell based biosensors
   ii. Enzyme based biosensors
   iii. Miscellaneous biosensors (cell components, DNA, antigens, etc.).

(b) Biosensors on the basis of transducer techniques used:
   i. Electrochemical biosensors (Potentiometric, Voltammetric, Conductometric, Miscellaneous)
   ii. Optical biosensors (Fluorimetric, Spectrophotometric)
   iii. Miscellaneous biosensors (Thermal, Mass).

2.1 WHOLE CELL BASED BIOSENSORS

Use of whole cell as biological recognition element has been extensively used as it provides a great deal of advantages (Wang et al, 2013) over other biological recognition elements as they provide proper environmental condition for other components like enzyme and cell components to work, no extra care has to be taken for storage, purification and use. They can be easily manipulated and adapted to consume and degrade new substrate under certain cultivating conditions (Leung et al, 2003). Additional progress in molecular biology and recombinant DNA technologies has opened endless possibilities in tailoring the microorganisms to improve their activity of an existing enzyme or foreign enzyme or protein in the host cell (Rensing et al,2003; Belkin, 2003).

Zeigler (2000), Lei et al (2005) and Su et al (2010) reviewed cell based biosensors and explained the set ups of various biosensor techniques for determination of various metabolic properties. Hansen et al (2001) reviewed use of whole cell bacterial biosensors for detection and quantification of various compounds and other conditions affecting genes expression of bacteria and categorized them in three different categories: non-specific, stress induced and specific biosensors, while D'Souza (2001); Castillo et al (2004); Mozaz et al (2004); Mozaz

et al (2006); Wanekaya et al (2008) reviewed their advantages, limitations, applications. Biosensors have also been projected as future techniques in environmental monitoring, bioremediation tools and real time monitoring tools. Marazuela et al (2002), Wolfbeis (2004) and Monk et al (2004) reviewed recent fiber-optic based biosensors on the basis of their recognition element, while explaining their characteristics and applications.

For sake of simplicity of classification whole cell based biosensors can be further classified on the basis of transducer employed for whole cell based biosensors and can be categorised into:

i. Electrochemical whole cell based biosensors
ii. Optical whole cell based biosensors
iii. Miscellaneous whole cell based biosensors.

2.1.1 Electrochemical whole cell based biosensors

Most biosensors use electrochemical detection as a transducer technique because of the low cost, ease of use, portability, and simplicity of construction (Eggins, 2002; Wang, 2006). Electrochemical detection being a surface technique, offer a great deal of advantages like; it does not depend upon reaction volume, even the small volume sample can be used for the measurement. Electrochemically monitored reaction typically generates a measurable current (amperometry), a measurable charge accumulation or potential (potentiometry) or alters the conductive properties of the medium between electrodes (conductometry) (Grieshaber et al, 2008).

So electrochemical biosensors can be categorised to:

a) Amperometric biosensors
b) Potentiometric biosensors
c) Conductometric biosensors
d) Miscellaneous biosensors

(a) Amperometric whole cell based biosensors

Amperometric whole cell based biosensors operate at fixed potential with respect to reference electrode and involves the detection of current generated by oxidation and

reduction processes taking place at the electrode surface (Lei et al, 2003;2004; Bartlett, 2008).

Ionescu et al (2006) studied the effect of immobilization of whole cell *Chlorella vulgaris* in alginate gel and pyrrole alginate matrix. Tag et al (2007) prepared a whole cell based biosensor using *Saccharomyces cerevisiae* for detection of Copper by using copper inducible CUP1 gene and transformed yeast strain having CUP1 gene transfused with lacZ gene from *E. coli*. Both species had their different inducible and detectable concentrations of Cu between 1.6-6.4 mgL^{-1}. An indirect amperometric system fitted with an oxygen electrode was used for detection. In 2008, Chong et al prepared a whole cell based environmental biosensor by immobilizing *Chlorella vulgaris* on the electrode surface and demonstrated unique properties of diamond biosensor to reduce problem of fouling of electrode. The detection limit of the proposed biosensor was 0.1 ppb for Zn^{2+} and Cd^{2+} ions. Donmez et al (2010) prepared a Pb (II) selective whole cell based biosensor with *Phormidium* sp. using various techniques like cyclic volammetry and differential pulse stripping voltammetry. They modified the Pb (II) binding carboxyl, sulphoxide and alcoholic groups in *Phormidium* sp. with formaldehyde, which led to enhanced Pb (II) binding. They had a detection limit of 2.5×10^{-8} M.

(b) Potentiometric whole cell based biosensors

Potentiometric whole cell based biosensors consist of an ion selective electrode (H^+ ions (Gaberlein et al, 2000), NH_4^+ ions (Verma et al, 2003), Cl^- ions (Han et al, 2002)) or gas sensing electrode (pCO_2, pNH_3) coated with microbial layer. Consumption of substrate from the analyte by microbial cells resulted in change in potential due to ion accumulation or depletion. Potentiometric transducer measures the difference in potential of working electrode and reference electrode and is related with concentration of analyte (Simonian et al, 1998).

Chee et al (2000) used *Pseudomonas putida* for preparation of an optical fibre biosesnors for evaluation of biological oxygen demand (BOD) of river waters. They calibrated the BOD sensor using artificial wastewater as standards with a response time of 15 minutes at 30° C and pH 7 by converting fluorescent signal to voltage, having no interference from various

heavy metal ions and high concentration of chloride ions. Liu et al (2007) showed the distinct advantage in using mammalian cells in understanding the physiological effect of various toxic analytes by preparing a light addressable potentiometric sensor (LAPS). They recorded the extracellular field potentials of spontaneously beating cardiomyocytes with LAPS (20-40 μV). Cardiomyocytes showed a clear change in beating frequency, amplitude and duration under the influence of various toxic metal (Hg, Pb, Cu, Zn, Cd, and Fe) ions in less than 15 minutes.

(c) Conductometric whole cell biosensors

Some microbes catalyse reactions causing change in total ionic concentration leading to change in conductivity of the solution. Even though the detection of solution conductance is non-specific, still it is extremely sensitive (Turner et al, 1992).

Chouteau et al (2004; 2005) prepared biosensors using *Chlorella vulgaris* for the detection of heavy metal ions and pesticides. Algae were immobilized using bovine serum albumin (BSA) cross linked in glutaraldehyde vapours. Alkaline phosphatase activity based biosensor showed the detection limit of 1ppb for Cd^{2+}, while bienzymatic biosensor showed a great deal of sensitivity to Zn^{2+}, Cd^{2+}, paraoxon-methyl on exposure of 30 minutes with a detection limit of 10 ppb. Tekaya et al (2013) prepared a conductometric biosensor for the determination of heavy metal ions like Cd^{2+} and Hg^{2+} using a cyanobacteria *Arthrospira platensis*, called spirulina by directly immobilizing biomaterial by physical adsorption on ceramic part of gold interdigited electrode. Michelis- Menton constant (K_m) was calculated as 0.75mM through calibration plot of conductance vs concentration with a detection limit of 10^{-20} M for both Cd^{2+} and Hg^{2+}.

(d) Miscellaneous whole cell based biosensors

Suzuki (2000) used microfabrication technique for the formation of lab on chip electrochemical cell using various sophisticated electrochemical techniques like amperometry, potentiometry, etc, which can be used for multi analyte determination and for in field determination of various heavy metal ions.

Gammoudi et al (2010) dealt with Love-wave bacteria based sensor platform for detection of heavy metal ions. Bacteria (*Escherichia coli*) were fixed on sensitive surface of sensor coated

with polyelectrolyte (PE) multilayer of poly (allylamine hydochloride) and poly (styrene sulfonate) using a simple and efficient layer by layer electrostatic self-assembly procedure. The real time characterization of PE multilayer and bacteria deposition is based on measurement of the resonance frequency perturbation due to mass loading during material deposition. Brett (2001) surveyed the strategies and applications of electrochemical sensors for environmental monitoring, their potential benefits and important designs.

2.1.2 Optical whole cell based biosensors

Variation in optical properties such as UV-Visible absorbance, bioluminescence, chemiluminescence, reflectance, and fluorescence brought by action of microbial biocatalyst with target analyte (Turner et al, 1992) forms the basis of optical whole cell based biosensors.

Vulkan et al (2000) used bioluminescent *Escherichia Coli* (HB101 pUCD607)and *Pseudomonas fluorescens* (10586r pUCD607) to find the soluble copper content in soil pore water samples and investigated threshold value of copper using biosensors for the first time. Preston et al (2001) showed the synergistic effect in toxicity of various metal ions like zinc, cadmium, cobalt and copper alone and in presence of each other on their luminosity. Horswell et al (2006) studied the effect of metal contaminants of sewage sludge on *Escherichia Coli* (HB101 pUCD607) and fungi (*Armilleria mellea*) dwelling on it. When Zn, Cu and Ni enriched sewage sludge was used to irrigate mature *Pinus radiata* forest. The bioluminescence of algae decreased as soluble Zn concentration increased in sludge, while fungi showed the decrease in presence of Cu. Presence of Ni in soluble or litter form doesn't cause any change or decrease in bioluminescence of the studied bacteria or fungi.

Riether et al (2001) fused metal inducible zntA and copA genes from *Escherichia Coli* were introduced with luxCDABE genes from *Vibrio fischeri* to get a metal induced change in luminescence. Biosensor required the incubation of 60-100 mins but showed a great deal of distinction between different metal ions, while Leth et al in same period genetically engineered *Alcaligenes eutrophus* with luxCDABE copper promotor bioluminescent gene from Vibrio fischeri and studied the bioluminescence as a function of concentration by immobilizing the reformed bacteria in polymeric matrix compatible with optical fibre and

studied it for sensitivity, selectivity, detection limit and storage conditions and achieved the detection limit of 1 μM in various broth media.

Biran et al (2003) prepared live cell array biosensor by immobilizing modified *Escherichia Coli* cells having lac Z reporter gene fused with heavy metal responsive promoter gene znt A on an optical imaging fibre containing large number of microwells, each microwell containing a bacterium. They used lac Z expression for measurement of mercury concentration and got a response of 100nM Hg^{2+} ions with an incubation period of 1 hr and discussed the flexibility and possibility of use of various reporter genes using fibre imaging technique. Nivens et al (2004) prepared a bioluminescent bioreceptor integrated circuit (BBIC) "laboratory on chip" by combining *Pseudomonas Fluorescens* containing bioluminescent reporter gene lux CDABE with optical application cation specific integrated circuits. Microenvironment created by the micro-organisms is capable of determining changes in various environmental pollutants. Bioreceptor was used to determine salicylate with a detection limit of $50\mu gl^{-1}$ with exposure time of 45 minutes, the same decreased to 20 minutes on increasing the concentration from $50\mu gl^{-1}$ to $1mgl^{-1}$.

Chu et al (2002) used nematode Caenorhabditis elegans to determine lethal toxicity of ten heavy metal ions and also the synergetic effect of those metal ions by using pairs and triple metal ion combinations. Chu et al (2005) used genetic mutation and transgenesis as a viable approach for identification of sensitive animals or organisms which were used as bioindicator for determination of various metal ions. Double mutant species showed 6, 3 and 2 times increase in sensitivity of above mentioned metal ions, respectively.

Horswell et al (2006) studied the effect of metal contaminants of sewage sludge on bacteria and fungi dwelling on it. When Zn, Cu and Ni enriched sewage sludge was used to irrigate mature Pinus radiata forest. The bioluminescence of *Escherichia Coli* (HB101 pUCD607) decreased as soluble Zn concentration increased in sludge, while fungi *Armilleria Mellea* showed the decrease in presence of Cu. Presence of Ni in soluble or litter form doesn't cause any change or decrease in bioluminescence of the studied bacteria or fungi. Park et al (2007) prepared a biosensor for cadmium by identifying a promoter gene for Cd through gene profiling of yeast *Hansenula Polymorpha*. Cadmium inducible gene drives the expression of

GFP on treatment with Cadmium and was used to monitor heavy metal contamination especially Cd in various water samples. Fu et al (2008) prepared mercury selective biosensors by introducing luciferase from bacterial origin as reporter gene into plasmid free *Pseudomonas Putida* X4 and *Enterobacter Aerogenes* NTG-01 as host strains. X4 and NTG1 had incubation time of 4 and 5 hrs, respectively with a detection limit of 100ppm at 28^{o}C and pH 7, having no interference from other metal ions like Cd^{2+}, Zn^{2+}, Co^{2+}, Cu^{2+}, and Pb^{2}.

Palomares et al (2009) studied the effects of various factors like pH, EDTA, anions like Cl^{-}, PO_4^{3-}, CO_3^{2-}, etc. on metal speciation toxicity of Zn, Hg, Cu, and Cd in *Anabeana* sp. modified with lux operon from *Photorabdus Luminescens*. They related the toxicity of various metal ions to their free concentration and also found that Zn-EDTA complex, complex of chloride with Hg are also toxic, while some increase in metal toxicity was found in presence of anions like PO_4^{3-}, CO_3^{2-}. Sarin et al (2009) studied the same using different resuscitation media, chloride ions on the bioluminescence of bacteria *Escherichia Coli* to Cd, Cu, Hg and Pb.

Babu et al (2012) prepared a bacterial biosensor for determination of toxicity caused by Zn^{2+}, Cd^{2+} and Hg^{2+} using zinc regulatory gene zntR and zntA promotor gene from znt operon of *E. Coli*. They also performed the studies in liquid media to quantify the GFP fluorescence using fluorimeter. At optimum culture incubation condition of 16 hrs, culture showed the maximum induction of 20, 0.005 and 0.002 ppm for Zn^{2+}, Cd^{2+} and Hg^{2+}, respectively.

Some researchers used the property of certain microbes to adapt and to absorb certain metal toxic more than their normal concentration as biomonitors.

Laughlin et al (2000) and Diels et al (2002) summarized various methods for analysis of soil extraction methodologies for heavy metal determination by their oxidation and reduction with various bioassays and also investigated new concepts and procedures for accessing the hazards due to metal contamination of soil, with their difference from bioavailable content of heavy metal ions. Diels et al (2002) put forward two approaches; first being adding soil additives to immobilize the metals and second was in situ precipitation of various metal sulphides.

Gutierrez et al (2003) discussed various experimental conditions and methodology for ciliates being used a biomarkers and biosensors for various environmental pollutants and also gave examples of various ciliates which can be used to determine bioavailable content of heavy metal ions in environmental samples. Gavrilescu (2004) discussed the significance and uses of unconventional method like use of biomaterials as a potential source of heavy metal removal by process called biosorption as a part of bioremediation and studied growth of various biomaterials in a bioreactor, Langmuir adsorption isotherm for adsorption and mass balance for measurement. Gonzalez et al (2006) reported cytotoxic effects of various metal ions like Zn, Cd and Cu using ciliates protozoa species due to bioaccumulation of Zn and Cd as a major reason of their toxicity, which was confirmed by TEM images of the cell having electron dense granules when exposed to sub lethal concentrations of these metal ions.

Choudhary et al (2009) used a Pseudomonas strain having phylogenetic similarity with Pseudomonas fluorescens extracted from uranium mine having high metal resistant property as bioremediation of metal ions. They found metal uptake of bacteria monophasic, fast saturating, concentration and pH dependent with uptake preference as $Cu^{2+} > Ni^{2+} > Co^{2+} > Cd^{2+}$ and preferential metal deposition was confirmed with techniques like TEM of cell fractions, FTIR spectroscopy and EDX analysis. Diels et al, 2009 prepared a metal sequestering system using highly resistant to environmental stressors Cupriavidus metallidurans having polysaccharides present on surface acting as metal binder and nucleation sites for crystallization of metal carbonates. They prepared inoculated sand filter bed to remove metal carbonates and other metal salts from the biomass. Heavy metal binding and floatation was combined in a biometal sludge reactor to seprate metal salts and heavy metals from metal contaminated soils.

Poli et al (2009) extracted six thermophilic extremophiles, Anoxybacillus amylolyticus, Geobacillus thermoleovorans, Geobacillus thermoleovorans subspecies stromboliensis, Geobacillus toebii subspecies decanicus, Bacillus thermantarcticus and Thermus oshimai and concentrated their study biosynthesis and activity of α-amylase producing-A. amylolyticus. When bacteria were grown in presence of heavy metal ions, decreased activity of α-amylase was related with decreased production of α-amylase was observed as function of metal ion concentration. Ravikumar et al, 2012, designed and applied molecular biosensors for zinc

and copper for use in bioremediation strategies. The selected zraP and cusC promoter from a genetic circuit and were fused to a dual-labeling reporter protein as an interactive component for zinc and copper, with a detection limit of 16 and 26 μM respectively. To extend the application of prepared biosensor they prepared a biosorption system to trigger bacteria to sense and absorb 13 ± 0.3 mg/L of zinc and 11.4 ± 0.42 mg/L of copper per gram of dry cell weight with induction concentration of 100mg/L of respective metal ions.

2.2 ENZYMATIC BIOSENSORS

The area of biosensors started with the development of enzyme electrodes for glucose detection prepared by Clark et al (1962). Enzyme system as biological recognition element in biosensor gives a great deal of selectivity with the substrate (Guilbault et al, 2004). Enzymes have been extensively used biocatalytic elements, enabling the determination of analytes in various formats like consumption, production of some species to be determined by transducers or their activation (Bontidean et al, 2000; Kamtekar et al, 1995; 1996) and inhibition (Amine et al, 2006; Sole et al, 2003). Castro et al (2003) gave an overview of various methods based on enzyme inhibition considering both biosensors and biosensing systems and concluded on the basis of various tables and examples that inhibition effect is not selective.

Enzymatic biosensor can be classified on the basis of transducer into electrochemical, optical and others

2.2.1 Electrochemical enzymatic biosensors

As discussed in whole cell based biosensors, the enzymatic assay based biosensors can also be categorized into four basic categories based on transducer employed:

a) Amperometric enzyme based biosensors
b) Potentiometric enzyme based biosensors
c) Conductometric enzyme based biosensors
d) Miscellaneous enzyme based biosensors.

(a) Amperometric enzyme based biosensors

Gogol et al (2000) synthesized an amperometric biosensor by immobilizing butyryl cholinesterase (Che) from horse serum onto a screen printed electrode coated with nafion layer, cross linked using glutaraldehyde vapours. Prepared biosensor showed better stability and sensitivity for organophosphate pesticides as compared with normal immobilized enzyme without nafion layer, with a detection limit of 3.5×10^{-7}M for trichlorfon and 1.5×10^{-7}M for coumaphos, when current was measured in a range of 200- 400 mV.

Svobodova et al (2002) studied the effects of immobilized glucose oxidase on the surface of dendrimer on gold support amperometrically and found that glucose oxidase immobilized on dendrimer by crosslinking with glutaraldehyde in vacuum had the stability of atleast eight weeks, response time of 1.3 min with detection limit of glucose was 25 μM and relatively lower michaelis menton constant as compared with glucose oxidase in solution. They tested the biosensor for detection of mercury on glucose oxidase activity and observed the detection of 100nM of it. They also studied the various kinetic parameters of enzymatic reactions, response time, sensitivity, detection limit, linear range and enzyme turnover number. Zeng et al, 2004 prepared a novel amperometric biosensor based on inhibition of glucose oxidase (GOx) for environmental chromium (VI) by crosslinking Gox with glutaraldehyde on electropolymerized aniline modified platinum electrode with ferrocene as electron mediator. The response of biosensor for glucose decreases with inhibition of chromium (VI) with a lower detection limit of $0.49 \mu gL^{-1}$ and linear response range divided in two parts one from 0.49-95.73 μgL^{-1} and other from $95.73 \mu gL^{-1}$ to 8.05 mgL^{-1} with minimal interference from various metal ions like lead (II), copper (II), cadmium (II), chromium (II), cobalt (II), nickel (II), tin (II) and showed interferences at trace levels from excess concentration of mercury (II) and silver (I).

