ISFET based biosensor for heavy metal ions

A

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Under the Supervision of

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Date: 10-07-13

Place: Patiala

Candidate's Declaration

I, hereby declare that the work being presented in the thesis entitled **"ISFET BASED BIOSENSOR FOR HEAVY METAL IONS"**, in partial fulfillment of the requirements for the award of the degree of Masters in Chemistry, School of Chemistry and Biochemistry, Thapar University, Patiala, is my own work during the period of Jan 2013 to July 2013, under the supervision of Dr. Susheel Mittal, Senior Professor, School of Chemistry and Biochemistry, Thapar University, Patiala. I have not submitted the matter embodied in this thesis for the award of any other degree.

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

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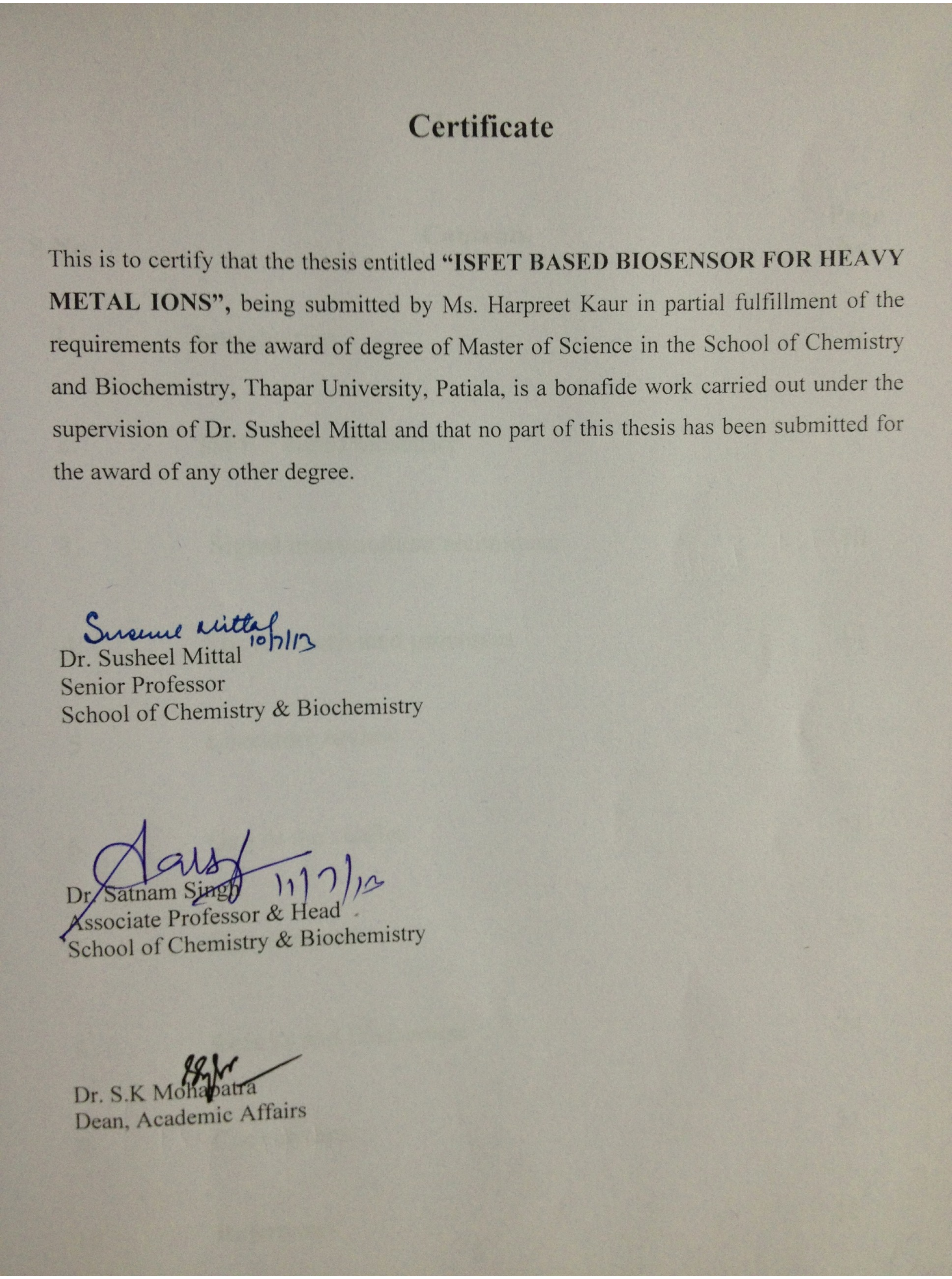

Certificate

This is to certify that the thesis entitled **"ISFET BASED BIOSENSOR FOR HEAVY METAL IONS",** being submitted by Ms. Harpreet Kaur in partial fulfillment of the requirements for the award of degree of Master of Science in the School of Chemistry and Biochemistry, Thapar University, Patiala, is a bonafide work carried out under the supervision of Dr. Susheel Mittal and that no part of this thesis has been submitted for the award of any other degree.