Rodriguez et al in 2004 prepared two separate amperometric biosensors for heavy metals determination in polluted samples using urease, glutamate dehydrogenase modified screen printed three electrode configuration and by immobilizing urease on screen printed electrode with alginate gel. Urease hydrolysed urea to produce NH_4^+, which is being determined using NADH- glutamate dehydrogenase coupled reaction system. NADH consumption is monitored amperometrically and correlated with urease activity. Presence of heavy metal ions causes inhibition of urease, which lead to decrease in consumption of NADH and

decrease in current. Prepared biosensor showed a linear detection range of 10-100 μgL^{-1} for Hg (II) and Cu (II) with a detection limit of 7.2μgL^{-1} and 8.5μgL^{-1}, detection range of 1-30mgL^{-1} Cd (II) and Zn (II) with detection limit of 0.3 mgL^{-1} for Zn (II) and 0.2 mgL^{-1} for Cd (II), while alginate gel immobilized urease gave the detection limit of 2.9 and 29.8 mgL^{-1} for Hg (II) and Cu (II). Negatively charged nafion membrane increased the cation concentration in proximity of the electrode causing the increase in detection limit 66.3μgL^{-1} and 53.6μgL^{-1} for Hg (II) and Cu (II). Accuracy of toxicity were verified using atomic absorption spectrophotometer (AAS) and inductively coupled plasma atomic emission spectroscopy (ICP-MS).

Malitesta et al (2005) immobilized the enzyme glucose oxidase in electropolymerized poly-o-phenylenediamine and employed it for the detection of Hg^{2+} ions amperometrically. This inhibition with metal ions appeared reversible and in agreement with data for enzyme in solution. The proposed method showed a low response time, rapid recovery of response with EDTA. Due to high response obtained for Cu^{2+} they proposed this biosensor as potential for total toxic content determination. Bucur et al (2005) identified two different strains of acetylcholinesterase from *Drosophila Melanogaster* named wild type Dm (wt-Dm) and E69W mutant to overcome the lower detection limit and selectivity of acetylcholinesterase system for detection of neurotoxic insectisides. Enzymes were immobilized on screen printed electrode by entrapping in photocrosslinkable PVA-SbQ polymer used with amperometric FIA system with three channels. In the same year Lapenaite et al prurified PQQ-dependent glycerol dehydrogenase from Gluconobacter sp. 33 and checked its activity and stability at various pH and temperature for determination of glycerol and triglycerides in various samples and concluded that immobilization of the enzyme by crosslinking with glutaraldehyde decreases the activity of glycerol dehydrogenase for glucose, sorbitol, mannitol and ethanol but increases the activity for methanol and dulcitol and investigated the inactivation of glycerol dehydrogenase with heavy metal ions like Pb^{2+}, Cd^{2+}, Co^{2+}, Ni^{2+}, Cu^{2+} and Hg^{2+}.

Ghica et al (2008) prepared an amperometric biosensor for determination of heavy metal ions based on enzymatic inhibition of glucose oxidase by immobilizing glucose oxidase by crosslinking with glutaraldehyde on electropolymerized poly (neutral red) having a detection

limit of 1μgL^{-1} for cadmium, 6μgL^{-1} copper, 3μgL^{-1} for lead and 9μgL^{-1} for zinc. Various plots and analysis showed competitive for cadmium, mixed for copper and lead, while non-competitive for zinc having an order of selectivity as $Cu^{2+} > Cd^{2+} > Pb^{2+} > Zn^{2+}$. Prepared biosensor can be regenerated using EDTA. In same period Guascito et al also prepared an amperometric biosensor for determination of metal ions based on inhibition of glucose oxidase immobilized on electropolymerized poly-o-phenylenediamine with platinum surface and was tested for various metal ions and CrO_4^{2-} for metal toxicity having being able to distinguish between Cr^{6+} and Cr^{3+} as it gets inhibited with CrO_4^{2-} only. Authors replaced glucose with hydrogen peroxide to study the effect of metal toxicity on decomposition of hydrogen peroxide.

(b) Potentiometric enzyme-based biosensors

Sodatkin et al (2000) modified the surface of an ion selective field effective transistor by immobilizing urease enzyme on its surface and covering it with negatively charged polymer layers to increase the sensitivity or inhibition effect due to accumulation of cations or heavy metal ions in the polymer matrix. They used the enzymatic biosensor for trace heavy metal ion analysis in different water samples with the detection limit of 1μM, while Krawczyk et al in the same year studied the inhibition effect of various salts of mercury like $HgCl_2$, PhHgCl and $Hg_2(NO_3)_2$ and some other heavy metal ions as potential interferents on urease enzyme entrapped in PVC matrix, based on measurement of initial rate of decrease of biosensor potential after addition of substrate after inhibition effect. Biosensor prepared is not selective for determination of any particular metal ion, so was used to determine total metal toxicity and was regenerated using Tris buffer solution with EDTA and thioacetamide for less than 10 minutes.

Hamlaoui et al (2002) prepared urea biosensor by covalent bonding urease directly on the surface of ammonium sensitive field effective transistor (zeolite incorporated polymeric membrane). ENFET showed a sensitivity of 15mV/purea as compared to nernstian response of 32mV/pNH_4^+ and stability of 15 days with detection limit of 3×10^{-5}M and tested the feasibility of prepared biosensor for environmental applications by exposing it to enzyme inhibiting heavy metal ions like Hg (II) and got the detection of less than 5×10^{-8}M for Hg (II).

Arkhypova et al (2001) proposed an original concept of enzyme mutibiosensor for determination of toxic substances based on enzyme inhibition analysis by using two types of transducers such as potentiometric pH sensitive field effective transistors and conductometric thin films interdigited electrodes having three enzymes namely urease, acetylcholinesterase and butyrylcholinesterase. They proposed a procedure for simultaneous determination of some heavy metal ions and pesticides with advantages using multivariate correspondence analysis treated experimental data. Renault in the same year presented state of the art of various biosensors for environmental control using inhibition of acyl cholinesterases by organophosphorus compounds or inhibition of enzyme phosphatases or direct determination of organophosphorus compounds by organophosphorus hydrolases. He emphasized on development of ENFET (enzyme field effective transistor) biosensors. Dzyzdevych et al (2003) reviewed their own publications based on ENFET prepared by immobilizing enzyme on the surface of ion selective field effect transistor (ISFET), used for determination of various substrates like glucose, urea, formaldehyde, creatinine are using their own prototype and allowed them to decrease the effect of buffer capacity and increase the sensitivity and used them for determination of various analytes like organophosphorus pesticides, heavy metal ions, glycoalkaloids. Dzyadevych et al (2006) discussed the theory of ion selective field-effect transistor (ISFET) in connection with their application in bioanalytical practice by concentrating on some specific modern microtechnologies for their creation, measurement schemes with set-ups.

Ogonczyk et al (2005) and Tymecki et al (2005) prepared a small sized, low cost potentiometric pH-urease electrode by screen printing a thick film of biocompoiste screen printable material composed of ruthenium oxide, urease, graphite and organic polymer. Electric circuits and electrode insulation were prepared using same thick film technology with inexpensive commercially available pastes. Mild conditions of paste are not dangerous for immobilized enzymes and used the developed disposable biosensor for determination of urease inhibitors. Ogonczyk et al found the detection in ppm level for silver and copper. Konkci et al (2006) developed a flow injection system for determination of alkaline phosphatase activity and adapted for determination of selected inhibitors of enzyme. Monofluorophosphate was used as substrate and fluoride ion generated is determined using

fluoride ion selective electrode. Fluoride ion generated defines baseline signal generated by the sensor. Various potential inhibitors were studied for interferences and allowed the determination of beryllium and vandate ions in ppb level highly selectively in less time. Koncki in next year (2007) reviewed the recent trends in potentiometric biosensors using biocatalytic and biosensing schemes as well as their uses as biomedical analysis was discussed in detail.

(c) Conductometric enzyme-based biosensors

Lee et al (2002) used sol-gel method to immobilize urease on screen printed IDA electrode using tetramethyl orthosilicate as sol-gel precursor to prepare a conductometric biosensor, responded linearly for urea concentration 0.05-10 mM in 5.0 mM imidazole-HCl buffer with a response time of 15 minutes. They also studied the inhibition effect of various metal ions like Hg^{2+}, Cu^{2+}, Cd^{2+}, Pb^{2+} at different incubation times. Suwansa-ard et al (2005) prepared a flow injection biosensor for determination of carbamate pesticides by immobilizing cholinesterase enzyme by covalent bonding in silica gel and studied the inhibition of enzyme with pH and conductivity electrodes. Prepared biosensor was put to test for determination of carbofuran and carbaryl pesticides.

Berezhetskyy et al (2008) prepared alkaline phosphatase conductometric biosensor by immobilizing alkaline phosphatase on interdigited gold electrode surface for assessment of various heavy metal ions in water samples in presence of tris nitrate as buffer and Mg^{2+} as enzyme activator. Various metal ions showed toxicity in the order $Cd^{2+} > Co^{2+} > Zn^{2+} > Ni^{2+} > Pb^{2+}$, with a detection limits of 0.5 ppm for Cd^{2+}, 2 ppm for Co^{2+} and Zn^{2+}, 5 ppm for Ni^{2+} and 40 ppm for Pb^{2+} ions. Soldatkin et al, 2012 prepared an conductometric biosensor by immobilizing three enzymes invertase, mutarotase, and glucose oxidase on the surface of planner thin film interdigited electrode deposited on ceramic pad. The developed biosensor showed the best sensitivity to Hg^{2+} and Ag^{+} ions and can be regenerated using EDTA or cysteine for inhibition caused by silver and mercury respectively.

(d) Miscellaneous electrochemical enzyme-based biosensors

Du et al (2007) prepared a new nanocomposite by functionalizing single walled nanotubes (SWNT) with poly (nile blue A), which in turn electroploymerized from Nb monomer

through cyclic voltammetry. Prepared nanocomposite had been characterized by various techniques like cyclic voltammetry, electrochecmical impedence spectroscopy, UV-visible spectroscopy and scanning electron microscopy. Alcohol dehydrogenase and nanocomposite were immobilized on glassy carbon electrode surface as biosensor, as it showed a great deal of electrocatalytic activity studied cyclic voltammetrically towards ethanol with good selectivity, sensitivity and biological affinity having Km value of 6.30 mM in a linear range of 0.1-3.0 mM and a lower detection limit of 50 μM. Kestwal et al (2008) also reported an electrochemical sucrose biosensor by immobilizing invertase and glucose oxidase on ultra-micro electrode in agarose-guar gum. Atomic force microscopy confirmed proper encapsulation of both enzymes during co-immobilization. Spectroscopic and electrochemical studied showed linear relation between enzymatic inhibition and metal concentration. The toxicity order as tested was $Hg^{2+} > Pb^{2+} > Ag^{+} > Cd^{2+}$, with a linear range of 5×10^{-10} to 12.5×10^{-10} M for mercury and detection limit of 5×10^{-10}M.

2.2.2 Optical enzyme-based biosensors

Sanchez et al (2003) prepared a biosensor by encapsulating alkaline phosphatase enzyme in sol-gel matrices derived from tetramethyl orthosilicate. Alkaline phosphatse hydrolysed 1-napthyl phosphate to fluorescent 1-napthol. The prepared biosensor was investigated in presence of pesticides like metham-sodium and tetradifon. In the very same year Tsai et al encapsulated urease with a fluorescent dye, FITC dextran by sol-gel immobilization and optimized various parameters like optical properties, relative enzyme activity, pH value and buffer concentration. They studied two analytical procedures incubated and un-incubated for comparison and understanding sensitivity and applicability to heavy metal analysis with a great deal of sensitivity and reproducibility having a standard deviation for Cu (II) and Cd (II), an analytical range of 10-230 μM and detection limit of 10 μM.

Kuswandi (2003) immobilized urease on an ultrabind membrane in optical fibre configuration for a biosensor. pH changes and spectroscopic changes were monitored before and after the exposure to various heavy metal ions using commercial pH indicator strip and wavelength of 615 nm, respectively with a range of 1×10^{-9}M to 1×10^{-5}M and a detection limit of 1×10^{-9}M for Hg (II) by flow method in preoptimized conditions. Exposed urease

was regenerated by L-cysteine. Krajewska et al (2004) performed multi-step analysis of inhibition of urease by Hg^{2+} including residual activity measurement after incubation of enzyme with metal ion, reactivation of Hg^{2+} inhibited urease, protection of urease with thiol agents prior to incubation with Hg^{2+}.

Nabok et al (2004) and Haron et al (2006) prepared a novel enzyme optical sensors by combining SiO_2/ Si_3N_4 /SiO_2 planar waveguide ATR (attenuated total reflection) transducer with composite polyelectrolyte self-assembled (PESA) coating containing both chromophores (cyclotetrachromotropylene) and enzyme (Urease and cholinesterase) molecules. Haron et al (2004) studied the inhibition in presence of cadmium and lead. Singh et al (2008) summarized the studies carried on development of biosensors for analysis of urea in different fields of application, immobilization techniques, stability and response time characteristics, transducer used like pH electrodes, ammonia gas sensing electrodes, ammonium ion sensing electorodes, optical, conductometric and amperometric transducers.

2.3 MISCELLANEOUS BIOSENSORS

Ramanathan et al (2001) reviewed principles, instrumentation, materials and methods of thermistor-based calorimetric measurement and its applications related to enzyme activity measurements, clinical monitoring, process monitoring, multianalyte determination, environmental monitoring, non-aqueous measurements.

May et al (2003) prepared a biosensor for determination of cadmium by taking into account the property of urease to get inhibited in presence of heavy metal ions. The changes in enzyme structure due to change in conformation of nickel site on binding with cadmium ions is monitored using surface plasma resonance (SPR). Enzyme modified with N-succinimidyl 3-(2-pyridithiol) propionate was spread as self-assembled monolayer on gold coated glass SPR sensor disk. The prepared biosensor had the detection range of 0-10 mg/L for cadmium.

Beside whole cell and enzymes, various other biological components like DNA, RNA, cell extracts, antibodies and proteins are being used as biological recognition element. All these recognition elements are covered here.

2.3.1 Electrochemical miscellaneous biosensors

Chow et al (2006) reviewed the use of peptides and amino acids as recognition elements of electrochemical sensors for heavy metal ions. They discussed complexation of metal ion with peptides and followed with their immobilization on the electrode surfaces. Subsequently, Application of peptide modified electrodes for detection of heavy metal ions, their challenges and future prospectus.

As discussed earlier, electrochemical miscellaneous biosensors can also be divided into four categories: amperometric, potentiometric, conductometric and other electrochemical miscellaneous biosensors.

(a) Amperometric biosensors

Babkina et al (2004) elaborated an amperometric biosensor based on stationary mercury film with silver support and cellulose membrane immobilized with single stranded DNA and calculated sorption and DNA-metal binding constants using biosensor. Babkina et al, in same year prepared another amperometric biosensor for heavy metal ions by immobilizing single stranded DNA on nitrocellulose membrane. Metal ssDNA binding constants showed the order of determination of various metal ions as Cu (II) > Pb (II) > Fe (III) > Cd (II). The proposed biosensor was based on preconcentration of heavy metal ion on the biosensor, then destruction of DNA-metal linkage with ethylenediaminetetraacetate and voltammograms were recorded.

(b) Conductometric biosensors

Adam et al (2005) prepared an electrochemical biosensor for heavy metal determination using adsorptive transfer stripping differential pulse voltammetery on phytochelatin adsorbed hanging mercury electrode. The proposed biosensor was used for determination of cadmium and zinc in human urine and platinum in pharmaceutical drugs. Oliveira et al (2009) studied in-situ interaction of metal ions like Pb^{2+}, Cd^{2+} and Ni^{2+} with double stranded DNA using differential pulse voltammetry, confirming that Pb^{2+}, Cd^{2+} and Ni^{2+} binds with dsDNA causing various conformational changes in dsDNA and electrochemically recognized these changes by changes in oxidative peaks of guanosine and adenosine bases. Pb^{2+} causes the oxidative damage to adenine forming 2,8-dihydroxyadenine. Cd^{2+} and Ni^{2+} caused the

conformational changes to dsDNA destabilizing the double helix enabling action of other oxidative agents on DNA.

2.3.2 Optical biosensors

Lu et al (2003) selected a catalytic DNA for selective determination of metal ions by an in-vitro selection process, discarding catalytic DNA having selectivity for interfering metal ions by negative selection. Sumner et al (2006) prepared a biosensor using a wild red fluorescent protein (DsRed) for determination of Cu^{+} and Cu^{2+} specifically and selectively even in the presence of other heavy metal ion.

Zuo et al (2009) immobilized DNA substrated (Cu-sub and Pb-sub) corresponding to metal dependent DNAzymes for copper (Cu- Enz) and lead (Pb- Enz) on aldehyde coated slides. On metal introduction cleavage of substrate on the action of DNAzyme gave dramatic change in fluorescent signal intensity. In the same year Koneswaran et al prepared l-Cysteine capped ZnS quantum dots for detection of Cu^{2+} by optical approach using functionalized ZnS QDs as fluorescence sensor for Cu (II) at an optimum pH of 5.0 at 2.5mgL^{-1} concentration of nanoparticles with a detection limit of 7.1×10^{-6}M, having no interference from other metal ions except Ag^{+} and Fe^{3+}. When compared with organic fluorophores, l-cysteine-capped ZnS QDs are brighter and more stable against photobleaching. While, in the same year Pazos et al presented several of the strategies that have been used for development of fluorescent-based peptide sensors like green fluorescent proteins, nanoparticle based peptides.

Wu et al (2010) developed sensitive and selective nanosensor based on DNAzyme modified quantum dots for detection of heavy metal ions. QDs coated with silica for increased stability and quantum yield. QD-DNAzyme biosensor was prepared by conjugating quencher-labelled DNAzyme on the surface of carboxyl-silanized QDs. In presence of metal ions emission is restored due to cleavage of DNAzyme, with a detection limit of 0.2 and 0.5nM for Pb^{2+} and Cu^{2+} respectively in a time of approximately 25 minutes with a single laser excitation. The proposed sensor is 50-70 folds better than the recently reported dye molecule based biosensors. Multiplexed detection was also demonstrated using two different colored QDs showing no interferences between Pb^{2+} and Cu^{2+}.

2.4 OTHERS

Bontidean et al (2000) prepared capacitance biosensor using metal regulatory and metal resistance proteins from cyanobacterium S*ynechococcus* for non-specific metal binding of Hg (II), Cu (II), Zn (II) and Cd (II) and . Metals can be detected at both low and high concentrations and the shape of the capacitance curves may reflect biologically relevant responses of the proteins to metals with a detection limit of 10^{-15} M. Bontidean et al (2003) prepared another capacitive biosensor using synthetic phytochelatin expressed in Escherichia coli by fusing to maltose binding protein domain and was purified. The new biosensor was able to detect various heavy metal ions in the sensitivity order Zn^{2+}> Cu^{2+}> Hg^{2+}> Cd^{2+}≥ Pb^{2+} with a detection range of 100fM to 10mM. Biological sensing element can be regenerated by using EDTA and can be stored for 15 days. Berggren et al (2001) reviewed various capacitive biosensor using dielectric properties of an electrode surface due to binding of analyte to immobilize affinity element without the need for a label or indicator reaction. The changes in electrical capacitance or impedence are measured either by using interdigited electrode or by using various potentiometric methods. Capacitive biosensors has been used since 80's for detection of antigens, antibodies, proteins, DNA fragments and heavy metal ions, giving a detection limit of 10^{-15}M with self-assembled recognition layers.

Cherian et al (2003) used microcantilever functionalized with metal binding protein AgNt84-6 as sensor for metal ions like Hg^{2+} and Zn^{2+}. AgNt84-6 responded for various metal ions like Ni^{2+}, Cd^{2+}, Cu^{2+}, Co^{2+}, Zn^{2+} and Hg^{2+}, which was attached to silicon nitride cantilever. On exposure to 0.1mM $HgCl_2$ the cantilever showed bending corresponding to expanding gold side, while no such change was observed when exposed to 0.1mM $MnCl_2$. Data was consistent and verified by electrophoresis carried out by SDS-PAGE gels. Saber et al in same year investigated complexation of metallothionins (MT) with zinc and cadmium ions by using an oligopepetide a part of MT immobilized on piezeoelectric crystals after treating with ethylenediamine plasma in glow discharge apparatus and then chemically reacted with glutaraldehyde. Complexation of MT with heavy metal ions like Zn^{2+} and Cd^{2+} causes the change in frequency of piezeoelectric crystals. The prepared sensor performed best at pH 7.4, increasing the pH causes the decrease in interaction of metal ions with immobilized MT. Zinc showed greater sensitivity than cadmium from solution containing single metal ions.

Exchange of ions and competition provoked adsorption of both the ion due to synergistic and antagonistic effects.

Wu et al (2004) immobilized metallothionin (MT) in carboxymethylated dextran matrix at an optimum pH of 4 with working temperature of 30°C for the detection of heavy metal ions by surface plasma resonance (SPR). The sensor chip binds with cadmium, zinc or nickel but not with magnesium, manganese or calcium and was able to differentiate clearly between cadmium and zinc or nickel. The binding affinity between metal and MT chip followed the order Cd > Zn > Ni with detection in micromolar range. Rica et al (2011) discussed use of functional peptide nanotubes, self-assembled from short peptides with recognition elements as building blocks to develop sensors. These peptide nanotubes form a bridge between microworld and nanoworld due to their length in micro range and diameter in nanometer range, which in-cooperate recognition element in them. By applying alternating current probes to detect impedence signal from peptide nanotubes makes them an excellent transducer material for detection of various pathogens, heavy metal ions with detection limit quite low and dynamic range can be improved by improving the device design.

Ueda et al (2003) reviewed immobilized metal ion affinity chromatograpy (IMAC), from its early usage as reliable purification procedure to its rapid expansion in number of preparative and analytical applications like protein refolding, evaluation of protein refolding status, protein surface topography studies and biosensor development. Xiang et al (2013) gave general methodology for metal ion detection using a low cost, simple and widely accessible personal glucose meter through invasive DNA approach i.e cleavage of DNAzyme generating a signal being quantified as concentration of heavy metal ion concentration.

Pijanowska et al (2005) reviewed their own work addressing the advancement in the field of biosensors as bioanalytical diagnostic devices using semiconductor technologies (ISFET) to reduce the cost, miniaturization as well as increase in sensitivity, selectivity, the detection limit and number of analytes to be determined. Verma et al (2005) summarized the use of biosensors for detecting and quantifying heavy metal ions in their mini review by categorizing the biosensors in two categories protein based which includes enzymes, metal-binding protein and antibody and whole cell based including both natural and genetically

engineered microorganism and also gave their advantages to various conventional techniques. In the same year Kroger et al gave an overview of measurement strategies currently employed, relevant available technologies, instrumentation required for marine sensors for various applications like eutrophication, organism detection, food safety, pollutants, trace metal ion and ecotoxicology. While, Kostal et al discussed DNA recombinant technology to synthesize pre-programmed nanoscale biopolymers, artificial protein polymers within a synthetic gene template and to use that protein based nano-biomaterials with both metal binding and tunable properties for heavy metal removal. Davis et al also during the same period discussed the introduction of thin films formed by Langmuir-Blodgett (LB) or self-assembly techniques in the field of biosensors with their applications. Daniels et al (2007) summarized the accomplishments of the past in impedance biosensors in which unlabelled DNA or protein targets can be detected by monitoring the surface impedance when a target molecule binds the immobilized probe.

Belluzo et al (2008) focused on designing electron-transfer based biosensors for clinical analysis, with emphasis on strategies currently used to improve the device performance, the present status of amperometric electrodes for biomedicine, trends and challenges envisaged for the near future as these provide a potential of low cost portable instrumentation. In the same year Palchetti et al discussed the use of nucleic acid based biosensors for detection of environmental pollution and toxicity. Where, nucleic acids can act as genosensors as they detect DNA/RNA components and they can also act specific receptor for biological or chemical species. Shao et al (2010); Kuila et al (2011) discussed excellent physiochemical properties of graphene and reviewed recent advances in graphene based electrochemical sensors and biosensors. In particular, direct electrochemistry of enzyme, its electrocatalytic activity towards small biomolecules and graphene based enzyme biosensors, graphene based DNA sensing and environmental analysis.

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3. Materials and Methods

3.1 CHEMICALS

Pikovskaya media, Beneck's media procured from HiMedia (India), ammonium molybdate, tin chloride from Loba Chemie (India) and hydrochloric acid from Merck (India) were used for identification of phosphate solubilizing microbes. Chemicals like sodium nitrate, dipotassium hydrogen phosphate, magnesium sulfate, calcium chloride, sodium carbonate, manganese chloride, zinc sulfate, sodium molybdate, copper sulfate, cobalt nitrate were procured from Sigma-Aldrich (India) , boric acid, ferric ammonium citrate, citric acid were purchased from HiMedia (India) and ethylenediamine tetra acetic acid (disodium salt) procured from Loba Chemie (India) were used for preparation of BG-11 media, while tris-hydrochloride from Sigma-Aldrich (India) was used for washing of the extracted biomaterial. Bovine serum albumin (BSA) and glutaraldehyde were procured from Sigma-Aldrich and were used for immobilization of biomaterial to the electrode surface. Ferrocene from Loba Chemie was used for checking the permeability of the immobilized electrode membrane.

Chronoamperometric study was carried out using p-nitrophenyl phosphate (p-NPP) (20mg tablet) as substrate, magnesium chloride as enzyme activator and Tris-HCl as supporting electrolyte. These chemicals of highest purity were procured from Sigma-Aldrich. pH of the solution was maintained with nitric acid and ammonia solution of AR grade purchased from from Merck (India)

Nitrate salts of metals zinc, cadmium, cobalt, nickel, copper, mercury, silver, sodium, calcium, magnesium were received from Sigma-Aldrich and were used as received for heavy metal testing.

3.2 INSTRUMENTATION

All chronoamperometric and cyclic voltammetric studies were recorded with a three-electrode system, immersed in a solution of substrate, activator and supporting electrolyte using Autolab Potentiostat/ Galvanostat model PGSTAT12 (Eco Chemie, Netherlands). Glassy carbon (GC) electrode (CH Instruments, USA, 3mm diameter) and Platinum (Pt) electrode (Metrohm, Netherlands, 2mm diameter) were used as working electrode. Platinum electrode (2mm diameter, CH Instruments, USA) and Ag/ AgCl (CH Instruments, USA and

Metrohm, Netherlands) were used as a counter electrode and reference electrode, respectively.

Scanning electron micrographs were recorded using JOEL scanning electron micrograph model JSM-6610LV, fluorescence was measured using a Perkin- Elmer LS45 Spectrofluorimeter, while spectrophotometric studies were carried out on Analytica Jena 205 spectrophotometer.

3.3 IDENTIFICATION OF MICROBES

Two methods were followed for identification of microbes having phosphatase solubilizing activity, which has been used further for preparation of biosensor as phosphate solubilizing microbe will cause the dephosphorylation of the phosphate substrate, generating an electrochemically active species, used for determination by various electrochemical methods.

i. We took the soil samples from beneath the trees were taken. 1g of sample soil was taken in 10 mL of autoclaved water as a stock and was further diluted 7-8 times by taking 1 mL and making it up to 10 mL using autoclaved water. Used 3^{rd}, 4^{th} and 5^{th} dilution for spreading on Pikovskaya media plates [prepared using Pikovskaya media (Pikovskaya, 1948)] and put them for incubation for three days at a temperature of 28°C. The developed phosphatase solubilizing strains of microbes were identified by the clear zones around the colonies formed and were transferred to Pikovskaya media broth for proper growth for 4 days at a temperature of 28°C with proper shaking using a shaker.
ii. Poured Beneck's liquid media (Aneja, 2003) in half-pint bottles upto 5 cm depth, plugged them with cotton and autoclaved for 20 minutes at 20 lb/in^2. On cooling added 1g of soil sample in each bottle and incubated at 30-35°C in an illuminated growth chamber for 15-20 days. Subcultured the microbial colonies by aseptically transferring a few cells or filaments by means of a platinum wire or nichrome loop on Beneck's agar media in petri plates.
iii. The identified and isolated colonies were further used for quantitative estimation of the phosphatase activity using 200 µL of microbial filtrate growing under mentioned conditions in 50 mL volumetric flask. Added 10 mL of chloromolybdic acid to the flask and diluted the contents to 40 mL. Followed by the addition of 1 mL Chlorostannous acid

and solution was made up to 50 mL. The developed blue colour was measured spectrophotometrically at 690 nm.

iv. Chloromolybdic acid solution was prepared by dissolving 15.0 g of ammonium molybdate in about 400 mL of warm distilled water followed by addition of 400 mL of 10N HCl. The solution was stirred, cooled and volume made up to 1litre with distilled water. Chlorostannous acid was dissolved in 10.0g of $SnCl_2.2H_2O$ crystals dissolved in 25 mL of concentrated HCl and kept in an airtight bottle as stock solution. The solution of chlorostannous acid for use was freshly prepared by taking 1 mL of the stock solution in 132 mL of distilled water.

3.4 CULTURE GROWTH AND EXTRACTION

Algae having maximum optical density was identified and used for culturing and sub culturing using BG-11 inorganic media (Stanier et al, 1971) prepared by the mixing the standard optimum concentration of its salt ingredients in distilled water. Added 1 mL of extracted identified algae in a 250 mL volumetric flask having 100 mL of autoclaved BG-11 media and kept it in growth room for three weeks at 30-35°C. After the optimum growth the culture was centrifuged at 10000 rpm and 4°C and washed the suspension with 0.1 M Tris-HCl before sub-culturing it using the same procedure.

Before being used for immobilization on the electrode surface and to induce maximum enzymatic activity, the optimally grown culture was extracted using the same procedure of extraction and starved by suspending the extracted pellet in phosphate free 100 mL BG-11 media in 250 mL volumetric flask for a week.

BG-11 media was prepared using standard protocol (Stanier et al, 1971) with optimum concentrations of $NaNO_3$, K_2HPO_4, $MgSO_4.7H_2O$, $CaCl_2.2H_2O$, ferric ammonium citrate, Citric acid, EDTA disodium salt, $NaCO_3$, H_3BO_3, $MnCl_2.4H_2O$, $ZnSO_4.7H_2O$, $Na_2MoO_4.2H_2O$, $CuSO_4.5H_2O$, $Co(NO_3)_2.6H_2O$ in one litre autoclaved distilled water.

3.5 IMMOBILIZATION OF THE MICROBES

Extracted microbes were immobilized on the surface of electrode (Platinum, Glassy Carbon) using bovine serum albumin and cross-linked with glutaraldehyde (Babu et al, 1991). The 1000µL of extracted biomaterial was mixed with 7.5% (w/v) of bovine serum albumin

(BSA). 30 µL of prepared mixture was cast on the electrode surface by drop coating method and was kept in saturated glutaraldehyde environment for 20 minutes, followed by drying in room temperature for 30 minutes. The glutaraldehyde caused the cross linking of BSA protein and the biomaterial get entrapped in the matrix. The glutaraldehyde would react with amines of BSA and would not affect the microbial proteins.

3.6 CHARACTERIZATION OF MICROBE MODIFIED ELECTRODE

The modified electrode was characterized for optimum composition for immobilization and response using various techniques like fluorescence, scanning electron micrograph (SEM), cyclic voltammetry and Chronoamperometry.

Membrane composition was tested for proper immobilization and entrapment using SEM, the cast membrane of the electrode was peeled off and was used for taking the micrograph.

3.6.1 Membrane permeability

The membrane was tested for permeability of the electrons by cyclic voltammetry. Cyclic voltammetry is an important technique to study electrochemical behaviour of system undergoing oxidation or reduction by exchange of electrons on the surface of the electrode. The cyclic voltammograms of 0.5 mM ferrocene solution in 0.1M Tris-HCl between the potential -0.2V to 0.8 V were run with blank. The algae modified electrodes, platinum and Ag/ AgCl were used as working, counter and reference electrodes respectively, using Autolab/ PGSTAT12/ Eco-Chemie/ Netherlands.

3.6.2 Biomaterial viability

In viable algae cells, if excitation wavelength lies close to Soret band (maximum absorption), then the florescence emission peak lies near to photosystem-II emission peak of algae (Hipkins et al, 1986). Viable algae cells immobilized on the coverslip were excited with a beam of wavelength 540 nm (Erokhina et al, 2004) using Perkin-Elmer LS45 spectrofluoremeter.

3.6.3 Chronoamperometric scan

Chronoamperometry involves biasing the potential of the working electrode from a value where no oxidation or reduction of the electrochemically active species takes place to a

potential where the current flows due to electrode reaction on the surface of the working electrode. The resulting current with respect to time is monitored.

p-nitrophenyl phosphate (p-NPP) used as substrate is acted upon by alkaline phosphatase in the membrane of algal cell and causes the dephosphorylation of the substrate to electroactive p-nitrophenol (p-NP). At a bias potential of 0.85 V (Hart et al, 2007) p-NP gets oxidised, releasing two electrons measured chronoamperometrically (Fig 1)

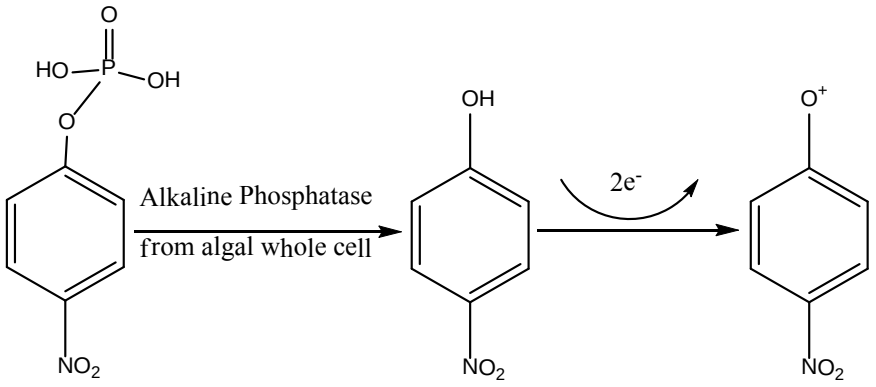

Fig 3.1: Detection principle of algal biosensor

Chronoamperometric study was performed by immersing the algae modified electrode as working electrode in 1mL of 20mM p-NPP as substrate, 1mL of 1mM $MgCl_2$ as enzyme activator in a total solution made up to 10 mL using 0.1M Tris-HCl as supporting electrolyte and buffer at a bias potential of 0.85V vs Ag/AgCl reference electrode and platinum electrode as counter electrode for 10-12 minutes.

The prepared biosensor was tested for optimum composition and response by changing the amount of biomaterial in the membrane, changing the pH of the solution.

3.6.4 Identification of transducer material

Identification of proper transducer material was made by immobilizing the biomaterial, using the above discussed method on the surface of various materials like glassy carbon, platinum and copper. The chronoamperometric scans were performed on modified electrodes of different composition and checked their response with respect to current generated by the system to see the maximum responding transducer material.

3.6.5 Variation of cell density

Number of cells getting entrapped in the membrane matrix was regulated by using various dilutions of the stock extracted and used for immobilization. The resulted biosensor was tested using the same chronoamperometric study for the optimum response.

3.6.6 Variation of pH

The pH of the experimental solution used for chronoamperometric study was varied using 0.1 M HNO_3 and ammonia solution of reagent grade before the start of the experiment, to study the effect of solution pH on the enzymatic activity of the immobilized biomaterial.

3.7 HEAVY METAL TESTING

Even the trace amounts of heavy metal ions inhibit the enzymatic activity (Lakshmi et al, 1991), as enzyme has greater tendency to complex with heavy metal ions to form stable metal-enzyme complex, resulting in decreased enzymatic activity. Lesser the activity lesser will be the production of electrochemically active species, lesser will be the oxidation current. Hence, the decrease in current due to decreased enzymatic activity can be co-related with the heavy metal ion concentration.

Having optimized the conditions of immobilization, the cast electrode was used for the determination of different heavy metal ions. The cast electrode was immersed in an aqueous solution containing 1 mL of metal ions (zinc, cadmium, cobalt, nickel, copper, mercury, silver, sodium, calcium, magnesium) solution of desired concentration, beside 1 mL of 20mM substrate pNPP, 1mL of 1mM $MgCl_2$ as enzyme activator and total solution made 10 mL using Tris-HCl as supporting electrolyte.

Stock solution of 0.1M of each metal ion was prepared by dissolving reagent grade nitrate salts procured from Sigma-Aldrich. Fresh solution of required concentration of metal ions was prepared by diluting the stock solution before start of the experiment.

3.8 ASSEMBLY FOR FLOW SYSTEM

An indigenously designed assembly was used for studying the experiments of mercury ion determination using the biosensor. A continuous but controlled flow of the electrolyte medium was maintained in the voltammetric cell, simulating a stream of waste water sample containing the heavy metal contamination. A peristaltic pump was used to flush the electrolyte through the channel opening in the voltammetric reactor vessel. Filtration system

was fitted in the channel to prevent any particulates entering the voltammetric cell. Supporting electrolyte and the heavy metal salt solutions were injected as when required through the openings in the teflon lid on voltammetric cell kept for the purpose. A stopcock and the pump were used to control the flow of liquid as per requirement.

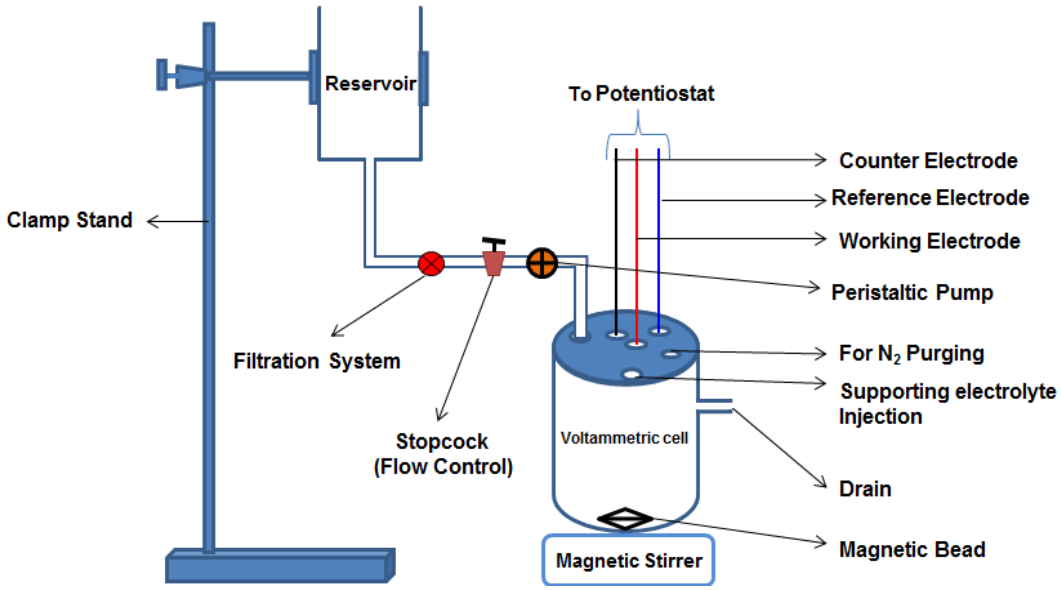

Fig 3.2: Schematic representation of assembly used for the of study of biosensor in a continuous flow system

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4. Results and Discussions

In an electrochemical biosensor a metabolic activity or the product of the cell is studied for exchange of electrons, oxidation or reduction using different techniques like cyclic voltammetry, chronoamperometry, conductometry, potentiometry, etc. The first step for preparation of whole cell based electrochemical biosensors was identification of microbes having some enzyme system available on the surface. Either of the reactant or product needs to be electro-active at the electrode surface. Alkaline phosphatase is one surface bound enzyme, which is released from the microbial surface on coming in contact with the phosphate substrate, causing its dephosphorylation by breaking phosphate-oxygen bond and releasing the phosphate. Hence, experiments for identification of whole cell microbes having alkaline phosphatase activity from various soil samples were carried out.