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1 INTRODUCTION

A biosensor is an analytical device which integrates a biological recognition element with a physical transducer to generate a measurable signal proportional to the concentration of the analyte [1]. The biological recognition element responds to the target compound and the transducer converts the biological response to a detectable signal, which can be measured electrochemically, optically, acoustically, mechanically, calorimetrically, or electronically, and then correlated with the analyte concentration [2]. The various biological recognition elements such as synthetic ionophores, supramolecular structures, micro-organisms, plant and animal tissues, cells, organelles, enzymes, antibodies, and various natural organic and inorganic molecules have been used in the fabrication of biosensors [3]. Among these biological elements, enzymes are the most widely used recognition elements due to their unique specificity and sensitivity [4]. However, the purification of enzyme is costly and time-consuming. In addition, the in vitro operating environment could result in a decrease of the enzyme activity. Microbes (e.g., algae, bacteria, and yeast) offer an alternative in the fabrication of biosensors because they can be massively produced through cell culturing. Also, compared to other cells from higher organisms such as plants, animals, and human beings, microbial cells are easier to be manipulated and have better viability and stability in vitro which can greatly simplify the fabrication process and enhance the performance of biosensors [5]. In order to effectively transform the biochemical response into a physical signal, the microbial cells, which serve as the recognition element in the biosensor, must be associated intimately with the transducer. Therefore, immobilizing microorganisms on transducers plays an important role in the fabrication of biosensors [4]. Transducers used are electrochemical (potentiometric, amperometric, conductimetric, voltammetric etc.), optical (dispersion, polarimetric, circular dichroism, scattering etc.), and colorimetric.

Biosensors are used for the analysis of different substances in the field of chemistry, biochemistry, medicine and biotechnology as well as for the control of the environment. They are characterized by compact dimensions, a short time of analysis, high sensitivity and simplicity in application, robustness, possibility of miniaturization and easy integration into hand-held devices. Their potential applications span from environmental and food analysis [12, 13], medical diagnostics [14], industrial process monitoring [15], to chemical and biological weapons detection [16].

Ion-sensitive field effect transistors (ISFETs) have found growing interest in the rapidly developing field of biosensors. Ion-selective field effect transistors or ISFETs were first developed in the 1970s as an alternative to the fragile glass electrode for the measurement of pH and concentrations of ions. ISFETs have the following significant characteristics and benefits:

(1) Resistant to breaking
(2) Can be stored dry and require little routine maintenance.
(3) Reduced acidic and alkaline errors in extreme pH ranges.
(4) Can be used over an extremely wide temperature range and are sterilizable for use in biological, medical and pharmaceutical applications.

In addition, ISFETs have very fast response, high sensitivity, and batch processing capability. What is most attractive is that ISFETs are a preferred transducing element for biosensors because the SiO_2 surface contains reactive SiOH groups, which can be used for covalent attachment of organic molecules and polymers. Also, ISFETs can be used to develop immunobiosensors.

The main problem, which should be solved when a biosensor is created, is the development of effective approaches for the immobilization of a biological material on the transducer surface. This is also applicable to the biosensors based on the ISFET. In this case an optimal way of immobilization should foresee not only preservation of the biological material activity but also its integration with the gate surface during the manufacture of the ISFET.

1.1 ISFET-based biosensors

Modification of the gate by various biological species makes it possible to construct biosensors [6]. A common development from ISFETs is enzyme-linked field effect transistors (ENFETs) [7]. Enzyme membranes are coated on the ion-selective gates of these electronic devices and the biosensor responds to the electrical potential change via the current output. Glucose oxidase was immobilized on the surface of ISFET for determination of glucose concentration in blood serum [8].

Advantages of immobilization for biosensors are:

- Biological stability, including plasmid stability
- Mechanical stability
- Chemical stability in the complex industrial effluent under consideration
- Thin films of entrapment matrix provide for rapid diffusion and short analysis times.

Algae have been immobilized successfully using bovine serum albumin and glutaraldehyde (GA) as a cross-linker [9]. The active membrane was formed by cross-linking algae with BSA in saturated GA vapours. This protocol was adapted from pure enzyme membranes used with some conductometric and ISFET biosensor [10]. Recent work has showed that yeast (*Saccharomyces cerevisiae*) can be of huge interest. Firstly as a source of enzymes (acid and alkaline phosphatases, esterases, dehydrogenases, etc.), secondly because they have their own sensitivity to pollutants and can be more adapted than algae for the detection of some compounds.