4.1 IDENTIFICATION OF MICROBES

Soil samples were taken from rhizospheric soil for identification of microbes having phosphatase activity. The serially diluted suspension of soil was spread on petri plates with a spreader, having Pikovskaya agar media(Pikovskaya et al, 1978) for bacterial culture and Beneck's media (Aneja et al, 2003) for algal culture separately. The phosphate solubilizing microbe grew on the petri-plates showing a clear zone around the colony (Fig: 4.1 a & c)

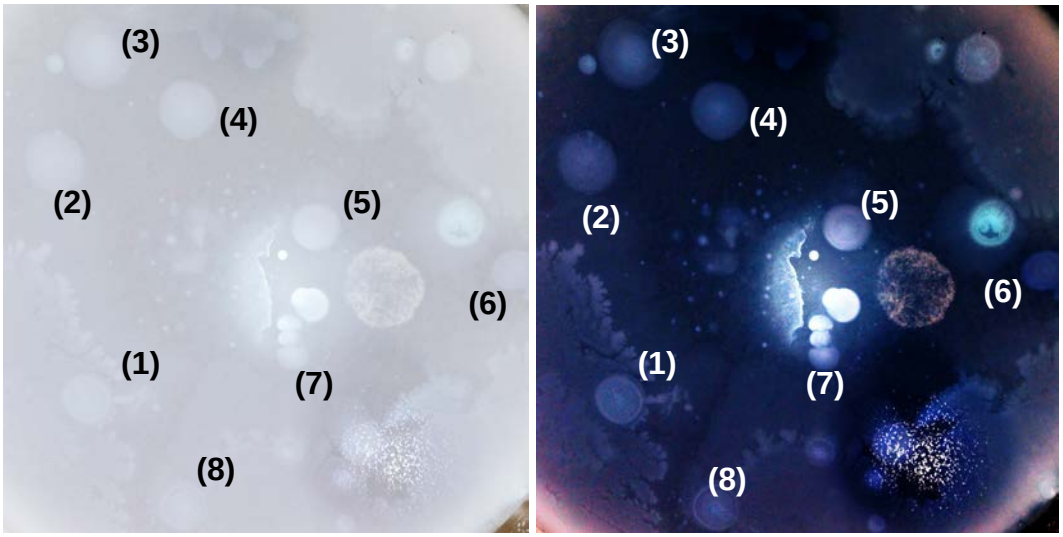


Fig 4.1 (a): Images showing colonies of phosphate solubilizing microbes in petri-plate having Pikovskaya media

Eight microbes identified for their phosphatase solubilizing capacity, were extracted from the petri-plate and transferred to broth media in a conical flask for further growth for 24 hrs. The isolated microbial colonies were again streaked on the petri-plates for purification and identification of the phosphate solubilizing microbes (Fig: 4.1 (b)).

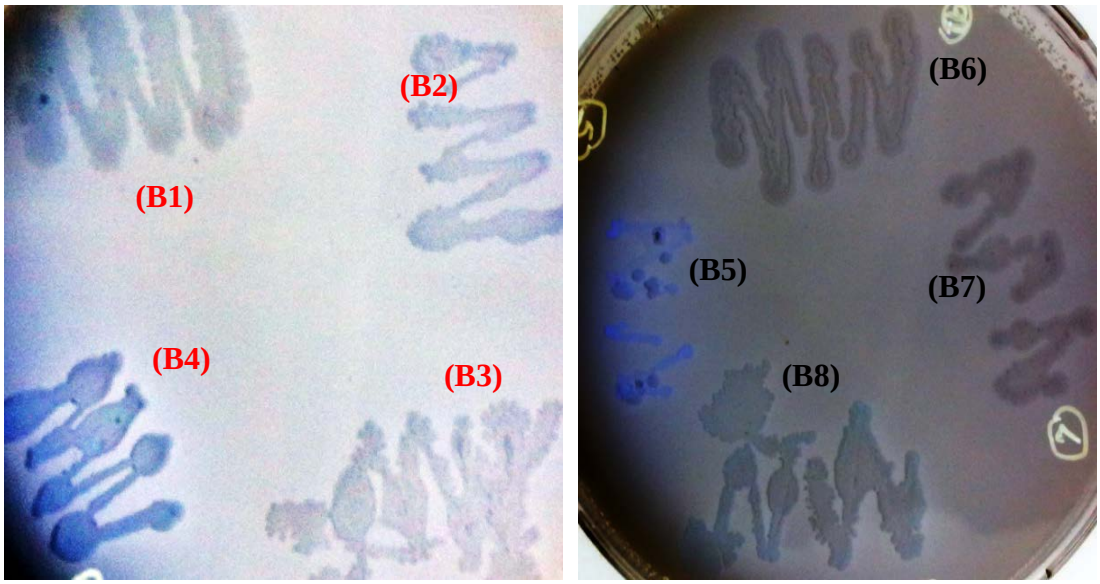


Fig 4.1 (b): Images showing colonies of phosphate solubilizing microbes in petri-plate having Pikovskaya media

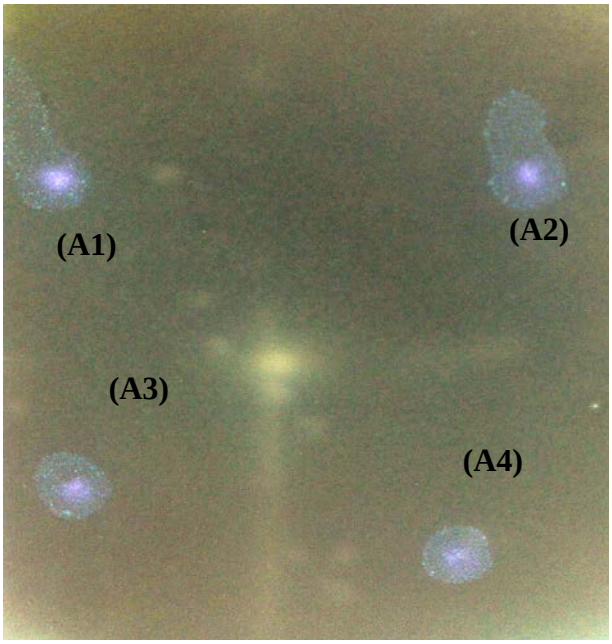


Fig 4.1 (c): Images showing algal colonies of phosphate solubilizing microbes in petri-plate having Beneck's media

The identified microbes were used for their quantitative phosphatase solubilizing capacity spectroscopically using chloromolybdic acid and chlorostannous acid. The developed blue colour was measured at 690 nm.

The various phosphatase solubilizing capacity of identified microbes are given in Table 4.1

Table 4.1: Quantitative phosphatase solubilizing activity of various identified microbes

S. No.	Microbial isolate	Absorbance at 690 nm	Concentration of phosphate (ppm)	% solubilization of the microbe
1	**B1**	**0.079**	**0.975**	**86.13**
2	B2	0.058	0.557	38.96
3	B3	0.049	0.451	24.61
4	B4	0.058	0.557	38.96
5	B5	0.052	0.475	28.42
6	B6	0.064	0.805	57.76
7	B7	0.049	0.451	24.61
8	B8	0.062	0.723	52.97
9	**A1**	**0.85**	**1.089**	**92.44**
10	A2	0.068	0.882	68.87
11	A3	0.054	0.513	29.59
12	A4	0.044	0.442	23.76

It is clear from Table 4.1, that microbial isolate A1 was having more than 90% phosphate solubilizing capacity, while A2, B6 and B8 had slightly more than 50% phosphate solubilizing capacity and other isolates have very low solubilizing capacity of around 30 %. The algal isolate A1 having more than 90 % capacity to solubilize phosphate from the substrate and was chosen for all the further studies (Harris et al, 1990; Fehrmann et al, 1993) by immobilizing on the electrode surface.

4.2 IMMOBILIZATION AND CHARACTERIZATION OF MICROBES MODIFIED ELECTRODES

A biosensor comprises of biomaterial closely interfaced to the transducer. Close proximity of these two components is highly required as the by-products of the reactions taking place can be easily identified and measured. It was achieved by immobilizing the biomaterial on the transducer surface. Immobilization can be done by different methods like physical adsorption, covalent interactions and entrapment in to a matrix media [Babu et al, 1991; Papageorgiou et al, 1986].

Identified phosphate solubilizing algal cells were used for immobilization with BSA in saturated glutaraldehyde on the Pt and GC electrode surfaces for closer proximity due to following reasons:

i. Being eukaryotic in origin can find more similarity in action with human cells.
ii. Easy to culture and handle.
iii. Lesser chances of getting contaminated as compared with bacterial and fungal cultures.

Glutaraldehyde plays a vital role in immobilization of the biomaterial on the electrode surface, because it causes the crosslinking of the protein bovine serum albumin (BSA) by linking with amines of the protein, without affecting the microbes and their viability. Time of exposure to glutaraldehyde holds the key to stability of the immobilization and membrane formed by it. Lesser than 20 minutes exposure to cast BSA-algae mixture electrode surface made the membrane unstable in aqueous media and solubilized in the media. Exposure of more than 30 minutes made the membrane brittle and got peeled off from the electrode surface. Hence, the stable immobilization could be achieved by exposing the algal-BSA cast electrode to saturated glutaraldehyde environment for 20-30 minutes.

The immobilized algal-BSA membrane was characterized for proper entrapment of biomaterial in the BSA membrane matrix, biomaterial viability and electron permeability across the membrane matrix. Optimization of the experimental conditions like composition of membrane matrix and solution for optimum response was done using various techniques

like scanning electron microscopy (SEM), fluorescence spectroscopy, cyclic voltammetry (CV), chronoamperometry etc.

4.2.1 Scanning electron microscopy

Freshly prepared cast membrane having algal cells entrapped in the BSA matrix cross-linked in glutaraldehyde environment was peeled off and was observed under scanning electron microscope (SEM) and micrographs were recorded to see the proper entrapment and availability of algal cells in the BSA matrix. Micrograph showed clear difference between the membrane containing biomass and membrane without biomass. Membrane containing biomass showed clear patches throughout the membrane, which indicated uniform spread of the biomass throughout the membrane. SEM image of membrane not containing biomass could be easily distinguished from the one with biomass, because of difference in appearance and absence of patches. {Fig: 4.2 (a), 4.2 (b) and 4.2 (c)}

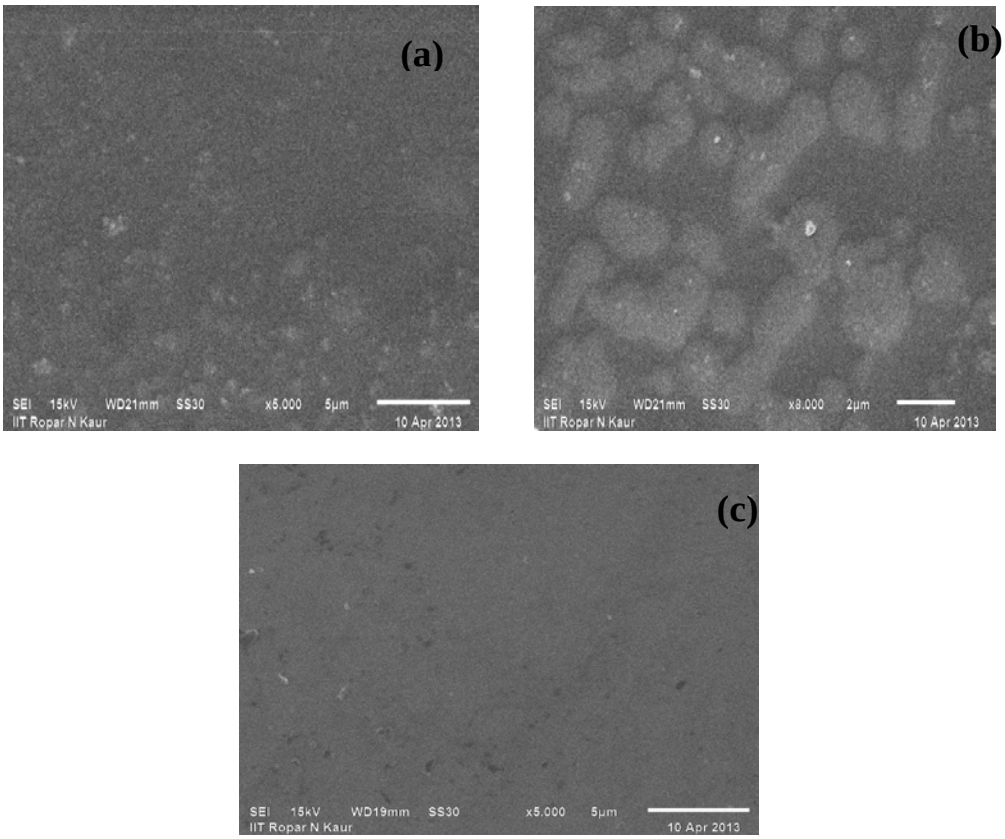


Fig 4.2: SEM images of membrane containing (a) & (b) BSA and Biomaterial (c) BSA only

4.2.2 Biomass viability

Alkaline phosphatase (AP) abundantly present in cell membrane of the whole cell was used to degenerate p-nitrophenyl phosphate (p-NPP) taken as a precursor to substrate i.e., p-nitrophenol (p-NP). For enzyme to be active, the biomaterial should remain viable in the membrane matrix environment. To check the viability of the immobilized algal cells fluorescence study of *Chlorella* sp. (A1) was carried out as free and immobilized biomass over the electrode surface using 540 nm light for excitation (Hipkins et al, 1986). The observed emission band of intensity 45 a.u at 680 nm (Fig: 4.3) corresponds to photosystem-II fluorescence emission peak of algae (Poryvkina et al, 2000, Erokhina et al, 2004). This low intensity of fluorescent light is quite common and the results are sufficient to accept viability of the biomass. The experiment was repeated at least 5 times and results were reproducible. The nearly similar magnitude of the emission bands at 680 nm for the free as well as immobilized biomass were observed and supports the viability of the biomass in immobilized form.

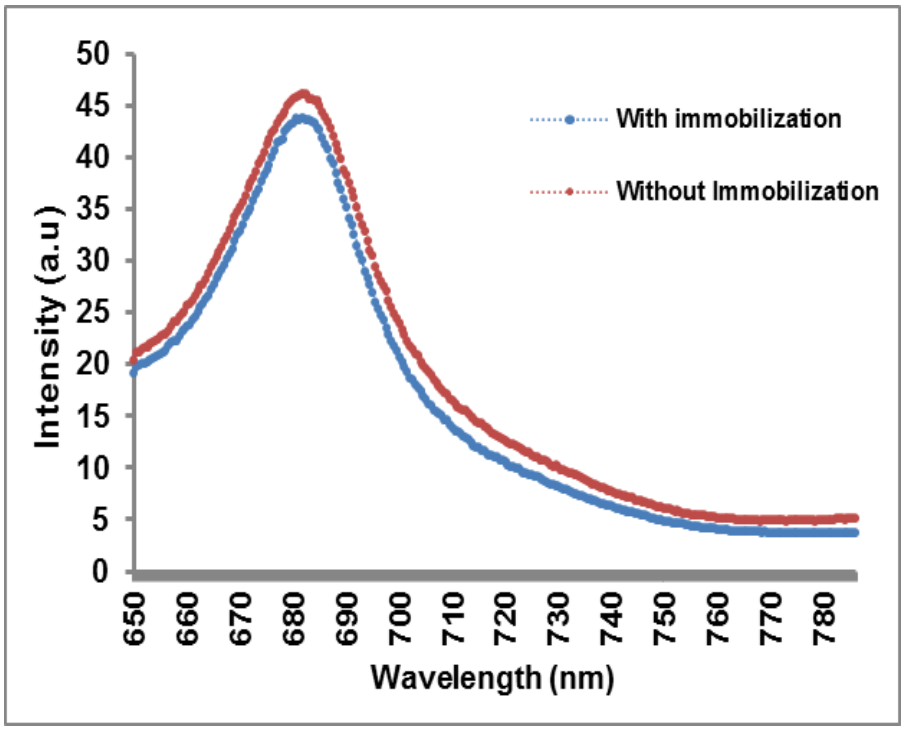


Fig 4.3: Fluorescence study of Chlorella sp. with (blue) and without immobilization (red) on electrode surface

4.2.3 Enzyme activity detection

Fluorescence study revealed that algal cells remain viable even after immobilization and enzyme also found to be active and breaks oxygen-phosphorus bond present in electro-inactive p-nitrophenyl phosphate resulting in dephosphorylation of the molecule and generating the eventual electro-active substrate p-nitrophenol for chronoamperometric studies (Lehmann et al, 2000) at a bias potential (Lakshmi et al, 1991, Hart et al, 2007). The p-nitrophenol thus formed undergoes electrochemical oxidation to form phenolate ion accompanied with the release of two electrons, hence, resulting in the flow of current.

The experiments were conducted to ascertain that current was generated in presence of substrate only and not by some other means. Two separate chronoamperometric scans were carried out; one in presence of p-NPP as substrate and other in absence of it with just Tris-HCl as supporting electrolyte. Fig: 4.4 clearly indicate that the flow of current is possible only when substrate is present and almost a flat line in absence of the substrate. These experiments established activity of the immobilized whole cells and the release of enzyme, which is responsible for oxidation and release of two electrons from the substrate i.e. p-NP, generated by enzymatic activity of alkaline phosphatase on substrate precursor i.e. p-NPP, and was measured chronoamperometrically.

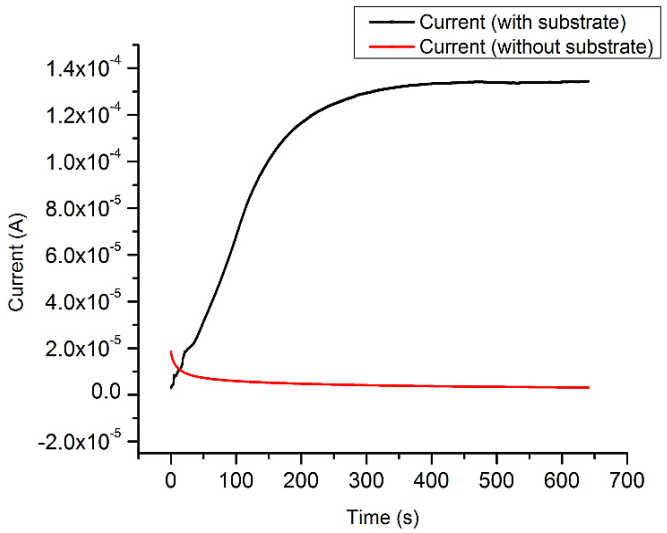


Fig 4.4: Electrochemical response of algae modified electrode in presence (black) and absence (red) of the substrate

4.2.4 Optimization of cell density and pH

It has been established that the current was generated in presence of p-NP, generated by cell membrane bound enzyme alkaline phosphatase from electro-inactive p-NPP. So, the magnitude of current generated at the electrode depends upon the amount of p-NP that undergoes oxidation, which further depends upon the amount of enzyme loaded on the surface of the electrode and hence, upon the number of cells per unit surface area of the membrane immobilized on the surface of the electrode. It is desirable to have high current output that can be achieved by increasing the cell density. However, overloading of the electrode surface with the cells can lead to the sluggish response of the electrode (Durrieu et al, 2002; Chouteau et al, 2004; Chong et al, 2008). Hence, optimization of the cell count per unit surface area of the electrode is highly desirable.

Experiments were conducted for optimization of cell density to be immobilized on the electrode surface. Oxidation of the substrate was carried out using different cell densities ranging from 1 million cells to 100 million cells per mL. Initial increase in cell density caused the increase in current, as increase in number of cells caused the increase in production of enzyme and ultimately leading to extra production of p-NP. The maximum current was obtained when cell density of 10 million cells per mL was used. Further increase lead to decrease in current due to overcrowding and over loading of the electrode surface causing the enzymatic activity to saturate after certain number of cells on the electrode surface, as shown in Fig: 4.5.

Performance of the electrode was also studied by varying pH of the solution to test the optimum pH environment for working of loaded enzyme in whole cell microbes entrapped in membrane matrix. The maximum current is an indicative of optimum environment in presence of an excess of the substrate, which was obtained at pH 9. Before and after this pH, there is a sharp decrease in output of the current, as shown in Fig: 4.6. It was observed that alkaline phosphatase works best in alkaline pH of around 9 beyond which the current decreased and can be attributed to denaturation of the enzyme at higher pH (Cannizares et al, 2004, Berezhetskyy et al, 2008, Chong et al, 2008).

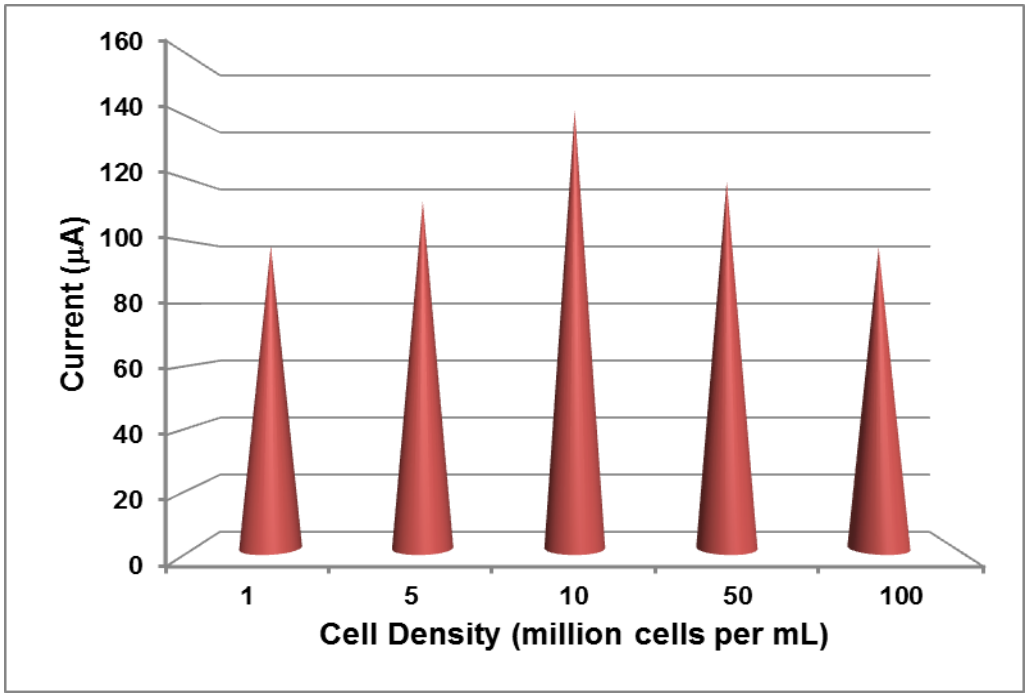


Fig 4.5: Amperometric responses of algae modified electrode at different cell densities

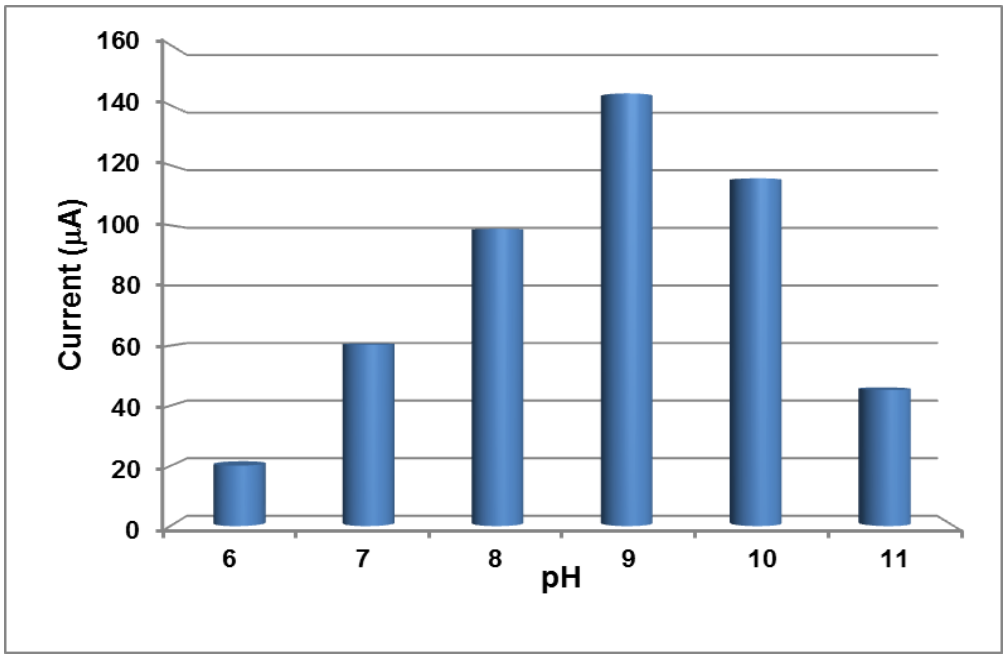


Fig 4.6: Amperometric responses of algae modified electrode at different pH conditions

4.2.5 Optimization of substrate concentration

The amount of current produced is also dependent upon the amount of substrate being acted upon by enzyme to produce the product, which is further oxidized to generate current. With higher amount of the substrate, more amount of product will be formed resulting in generation of higher magnitude of current. As the amount of substrate is increased beyond the capacity of the enzyme, a steady state or saturation is achieved. So the amount of substrate to be added to the reaction media has to be optimized. Experiments were conducted by adding different amounts of the substrate to know its optimum amount to be added in the reaction media. The concentration range was varied from 4.0×10^{-9} M to 20.0×10^{-9} M. It was observed that the current increased almost linearly from 4.0 to 14.0 nM with a very minimal but gradual change and became stable after 16.0 nM concentration of the substrate. Hence, the optimum saturation level of the substrate concentration was achieved at 16.0 nM concentration (Fig: 4.7). This optimized concentration of the substrate was used for further studies on the modified electrode.

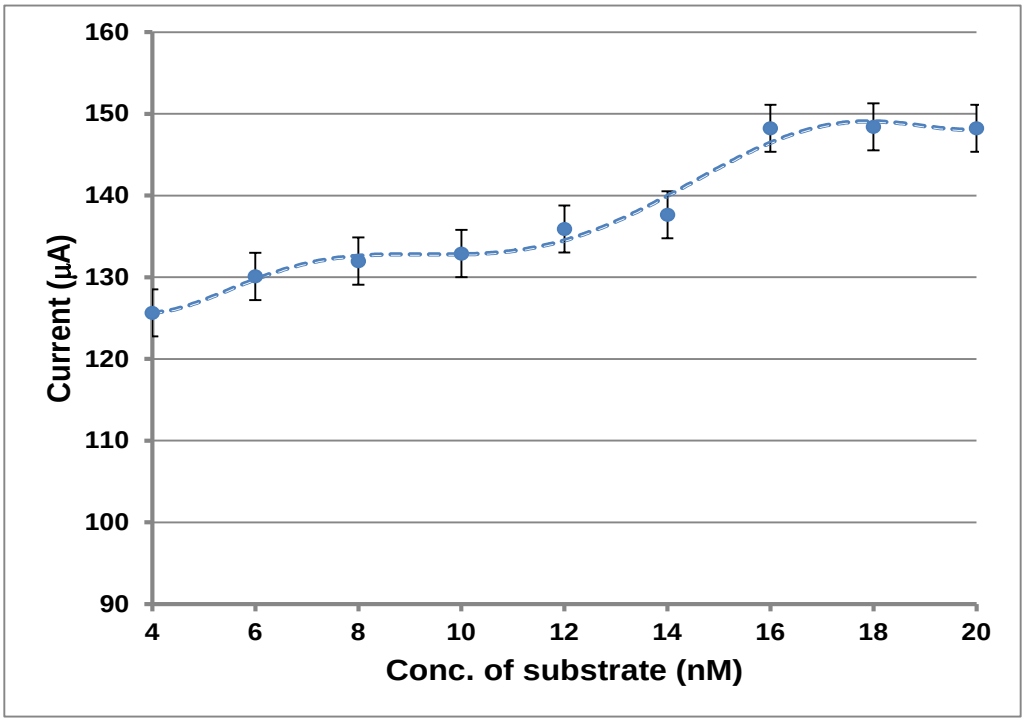


Fig 4.7: Amperometric responses of algae modified electrode at different substrate concentrations

4.2.6 Identification of electrodes

Experiments were conducted to check the surface material of transducer for immobilization of the identified microbe for best performance under the optimized experimental conditions. The microbes mixed with bovine serum albumin (BSA) were drop coated on the electrodes of three different materials i.e. platinum (Pt), copper (Cu) and glassy carbon (GC). Each of these was put in saturated glutaraldehyde environment to get a proper crosslinking of the BSA and entrapment of the biomaterial on to the electrode surface.

The immobilized electrodes were used for their respective chronoamperometric scans at a bias potential of 0.85V vs Ag/AgCl electrode in excess of p-NPP as substrate. The overlay of the scans is given in Fig: 4.8.

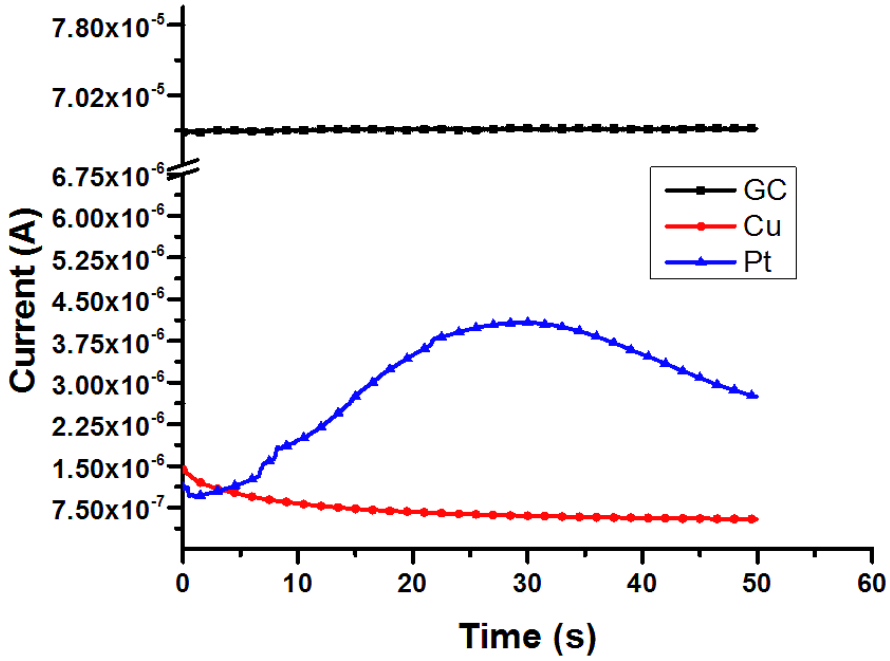


Fig 4.8: Current response of various algae modified electrode surfaces in presence of excess of substrate

The chronoamperometric scans showed that the glassy carbon electrode responded very well with immobilization giving current in milliampere range, which can be attributed to closer proximity of the biomaterial with the electrode surface. GC is also resistant to fouling, but other electrodes like platinum (Pt) and copper (Cu) have very less current generated as compared to GC electrode.

For an electrochemical reaction at the working electrode, the stability of current is different for different electrodes. It is also evident from the scans that on Pt electrode the current rises initially and then decreases slowly, but in case of Cu current falls continuously

4.2.7 Durability of the membrane

Biomembranes cast on the electrode surface are likely to undergo fouling on repeated use, particularly, when the substrate used is in excess. Modified electrodes (Pt and GC) coated with BSA and biomaterial based membrane were used repeatedly in the presence of excess of substrate to test the workability and durability of the immobilized membrane.

Workability of the algae modified electrode was tested by running repeated chronoamperometric scans after 5 minutes of gap in between. The working electrode was washed thoroughly with double deionized water before and after the scan. Fig: 4.9 (A) & (B) showed that the current stays almost constant up to 8 scans for Pt electrode and 10 scans for GC electrode, and thereafter, the current becomes unstable and decreases irregularly. Hence, the algae modified electrodes can be used repeatedly up to eight scans and ten scans for Pt and GC electrodes, respectively, without any loss in current magnitude.

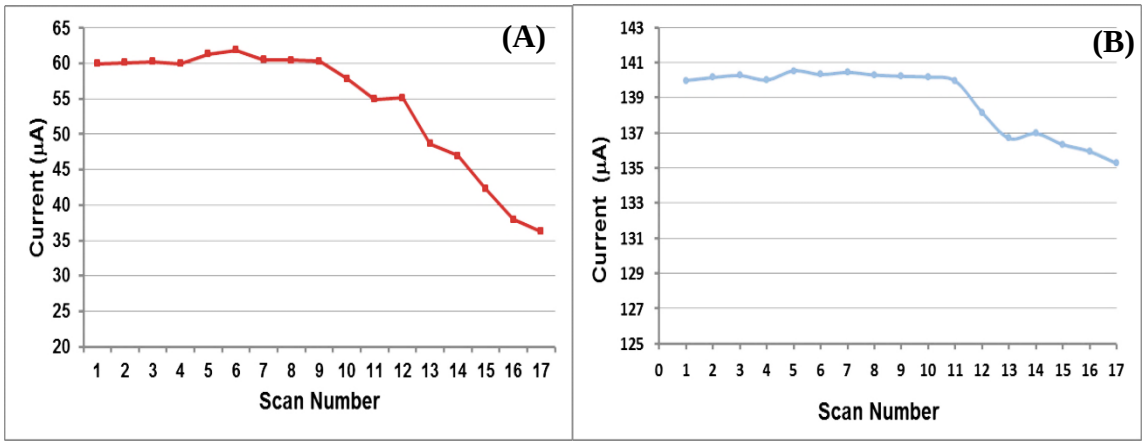


Fig 4.9: Amperometric responses of immobilized algae modified platinum (A) and glassy carbon (B) electrodes with repeated scans

The decrease in current for Pt and GC electrodes, respectively after 8 and 10 scans is due to fouling of the electrodes, more so on metallic (Pt) electrode than on non-metallic (GC) electrode.

Durability of the biosensor was also studied with respect to time i.e. shelf life and its reusability. Chronoamperometric scans were recorded to see reproducibility and shelf life of the modified Pt and GC electrodes at different time intervals in presence of excess of p-NPP as substrate, $MgCl_2$ as enzyme activator and Tris-HCl as supporting electrolyte. Amperometric scans were drawn after gaps of 1 day, 2 days, 8 days, 14 days and 21 days after the immobilization of the membrane on GC and Pt electrodes, shown in Fig: 4.10 and 4.11, respectively, to know shelf life of the electrodes. It was observed from the overlaid Amperometric scans (Fig: 4.10 & 4.11) that the current remained stable and reproducible up to 8 days in case of Pt electrode and 14 days in case of GC electrode. It was also observed that current fell sharply after 8 days with Pt electrode (Fig: 4.10), while a small decrease of about 5% up to 21st day in case of GC electrode (Fig: 4.11).

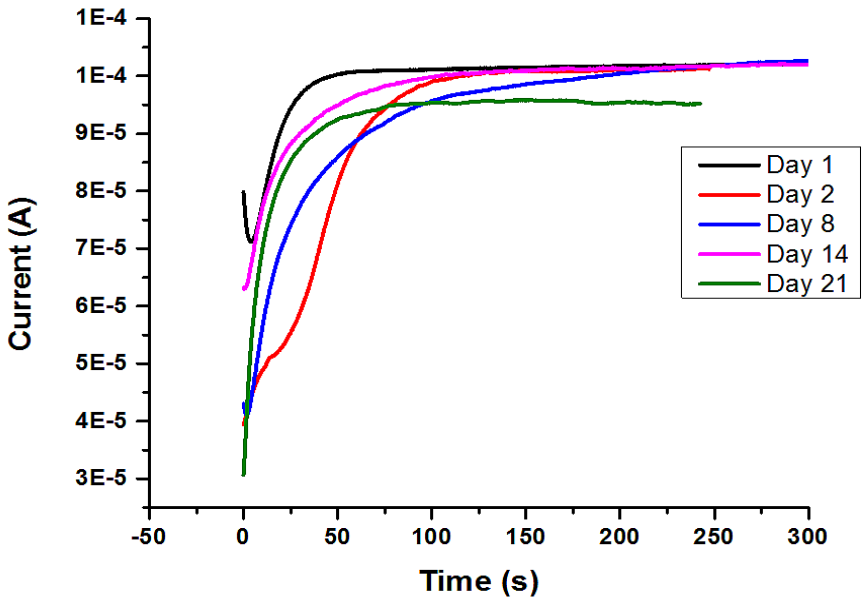


Fig 4.10: Chronoamperometric scans on algae-glassy carbon electrode for substrate (p-NPP) with enzyme activator and supporting electrolyte after different periods of immobilization

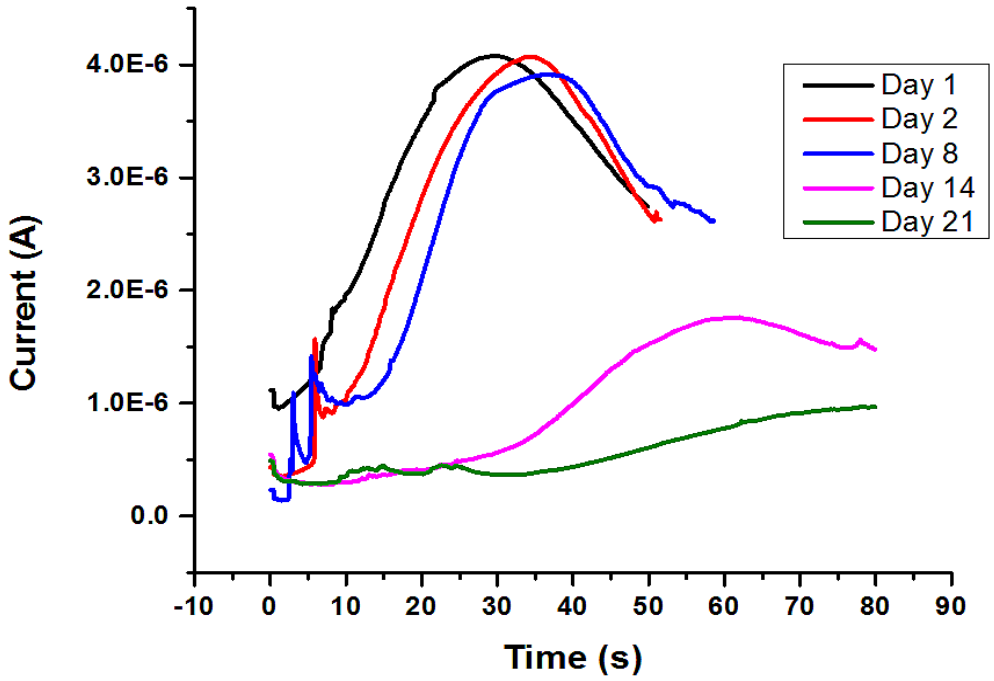


Fig 4.11: Chronoamperometric scans on algae-platinum electrode for substrate (p-NPP) with enzyme activator and supporting electrolyte after different periods of immobilization

Experiments were repeated with fresh solutions every day to record chronoamperometric scans for an excess concentration of the substrate. The electrodes were cleaned thoroughly with doubly distilled deionized water before and after the experiment, were covered with a rubber electrode cap and kept at room temperature before any further experiments. Hence, the stability, reproducibility and shelf life of the algae modified Pt electrode could be taken as 8 days and 14 days for modified GC electrode.

4.3 BIOSENSORS FOR HEAVY METAL IONS

In presence of heavy metal ions, magnitude of the current decreases due to their interaction with enzyme alkaline phosphatase from the immobilized whole cells (*Chlorella* sp.) (Guilbault, 1984), which decreases the electro-active p-NP generation. The metal ions interacting with biomaterial can be taken as of bioavailable content (Gadd et al, 1978; Slama et al, 1996) of heavy metals in the sample solution. The same principle is being used for determination of bioavailable content of heavy metal ions using algae modified Pt and GC electrodes as biosensors.