The use of micro-organisms for multi-detection is a good alternative, each living cell containing a large number of enzymes. For electrochemical detection, membrane bound enzymes are of particular interest since enzymatic reactions occur on cell surface. It has been shown that for *Chlorella vulgaris* microalgae, some alkaline phosphatases [11] and esterases [12] belong to the cell wall, their activities can then be monitored rapidly. They also enable electrochemical detection using conductometric biosensors as these two enzymatic reactions involve either consumption or production of charged species and, therefore, lead to a global change in the ionic composition of the sample.

One of the most important reasons for using living cells as biological component in biosensor is to obtain information about the effect of a stimulus on a living system. Whole cell biosensors provide the opportunity to elicit such information for applications such as pharmacology, cell biology, toxicology, and environmental measurements.

An advantage of using the whole cells instead of purified enzymes is that there is no necessity of isolation and purification of enzymes, the steps which are usually complicated and expensive. Another advantage is the low stability of some pure enzymes in comparison to those involved in their natural environments (in cytoplasm or cytoplasmic membranes, where better stability of enzymes is secured naturally). Also, whole cells have the capability to convert complex substrates using specific metabolic pathways. Whole cell biosensors are also employed for the monitoring of typical sum parameters, which cannot be monitored using enzyme-based sensors, such as toxicity, biological oxygen demand, nitrification inhibitors, xenobiotic compounds or heavy metals. Also, living cells are continuously repairing their enzyme activities and enzyme-cascades, accounting for long term stability of labile biological recognition element.

In order to improve the biosensors' response, living cells are suggested instead of purified enzyme. In fact, considered as complex biochemical plants, these cells grow, reproduce and communicate with the environment using biochemical and biophysical processes. Treated with drugs or toxic substances, they provide more information than enzyme-based biosensors and could be greatly useful for basic research and various biomedical and biotechnological applications [13]. This is why, in recent years, the development of whole-cell biosensors has been met with an increasing interest [14-17]. Whole cells have actually the possibility of converting complex substrates using specific metabolic pathways [18-20]. Unlike enzyme-based biosensors, whole-cell biosensors can be used to monitor typical parameters, such as toxicity [21-24], biological oxygen demand, nitrification inhibitors [25], xenobiotic compounds [26] or heavy metals [27]. Thus, they provide convenient early warnings or environmental screening capabilities that would otherwise require either a large number of specific sensors or time-consuming laboratory analyses [28]. Whole cell biosensors, based on living cells as molecular recognition elements, provide functional information, e.g., about signaling events, protein synthesis, apoptotic or necrotic cell death, that cannot be achieved with more conventional analytical techniques or with immunosensors and genosensors. Since the cell is a tightly regulated system, resulting biosensors give robust and reproducible signals [29] living cells are continuously repairing their integrated enzyme activities and cascades. This is undoubtedly an advantage with respect to an improved long-term stability of reliable biological recognition elements. Whole cells also provide a multipurpose catalyst especially when the process requires the participation of a number of enzymes in sequence. Whole cells can also measure the bioavailable concentration of a target analyte, i.e., analyte concentration able to pass through a cell membrane. In addition cells can be grown in two dimensional (2D) or three-dimensional (3D) cell-culture systems that can mimic in-vivo responses of animal tissues and can be incorporated in a sensor for multianalyte detection in an array format [30].

1.2 Signal measurement techniques

1.2.1 Electrochemical techniques

Amperometry is operated at a given applied potential between the working electrode and the reference electrode and the current signal is recorded and correlated with the concentration of target compounds. In the amperometric detection, the current signal is generated due to the reduction or oxidation of an electro-active metabolic product or intermediate on the surface of a working electrode [31].

Conductometry is a technique depending on the conductivity change in the solution due to the production or consumption of ionic species, for example, by the metabolic activity of the microorganisms [3, 32]. The measurement of conductance is extremely fast and sensitive. It is also worth noting that such biosensors are suitable for miniaturization since it requires no reference electrode in the system [32]. However, all charge carriers could result in the change of conductivity, thus the selectivity of conductometric biosensors is relatively poor [33].

Potentiometry involves the measurement of the potential difference between the working electrode and the reference electrode and the potential signal exhibits concentration-dependent behavior. The transducer employed in the potentiometric technique is usually a gas-sensing electrode or an ion-selective electrode [3, 34]. The sensitivity and selectivity of potentiometric biosensor are outstanding due to the species-selective working electrode used in the system. However, a highly stable and accurate reference electrode is always required and challenging to maintain, which may potentially limit the application of potentiometry in microbial biosensors.