4.3.1 Platinum (Pt) electrode modified biosensor

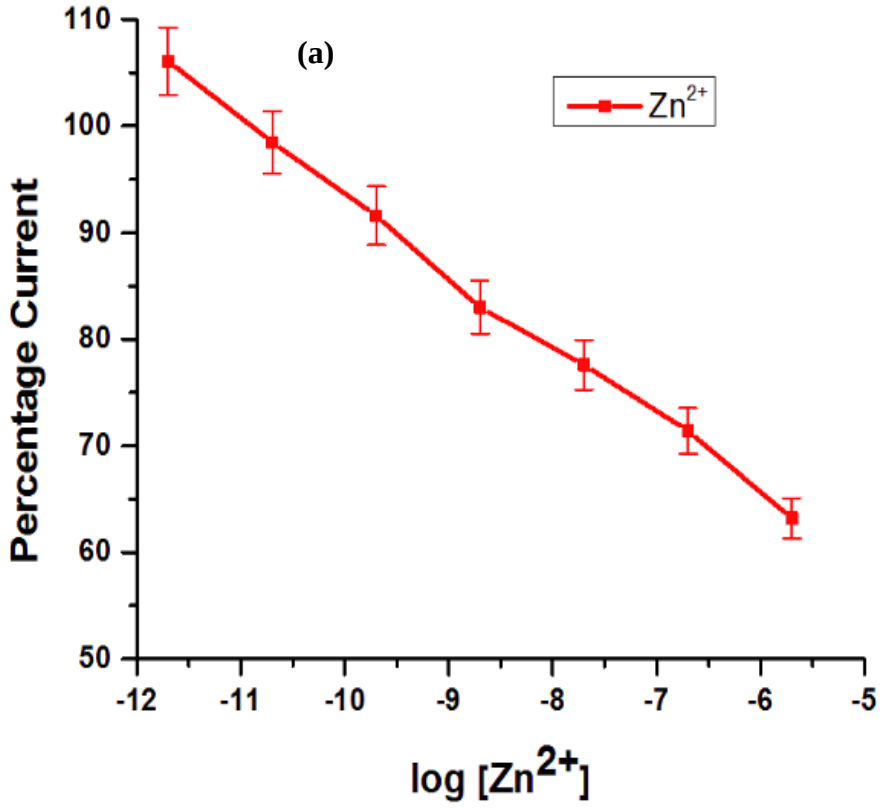
It is already been discussed that the enzymatic activity of alkaline phosphatase is inhibited (Blake et al, 2001) in the presence of heavy metals, resulting in the decreased production of p-nitrophenol and the subsequent lower oxidation current.

An algae modified Pt electrode with optimized composition algae modified Pt electrode was used for the determination of different heavy metal ions in aqueous samples based on the above mentioned property of surface bound enzyme alkaline phosphatase of algae entrapped in BSA matrix over the electrode surface. The algae modified Pt electrode was immersed in an aqueous solution containing zinc, cadmium, cobalt, nickel and copper separately, with Tris–HCl as a supporting electrolyte and $MgCl_2$ as an enzyme activator in excess of substrate p-NPP to get the maximum APA of the enzyme. Under optimum conditions, the working electrode was impressed a bias potential of 0.85V applied across the reference electrode, resulting in the oxidation of electroactive substrate (p-NP) the species.

The whole cell algae modified platinum electrode was used for determination of Zn^{2+} ions in an aqueous solution containing excess of substrate. Chronoamperometric scans were run for p-NP in presence of zinc ions in a range of 10^{-12} M to 10^{-5} M in aqueous solution. These experiments were repeated 5-6 times and a representative plot between current and concentration of heavy metal ion is shown in Fig: 4.12 (a).

Algae modified Pt electrode responded in a linear fashion for Zn^{2+} ions in the concentration range 10^{-12} M to10^{-6} M with a correlation coefficient of 0.9975 {Fig: 4.12 (a)}. Zn^{2+} acts as an inhibitor of the enzyme alkaline phosphatase bound on cell membrane of whole cell algae,

resulted in decrease of alkaline phosphatase activity (APA). Above and below the linear concentration range the modified electrode behaved weirdly and deviated from the linear behaviour. Hence, the linear range of detection of Zn^{2+} ions can be taken as 10^{-12} M to10^{-6} M for algae modified Pt electrode biosensor. The algae modified platinum electrode was also tested for different metal ions like cadmium, cobalt, nickel and copper. The response of the electrode was more or less the same as that for zinc ions (Paddle et al, 1996: Krawczyk et al, 2000) {Fig: 4.12 (b)}. It is very difficult to prepare a whole cell based biosensor which responds to heavy metal ions in a specific manner as all metals complex with enzymes of the cells to more or less the same extent. Yet, the relative performance of the biosensor in the presence of different heavy metal ions can be studied.



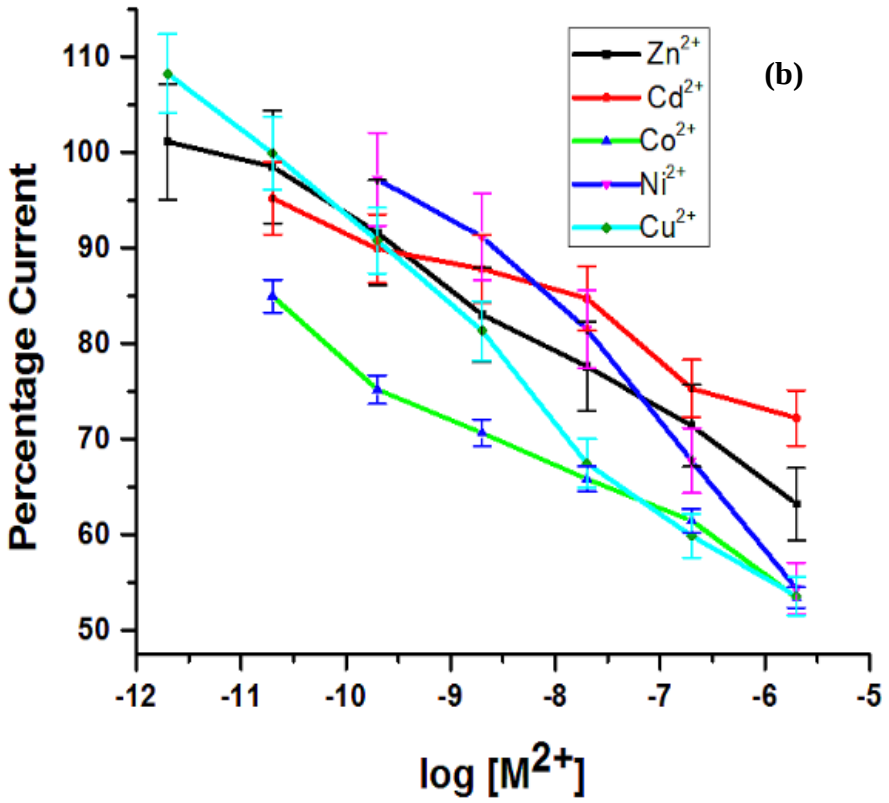


Fig 4.12: Calibration curve for Zn^{2+} ions (a) & Heavy metal detection by algae-platinum biosensor (b)

Hence, experiments were performed on the biosensor to study its linear relative response towards these metal ions in the presence of some other metal ions like copper, nickel, cobalt, cadmium at a constant concentration of 10^{-6} M. Therefore, the five sets of calibration curves (Fig: 4.13 and Fig: 4.14) generated are discussed below:

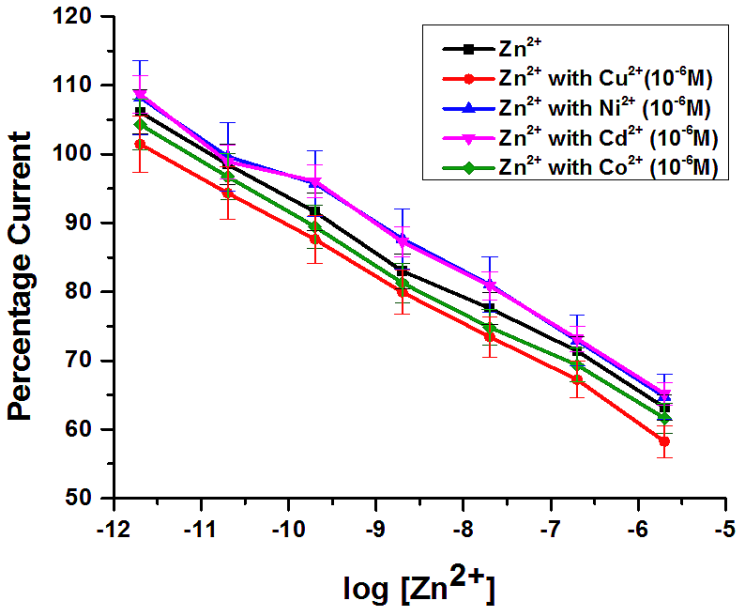


Fig 4.13: Calibration curves for Zn^{2+} ions and their interference in presence of metal ions like copper, cobalt, cadmium and nickel

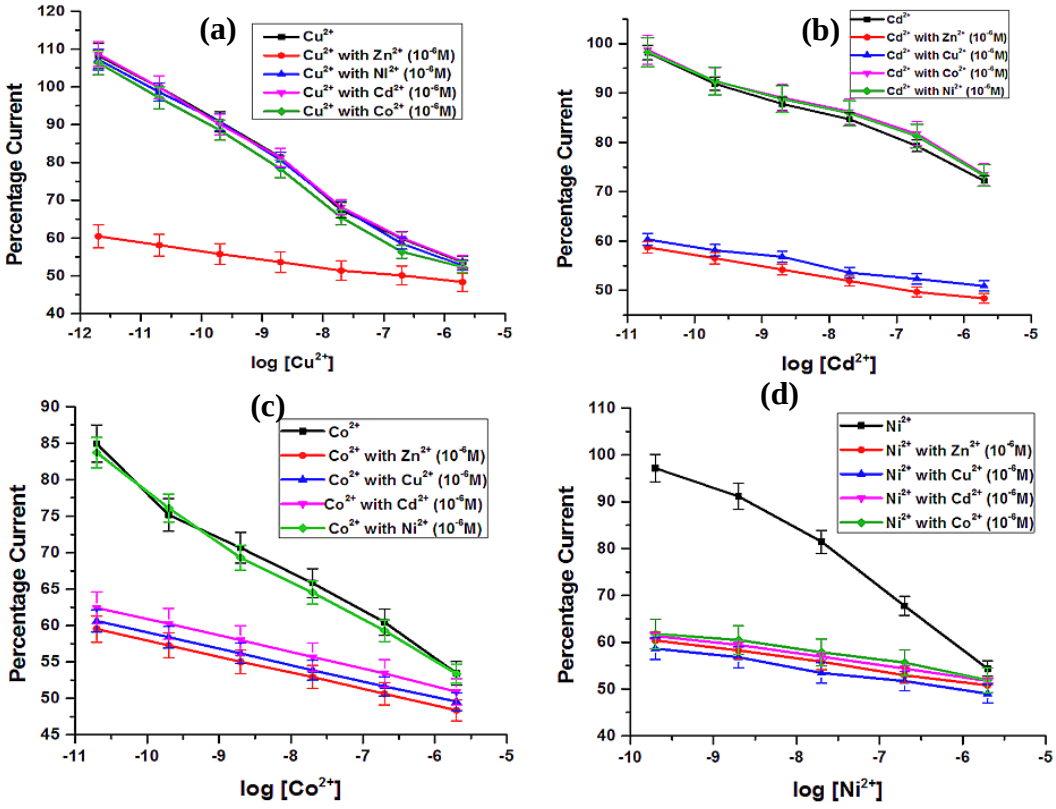


Fig 4.14: Calibration curves for Cu^{2+}, Cd^{2+}, Co^{2+} and Ni^{2+} ions and their interference in presence of different metal ions as secondary interferent ions

Experiments were performed to study the current magnitudes resulting with the modified Pt electrode in the presence of heavy metal ions over a wide concentration range 10^{-12} M to10^{-6} M in the presence of a fixed concentration (10^{-6}M) of secondary interfering ions. The decrease of the current so obtained gave a clear idea about the relative toxicity of the metal ions in presence of other metal ions acting as secondary interfering ions.

As observed in Fig: 4.12 (a) the fall in current with an increase in the concentration of the zinc ions is linear, thereby indicating the increased toxicity for the biomass. The trend of change in the current is very peculiar in the case of the zinc ions in presence of other metal ions (Fig: 4.13), which shows a high toxicity even at a concentration of 10^{-6} M, when added as a secondary interfering ions. Rate of decrease in the magnitude of current so obtained gave a clear idea about the relative toxicity of the metal ions in presence of some metal ions acting as secondary interfering ions, when added to solution of primary metal ions. All the other metal ions show linearly decreasing current values in the presence of each other, while the decrease due to zinc dominates and results in an almost flat curve. This dominance of zinc is also supported by the literature. Further, the toxicity due to heavy metal ions can be ordered qualitatively depending upon the response of the biosensor in the presence of different combinations of heavy metal ions, as shown in Fig: 4.14. The relative selectivity of the biosensor for heavy metal ions can be put in the following order: $Zn^{2+} > Cu^{2+} > Cd^{2+} > Co^{2+} > Ni^{2+}$.

Metal ions like copper, nickel, cadmium and cobalt (1 x 10^{-6} M) when added to different concentrations of Zn^{2+} ions as secondary interfering ions did not change either the slope or magnitude of the decrease in the percentage current as was observed for the pure Zn^{2+} ions in absence of any other interfering ions (Fig: 4.13). While the calibration curve for copper showed a sharp decrease in both the slope and the magnitude of the percentage current in the presence of Zn^{2+} ions (1 x 10^{-6} M). The presence of metal ions like nickel, cadmium and cobalt did not influence the linear trend (correlation coefficient 0.9926) or the slope of the calibration curve {Fig: 4.14 (a)} for copper ions. The calibration curve drawn for cadmium is strongly inhibited by the presence of zinc and copper, while cadmium and nickel did not influence the slope of the calibration curve (correlation coefficient of 0.975). The presence of zinc, copper and cadmium resulted in a sharp loss in the slope of cobalt, but nickel did not

have any effect on the linear behavior of the curve (correlation coefficient of 0.9854). All the metals i.e. zinc, copper, cadmium and cobalt showed a strong interference for the calibration curve of nickel (correlation coefficient of 0.9776).

4.3.2 Conclusions

The following conclusions can be inferred from the experimental detail given above:

1. The presence of zinc dominates the activity of the microbes with respect to the other metal ions. As metal ions like cadmium, cobalt, copper and nickel did not affect the biosensor response towards the zinc metal ion concentration in terms of slope and correlation coefficient of calibration curve. Moreover, the presence of zinc with other heavy metal ions certainly reduces the slope of the calibration curve by a significant degree.
2. The calibration curves for each heavy metal ion are linear in the absence of any other metal ion and the decrease in the percentage current is about the same, i.e. by about 40% except for cadmium, where it is decreased to only 30%.
3. On the basis of the interference studies indicated in terms of the decrease in the percentage current (72pprox.. 40%), the interference level of the metal ions can be arranged as follows: $Zn^{2+} > Cu^{2+} > Cd^{2+} > Co^{2+} > Ni^{2+}$.

The trend in the interference as observed above can be looked at as if the metal ions with filled d-orbitals, i.e. Zn^{2+},Cd^{2+} and Cu^{2+}, complex easily with the microbial enzyme in comparison to Ni^{2+}. On the basis of linear response range of the algae modified Pt electrode the lower detection limit was also calculated which comes out as: 10^{-12} M for Zn^{2+} and Cu^{2+}, 10^{-11} M for Co^{2+} and Cd^{2+} and 10^{-10} M for Ni^{2+}.

4.3.3 Glassy carbon (GC) electrode modified biosensor

Algae modified GC electrode was used for determination of different heavy metal ions in aqueous media. The whole cell based GC modified microbial biosensor responded in a linear fashion for Zn^{2+} ions in concentration range of 10^{-14} M to 10^{-6} M {Fig: 4.15 (a)}. So, the lower limit of detection can be taken as 10^{-14} M.

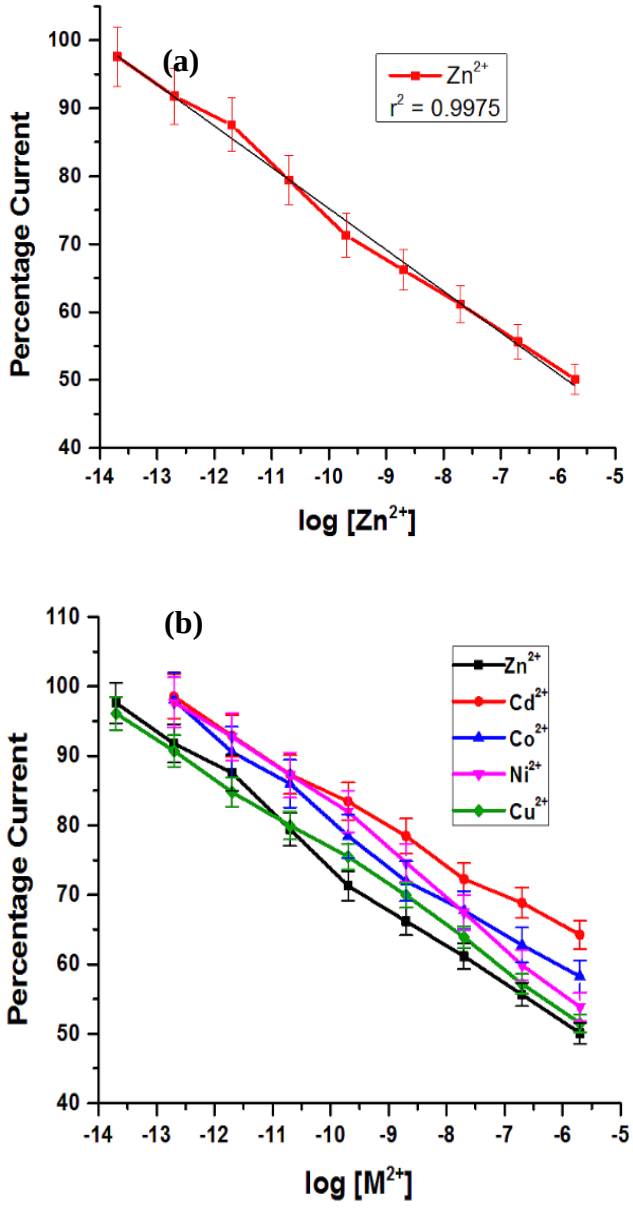


Fig 4.15: Calibration curve for Zn^{2+} ions (a) & Heavy metal detection by algae-glassy carbon biosensor (b)

However, modified electrode reaction was fairly similar for other heavy metals ions i.e., copper, cadmium, cobalt and nickel. It is very difficult to prepare a whole cell based biosensor selective for heavy metal ions, as metal complexation of enzyme of whole cell with most of these metals remains the same. When the algae modified GC electrode was exposed to different metal ions like Cd^{2+}, Co^{2+}, Ni^{2+} and Cu^{2+}, the results were similar to those obtained with Zn^{2+} ions. The lower detection limit, as observed from corresponding calibration curves {Fig: 4.15 (b)} as 10^{-14} M for zinc and copper, 10^{-13} M for cadmium, cobalt and nickel ions. Biosensor was studied for its relative selectivity response to these metal ions in the presence of some other metal ions as interferents, added separately at a constant concentration of 10^{-7} M. Five sets of calibration curves were drawn for each one of these metal ions as a primary ion and the representative curves are shown in Fig: 4.16 and Fig: 4.17.

During relative selectivity studies for Zn^{2+} ions, both magnitude of the slope and the decrease in percentage current did not change even on addition of copper, nickel, cadmium and cobalt solutions (1×10^{-7} M) to different concentrations of Zn^{2+} ions (Fig: 4.16).

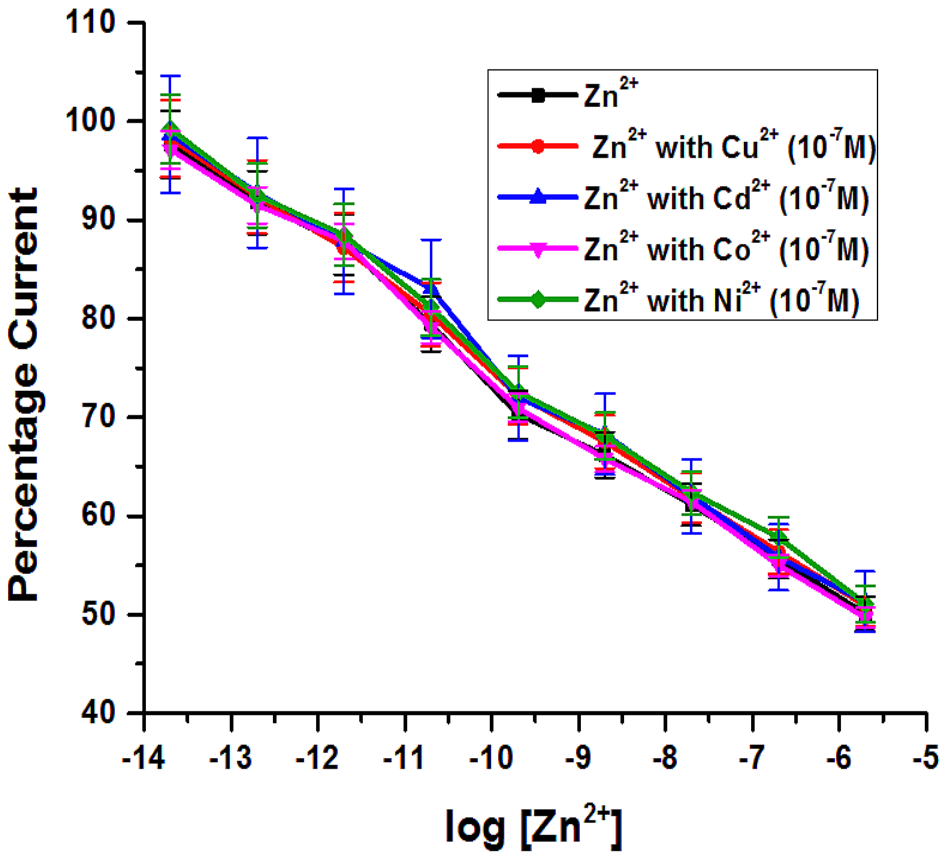


Fig 4.16: Calibration curve for Zn^{2+} ions and its interference in presence of metal ions like copper, cobalt, cadmium and nickel using algae modified GC electrode

Calibration curves for copper and cadmium showed sharp decrease in both slope and magnitude of current in presence of Zn^{2+} ions (1×10^{-7} M), added as interferent. The presence of cadmium in copper and vice-versa showed no influence on linearity and calibration of the respective metal ions, while nickel and cobalt ions did not affect the linear trend or slope of the calibration curve (Fig: 4.17) for both copper and cadmium ions.

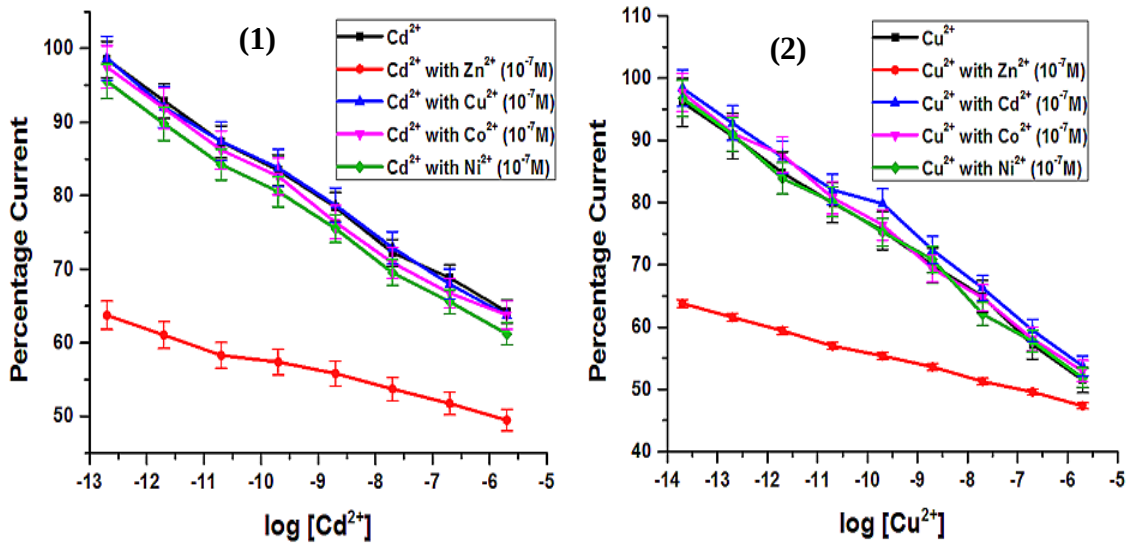


Fig 4.17: Calibration curves for Cd^{2+} (1) and Cu^{2+} (2) ions and their interference in presence of different metal ions as secondary interfering ions

Calibration curve drawn for cobalt ions is strongly inhibited by the presence of zinc, copper and cadmium, while nickel ions did not influence slope of the calibration curve. Metal ions like zinc, copper, cadmium and cobalt showed strong interference in the calibration curve for nickel (Fig: 4.18).

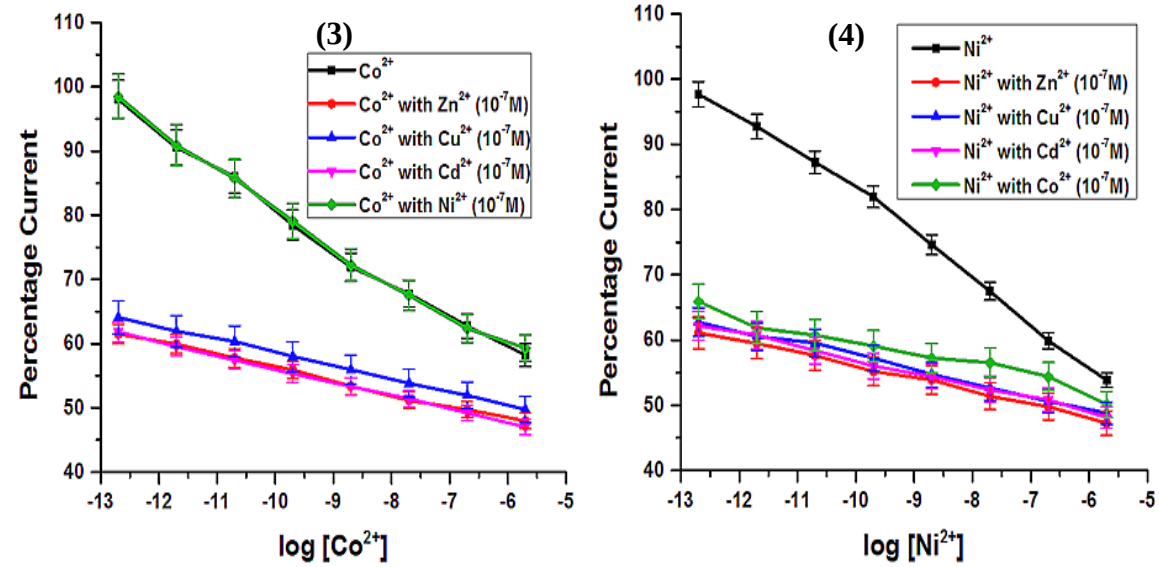


Fig 4.18: Calibration curve for Co^{2+} (1) and Ni^{2+} (2) ions and their interference with different metal ions as secondary interferent ions

The modified GC based biosensor electrode was taken as sensitive to heavy metal ions particularly to the zinc, copper and cadmium than the cobalt and nickel. Experiments conducted using alkali and alkaline earth metal ions as inhibitors, showed no such inhibition as was seen with heavy metal ions. The response of biosensor for some s-block elements is reported in Fig: 4.19. It is quite evident that there is only 2% decrease in percentage current in comparison to approx. 40% observed with heavy metal ions. Hence, the algae modified electrode can be taken as biosensor for heavy metal ions, having no interference from alkali and alkaline earth metal ions.

The trend of interference as observed above can be looked into as if metal ions with filled d-orbitals i.e. Zn^{2+}, Cd^{2+} and Cu^{2+} complex easily with microbial enzyme in comparison to Ni^{2+} and Co^{2+}, which are less known to form stable complexes with enzymes.

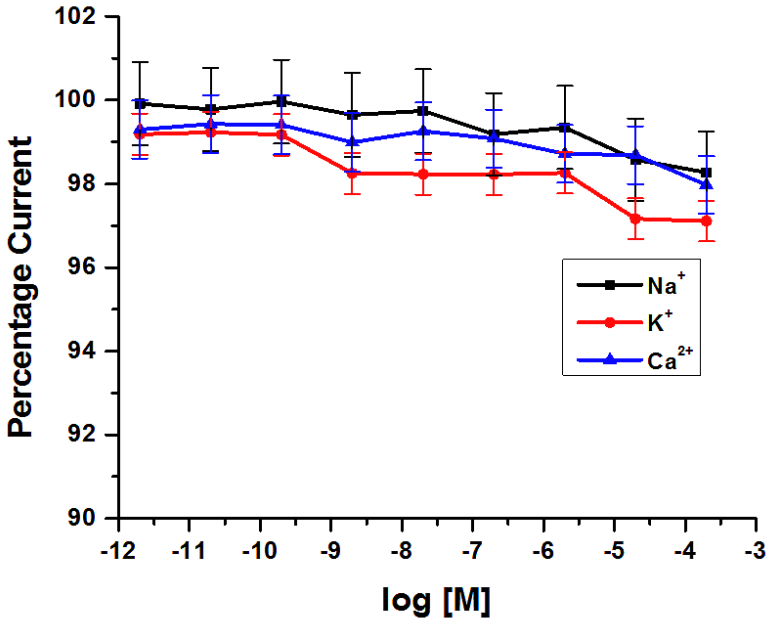


Fig 4.19: Calibration curves for some s-block elements (Na, K, Ca) using algae modified GC electrode

So it can be further concluded from the above discussion that oxidation current decreased linearly with increase in the concentration of heavy metal ions from 10^{-14} M to 10^{-7} M with calibration curves showing a correlation co-efficient of 0.99. The detection limit of the

modified glassy carbon electrode can be taken as 10^{-14} M, with a linear detection range of 10^{-14} M to 10^{-7} M and relative selectivity order $Zn^{2+} >> Cu^{2+} \approx Cd^{2+} > Co^{2+} > Ni^{2+}$.

4.3.4 Conclusions

Following inferences emerge out of the experimental details given above:

1. Presence of zinc dominates the inhibition activity of microbes with respect to other metal ions like cadmium, cobalt, copper and nickel.
2. Slopes of calibration curves for both copper and cadmium are influenced by the presence of zinc metal ions causing sharp decrease in the slope. However, the presence of copper and cadmium ions does not affect the calibration curve for zinc metal ions.
3. On the basis of interference studies indicated in terms of decrease in current (approx. upto 40%), interference levels of metal ions can be arranged as follows: $Zn^{2+} >> Cu^{2+} \approx Cd^{2+} > Co^{2+} > Ni^{2+}$.
4. Copper and cadmium do not interfere in the detection of each other although having a strong interference from zinc.

Table 4.2: Comparison of response and properties of algae modified platinum and glassy carbon electrodes

S. No	Parameter	Platinum Electrode	Glassy Carbon Electrode
1	Modification	Algae (Chlorella sp.)	Algae (Chlorella sp.)
2	Current	Falls after reaching a maxima	remains stable after reaching the maxima
3	Detection limit	10^{-12} M	10^{-14} M
4	Relative selectivity for heavy metal ions	Zn^{2+}	Zn^{2+}
5	Interference behavior	Cu^{2+} interferes in detection of Cd^{2+}	Cu^{2+} & Cd^{2+} show no interference with each other and respond independent of each other
6	Relative selectivity order	$Zn^{2+} > Cu^{2+} > Cd^{2+} > Co^{2+} > Ni^{2+}$	$Zn^{2+} >> Cu^{2+} \approx Cd^{2+} > Co^{2+} > Ni^{2+}$

It is also evident from the above discussion that both modified Pt and GC electrodes show relative selectivity towards zinc, but they respond quite differently from each other, in terms of their selectivity order, detection limit etc. The comparison of both modified electrodes has been given in Table 4.2.

4.3.5 Biosensors for Mercury ions

The *Chlorella* sp. Modified GC electrode showed greater degree of sensitivity, selectivity and resistance to fouling in the experiments and were used for repeated measurements without any decrease in current output with in the optimized conditions of life time and reproducibility. Hence, algal cells immobilized on the GC electrode in the optimum conditions and composition of pH, exposure to glutaraldehyde and cell density was used for bioavailable mercury determination in aqueous media. Mercury is known as extremely dangerous and poisonous metal ion for living beings and poses a potential threat to various enzymatic activities (Dorst et al, 2010; Omura et al, 2004) and causes an irreparable damage to enzyme molecule rendering it ineffective.

On the same terms, algae modified GC electrodes when exposed to mercury ions showed decrease in current generation, because of the reason that mercury inhibited the activity of alkaline phosphatase present on cell wall of the whole cell algae, causing a decrease in the production of electro-active p-NP. The modified electrode responded linearly in the concentration range of 10^{-14} M to 10^{-6} M of mercury, with gradual decrease in the current generated by oxidation of enzymatically generated electro-active p-NP from electro-inactive p-NPP. Hence the lower limit of detection can be taken as 10^{-14} M for mercury ions.

Silver is the most compelling threat as interferent in the determination of mercury, so the interference of silver ions was studied in the determination of mercury ions. A constant concentration of silver ion was added in mercury solution and studied the changes made to APA of surface bound alkaline phosphatase enzyme was studied varying the concentration of mercury ions in the solution. It can be noted from Fig: 4.20 that there is hardly any decrease in the slope of the calibration curve for mercury determination even in the presence of silver ions. Hence, silver even being most impending interferent for mercury has not affected the slope of calibration curve for mercury ions. Therefore, it can be said that the algae modified GC electrode is selective and ultra-sensitive for mercury ions and can be used for mercury determination in aqueous media with no interference from silver ions.

To test the selectivity of algae modified GC electrode for mercury ions, experiments were conducted by varying the fixed concentration of silver ions from 10^{-8} M to 10^{-5} M in the

solution with varying concentration of mercury ions (Fig: 4.20). Calibration curves drawn for mercury ions in presence of different fixed amount of silver ions in fresh experiments showed no change is slope or linearity when compared with calibration curve drawn for mercury ions in absence of any other potential interferent metal ions. Hence, the algae modified GC electrode was taken selective for determination of mercury, having no interference from its potential interferents (Band et al, 1972; Bernth et al, 1984; Pick up et al, 2001) like silver ions, even at a high concentration of 10^{-5} M silver ions in the solution.

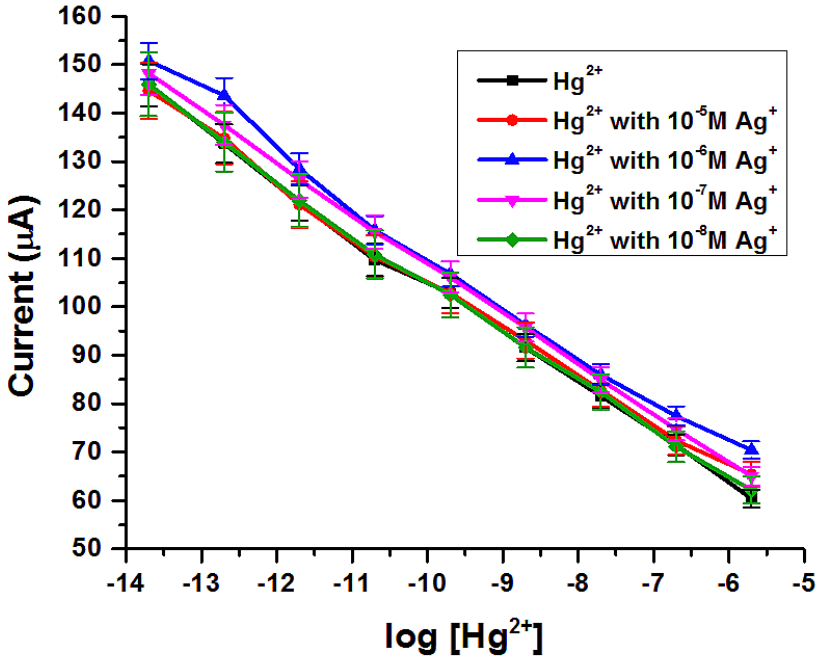


Fig 4.20: Calibration curve for Hg^{2+} ions and its interference in presence of varying concentration of Ag^{+} ions for *Chlorella* sp. Modified GC electrode

To establish the selectivity of the algae modified electrode for mercury ions interference studies were carried out using different representatives from various periodic table groups like transition metals (Zn), alkali metals (Na) and alkaline earth metals (Ca). A constant concentration of 10^{-7} M Zinc, sodium and calcium were added as interferent to the solution for mercury determination. Barely any change in slope of calibration curve for mercury determination (Fig: 4.21) showed that the algae modified GC electrode is selective for mercury and having no interference from alkali, alkaline earth and transition metal ions.

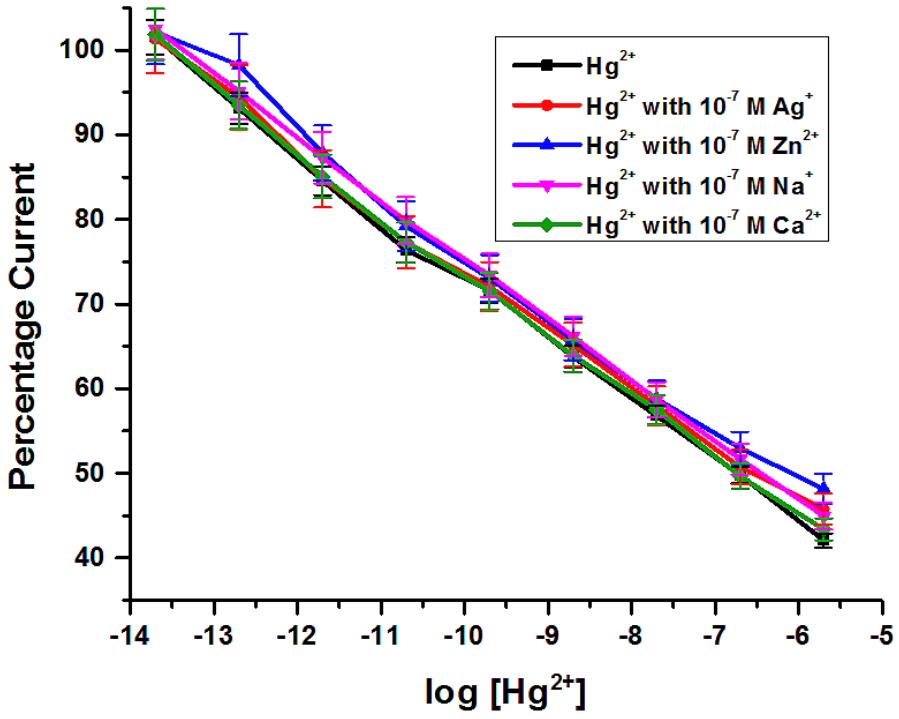


Fig 4.21: Calibration curve for Hg^{2+} ions and its interference in presence of representative metals from various groups of periodic table for *Chlorella* sp. Modified GC electrode

Hence, it can be concluded from the above experiments that the algae modified GC electrode can be taken as mercury selective biosensor, having a detection limit of 10^{-14} M for determination of mercury ions in aqueous media. The discussed biosensor is having no interference from any of the potential interferents even in the presence of very high concentration of its potential interferents.

4.3.6 Continuous flow analysis

Heavy metal ions are known as obnoxious pollutants causing various kinds of metabolic anomalies to both flora and fauna (Dewhurst et al, 2002). These pollutants or anthropogenic origin are either dumped into to various fresh water resources or they find their way to these water resources through leaching and rain water drainage. So, the next hurdle in determination of these heavy metal ions is their in-situ and online determination (Rawson et al, 1987; Biran et al, 2000; Tag et al, 2007, Komaitis, 2010; Mimendia et al, 2010). Keeping that objective in mind an experimental set up with an indigenously designed assembly for the variable flow rate ranging between 0.25 mL/min to 5.0 mL/min was used. A continuous stream of aqueous medium consisting of the substrate, supporting electrolyte and enzyme activator was varied from 0.25 mL/min to 5.0 mL/min to optimize the flow rate for chronoamperometric experiments, which were to be conducted to optimize response time of the set up for each flow rate (Fig: 4.22).

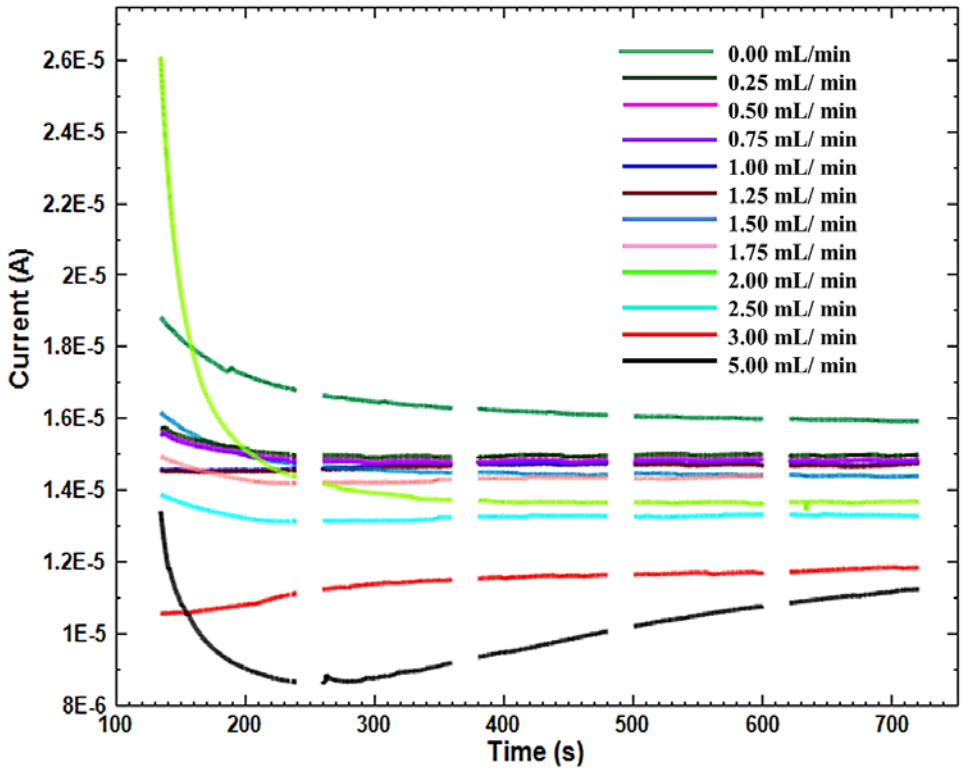


Fig 4.22: Overlay of chronoamperometric plots of mercury biosensor at different flow rates of electrolyte solution

From the overlay of chronoamperometric plots (Fig: 4.22) it can be seen that the current reached a steady state and is optimized after about 5 minutes from start of the experiment and was used to quantify the current produced in all further experiments.

Similar experiments were performed for current measurement as a function of flow rate to find out the optimum flow at which the current is sustained and stable. It was observed (Fig: 4.23) that after an initial decrease in current, it became more or less stabilized upto 1.5 mL/min flow rate and followed by a sudden drop in current on further increment in flow rate. Hence, it is established that the biosensor can be used with in a range of flow rate of 0.5 mL/min to 1.5 mL/min for mercury determination from a flowing stream of aqueous media and optimum flow rate can be taken upto 1.5 mL/min.

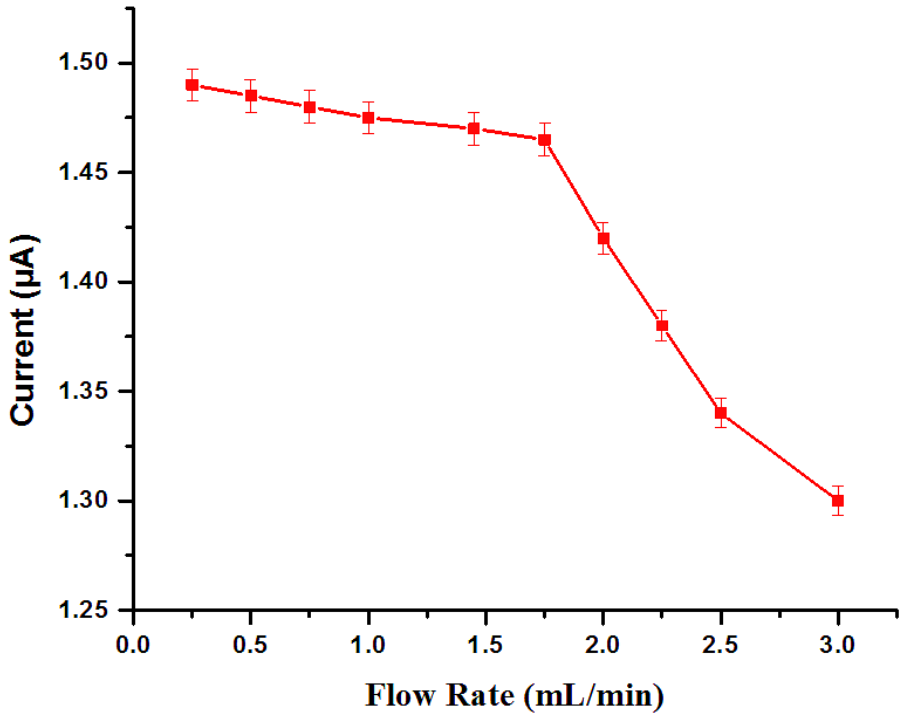


Fig 4.23: Plots of variation in current with varying rates of flow of electrolyte solution for mercury biosensors

Applying the optimized conditions of response time and flow rate, a calibration curve for Hg^{2+} ions was drawn in a concentration range of 10^{-14} M to 10^{-6} M using mercury selective

algae modified GC electrode. Experiment was repeated 5 times and a representative response of the electrode system in terms of current plotted against log [Hg^{2+}] is shown in Fig: 4.24.

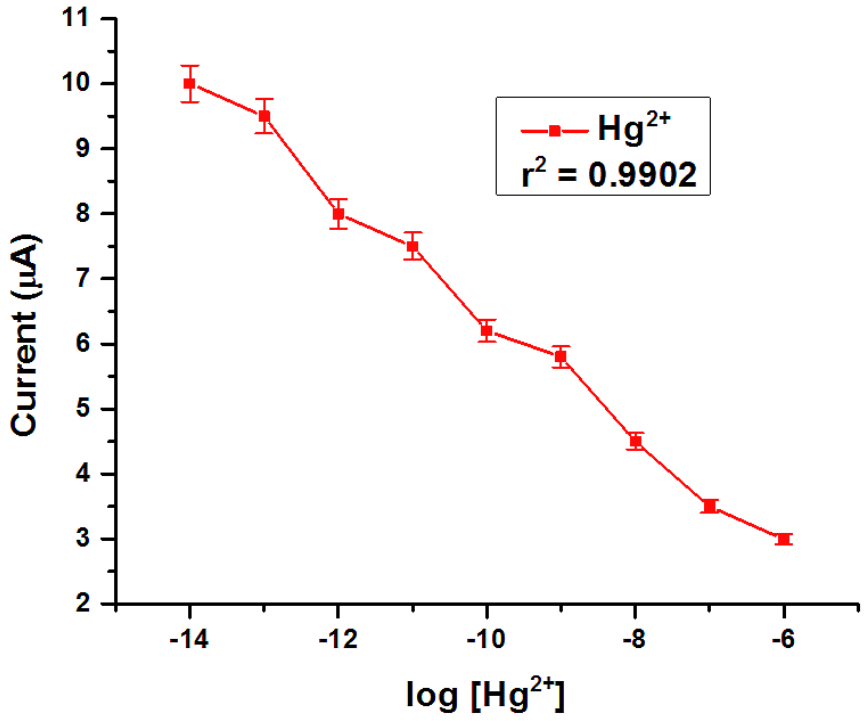


Fig 4.24: Calibration curve for mercury ions at a flow of 1.5 mL/min using mercury biosensor

From the calibration curve it can be seen that the mercury selective algae modified GC electrode can also be used for the determination of mercury in a flowing stream of aqueous media with a moderate flow rate of 1.5 mL/min. The calibration curve had a correlation co-efficient (r^2) value of 0.99. As discussed earlier also the algae modified GC electrode had no interference from any of the potential interferents and members of various groups of periodic table and can be used for Hg^{2+} ion determination in a continuous flow system.

Another experiment was conducted to check consistency of the current response of the biosensor for Hg^{2+} ion on successive addition of Hg^{2+} ions after regular intervals of time for in-situ and online determination of mercury ions in a flowing stream of aqueous media. Chronoamperometry was done on solutions having mercury ions running concentration of 10^{-14} M at a flow rate of 1.5 mL/min, the electrolyte medium was spiked with 100µL of 10^{-14}

M Hg^{2+} ion solution after every 120 seconds. The results were plotted (curve 'b' in Fig: 4.25) and compared with those containing no mercury ions (curve 'a' in Fig: 4.25).

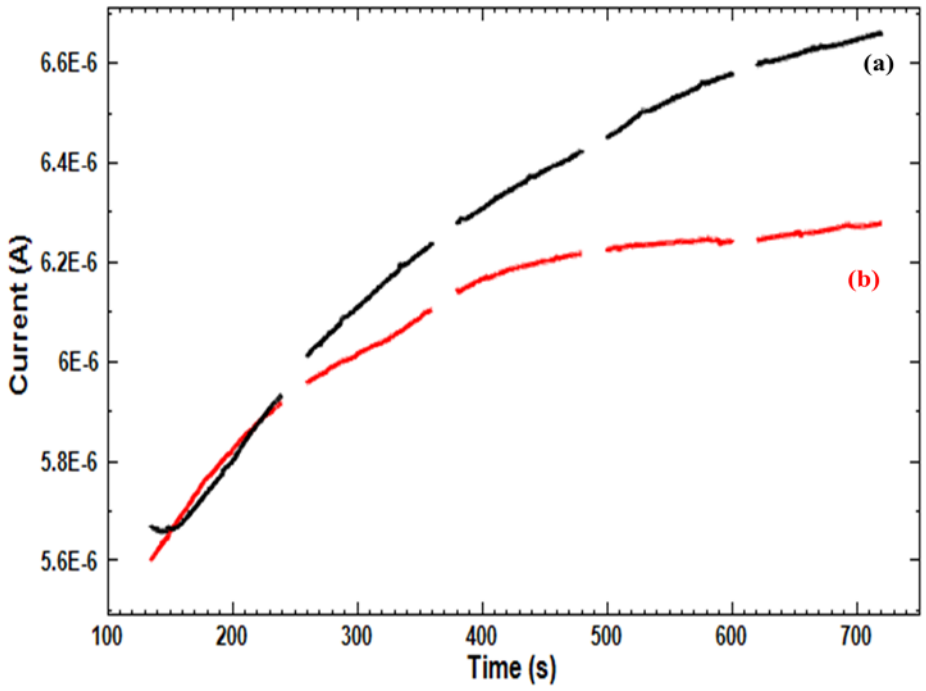


Fig 4.25: Chronoamperometric plots of mercury biosensor for electrolyte system with (b) and without (a) spiked concentration of mercury ions

The curves show a successive decrease in current with successive increase in Hg^{2+} ion content in the medium. This shows that the electrode can be used for determination of mercury at any given stage of the experiment and biosenstivity of the biosensor electrode for Hg^{2+} ions remain active throughout the experiment and algae modified GC electrode can be successfully employed for online and in-situ determination of mercury in flowing stream of aqueous media at moderate flow rate of 1.5 mL/min.

4.3.7 Conclusions

Following conclusions can be withdrawn from the above discussions:

1. The *Chlorella* sp. Modified GC electrode is selective for determination of mercury, having no interferences from its potential interferents like silver and heavy metal ions like zinc etc.
2. Prepared biosensor can detect ultra-trace quantity of mercury ions in aqueous media, having a lower detection limit of 10^{-14} M and detection range of 10^{-14} M to 10^{-6} M for mercury ions.
3. Prepared biosensor can also be used for continuous flow system for determination of mercury with same selectivity and sensitivity, with a variable flow rate of 0 – 1.5 mL/min. Current remains stable with a very small change in its magnitude upto 1.5 mL/min and decreases quite sharply with further increase in flow rate.

Similar trends in selectivity and sensitivity is achieved even in flowing stream of aqueous media having mercury ions and it can also be used for in-situ determination of mercury ions as biomaterial remains active throughout the experiment and shows decrease in magnitude of current with successive additions of mercury ions.

4.4 APPLICATIONS OF DEVELOPED BIOSENSORS

The developed biosensors can be used as sensitive and selective alternates to various traditional techniques (Webb et al, 1995; ckson et al, 1996; Burlingame et al, 1996)for measurement of various heavy metal ions. Beside these, the biosensor was tested with various real-time samples and cross verified using atomic absorption spectroscopy.

The prepared biosensors were put to use for analysis of some artificially prepared and real time samples collected from various sources. The results obtained from the different analyses are reported in following tables.

4.4.1 Real sample analysis for zinc using algae modified platinum biosensor

Some real time samples were collected from the laboratory drain and were filtered using fine filter paper for any kind of insoluble impurities. The real time samples were mixed with Tris-HCl as supporting electrolyte and $MgCl_2$ as enzyme activator and were analyzed by biasing the electrode at a biasing potential of 0.95V vs Ag/AgCl reference electrode. The results of metal determination using biosensors were compared with that using atomic absorption spectrometry.

It can be seen from the Table 4.3 that the algae modified Pt biosensor has a great deal of better sensitivity than atomic absorption spectrometer and results obtained by Pt biosensor are quite reproducible. The results clearly show that the sample one and two were having very high concentration of zinc, which can be due to dumping of various zinc salts being dumped in drainage after being used in the laboratories. The rest concentration of various heavy metal ions is quite negligible and can be linked with the same source.

4.4.2 Real sample analysis for zinc using algae modified glassy carbon biosensor

Some artificial samples were prepared by dissolving known concentration of various heavy metal ions and filtered insoluble impurities from the artificial samples using fine filter papers. The samples were mixed with Tris-HCl as supporting electrolyte and $MgCl_2$ as enzyme activator and were analyzed by biasing the modified electrode at a biasing potential of 0.85V vs Ag/AgCl reference electrode. The legitimacy of the results was tested using standard technique for heavy metal measurement technique like atomic absorption spectrometry.

It can be seen from the Table 4.4 that the algae modified GC biosensor has a better sensitivity than atomic absorption spectrometer and results obtained by GC biosensor are quite reproducible and comparable with the results obtained from atomic absorption spectrometer. The results clearly show that the sample one and two were having very high concentration of zinc, sample 3 is having high concentration of nickel, sample 5 is having copper, while sample 7 which can be due to dumping of various zinc salts having high concentration of cadmium ions. The concentration of samples for various ions obtained using GC based biosensor is almost in comparison with the one obtained with atomic absorption spectrometer. Hence, algae modified glassy carbon biosensor can be used for determination of various heavy metal ions, depending upon its relative selectivity, as discussed earlier.

4.4.3 Real sample analysis of mercury using algae modified glassy carbon biosensor

The reported glassy carbon electrode modified with algal cells is found selective for mercury, having no interference from any of its potential interferents like silver, copper, zinc, alkali and alkaline earth metals. So the reported biosensor can be used for determination of mercury in real time and artificially prepared samples.

Table 4.5: Real time samples collected from laboratory drainage and artificially prepared samples from mercury salts analysed using algae modified GC electrode and their comparison using atomic absorption spectrometry

S. No	Sample	Hg^{2+} (10^{-8} M)	
		AAS	Biosensor
1	AS1	450 ± 3	424 ± 5
2	AS2	380 ± 4	376 ± 2
3	AS3	420 ± 6	418 ± 3
4	AS4	450 ± 4	448 ± 3
5	RS1	3.5 ± 0.04	3.59 ± 0.05
6	RS2	8 ± 0.34	7.68 ± 0.15
7	RS3	3.6 ± 0.09	3.74 ± 0.14
8	RS4	4.6 ± 0.064	4.61 ± 0.02

The samples were collected from the laboratory drain (RS) and artificially prepared using known concentration of the mercury ions (AS) in them. Both the samples were mixed with

Tris HCl and $MgCl_2$ and were analyzed using algae modified GC biosensor. The results illustrated that the algae modified biosensor can be used for accurate and repeated determinations of mercury ions in given samples even at a very low concentration of metal ions. The results are quite in agreement with those from atomic absorption spectrometer. Hence, the developed biosensor can selectively sense mercury even at very low concentration without having any interference from any other metal ions.

The prepared biosensor was also tested for determination of mercury in a flowing stream. Artificially prepared sample were injected into an indigenously designed assembly having flowing system of Tris-HCl as supporting electrolyte, $MgCl_2$ as enzyme activator depicting a flowing stream having a moderate flow rate of 1.5 mL/min. The results thus obtained for determination of mercury ions in an aqueous sample were compared for authentication with atomic absorption spectrometer.

Table 4.6: Comparison of the reported mercury sensors based on different techniques with the proposed method

S. No	**Sample No.**	**AAS (10^{-8} M)**	**Batch method determination (10^{-8} M)**	**Flow system determination (10^{-8}M)**
1	Hg-1	2.5 ± 0.02	2.49 ± 0.05	2.46 ± 0.01
2	Hg-2	1.0 ± 0.02	1.02 ± 0.04	0.99 ± 0.03
3	Hg-3	0.35 ± 0.02	0.36 ± 0.02	0.34 ± 0.04

It is very clear from Table 4.6 that the biosensor is very sensitive and can determine even very low concentrations of mercury ions even in a flowing stream. The results using biosensor in a flow system are in complete agreement with those obtained from atomic absorption spectrometer and those using biosensor in a batch method. Hence, it can be said that the proposed biosensor can be used for determination of ultra-trace levels of mercury in a flowing stream of aqueous media and can solve a major problem of heavy metal determination i.e. in-situ determination of metal ions like mercury.

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4.6 LIST OF PUBLICATIONS

Accepted Papers

1. **S. K. Mittal, J. Singh**, Ashok Kumar SK, Chlorella modified glassy carbon electrode as whole cell microbial sensor for heavy metal ions, **Current Analytical Chemistry**, 365-372, 8, 2012. **(Impact Factor = 1.0)**
2. **J. Singh, S. K. Mittal,** Whole cell based amperometric sensor with relative selectivity of zinc ions, **Analytical methods**, 1326-1331, 4, 2012 **(Impact Factor = 1.855)**
3. **J. Singh, S. K. Mittal,** Chlorella sp. Based biosensor for selective determination of mercury in presence of silver ions, **Sensors and Actuators B**, 48-52, 165, 2012 **(Impact Factor = 3.896)**

Communicated Papers

4. **J. Singh, S. K. Mittal**, Modified glassy carbon biosensor for online monitoring of ultra-trace level mercury in continuous stream, **Biosensors and Bioelectronics**, Communicated **(Impact Factor = 5.602)**

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