Voltammetry is the most versatile technique in electrochemical analysis. Both the current and the potential are measured and recorded. The position of peak current is related to the specific chemical and the peak current density is proportional to the concentration of the corresponding species. A remarkable advantage of voltammetry is the low noise which can endow the biosensor with higher sensitivity. In addition, voltammetry is able to detect multiple compounds, which have different peak potentials, in a single electrochemical experiment (or scan), thus offering the simultaneous detection of multiple analytes. Furthermore, an effective pre-concentration step (electrochemical stripping analysis) makes the voltammetric technique one of the most sensitive electroanalytical methods [35].

1.2.2 Optical techniques

Optical detection is usually based on the measurement of luminescent, fluorescent, colorimetric, or other optical signals produced by the interaction of microorganisms with the analytes and correlates the observed optical signal with the concentration of target compounds. Optical sensing techniques are especially attractive in high throughput screening since they enable biosensors to monitor multiple analytes simultaneously [36].

Fluorescent sensing technique is based on the measurement of fluorescence intensity which is proportional to the concentration of the target analyte. Fluorescence can be detected at a longer wavelength after the excitation of the fluorescent substance at a shorter wavelength. Fluorescent biosensors have been widely applied in analytical chemistry due to their easy construction using standard molecular biology techniques [37].

Bioluminescent sensing technique used in microbial biosensors relies on detecting the change of luminescence emitted by living microorganisms which responds to the target analyte in a dose-dependent manner.

Colorimetric sensing technique in microbial biosensors involves the conversion of a chromogen substrate into a colorful compound by the metabolic activity of the microbial sensing element. The colored product can be distinguished by the naked eye or a spectrophotometer. Because of its simple and inexpensive measurement setup, colorimetric technique has been widely applied in the fabrication of cost-effective microbial biosensors [38].

1.3 Enzyme activated processes

Various microbes such as bacteria (*E.coli, Chlorella vulgaris)*, yeast (*Saccharomyces cerevisiae*), and fungi (*Aspergillus niger*) are employed in the fabrication of whole cell biosensors as their cell walls secrete certain useful enzymes such as phosphatases, pectinases, glucose oxidase, lactases, proteases, amylases, and cellulases. These enzymes are involved in conversion of various substrates to products yielding specific ions of particular concentration which are then detected by biosensor. The principle of operation of biosensor is based on the following type of reaction:

$$\text{Substrate} \xrightarrow{\text{phosphatase}} \text{Product} + PO_4^{3-}$$

ISFET with immobilized urease detects pH changes around the gate surface as a result of enzymatically catalyzed hydrolysis of urea according to the reaction:

$$(NH_2)_2CO + 2H_2O + H^+ \xrightarrow{\text{urease}} 2NH_4^+ + HCO_3^-$$

The pH changes induced by a spontaneous hydrolysis of D-glucono-d-lactone (produced from oxidation of D-glucose with the help of enzyme glucose oxidase) to gluconic acid can be registered with an ISFET.

$$\text{D-glucose} + O_2 \xrightarrow{\text{Glucose oxidase}} \text{D-glucono-d-lactone} + H_2O_2$$

Consumption of ascorbic acid during peroxidase catalyzed H_2O_2 reduction causes a local pH increase in the biomembrane which is registered with ISFET.

$$\text{Ascorbic acid} + H_2O_2 \xrightarrow{\text{peroxidase}} \text{dehydroascorbic acid} + H_2O_2$$

2 LITERATURE REVIEW

Ion-sensitive field effect transistors (ISFETs) have found growing interest in the rapidly developing field of biosensors. ISFETs were first developed in the 1970s as an alternative to the fragile glass electrode for the measurement of pH and concentrations of ions. ISFETs are not directly meant to replace the conventional pH glass electrodes, but to develop alternatives for measurement circumstances where glass electrodes could not be applied; measuring in ultra small volumes and with an extremely fast response. The selectivity and chemical sensitivity of the ISFET are completely controlled by the properties of the electrolyte/insulator interface. For example, many different types of oxide coatings of inorganic materials (e.g., SiO_2, Si_3N_4, Al_2O_3 or Ta_2O_5) can be used for obtaining a pH response. Protonation/deprotonation of the gate material is influenced by the pH at the gate area [39, 40], which controls the surface potential. The sensor response obeys the Nernstian law (59.2mV/pH). The response of an ion-selective electrode is given by:

$$E = E^0 + RT/zF \ln[i]$$

where E is the measured potential (in volts), E^0 is a characteristic constant for the ion selective/external electrode system, R is the gas constant, T is the absolute temperature (K), z is the signed ionic charge, F is the Faraday constant, and $[i]$ is the molar concentration of the free uncomplexed ionic species.

2.1 ISFET based biosensors

Modification of the gate by various biological species makes it possible to construct biosensors [6]. A common development from ISFETs is enzyme-linked field effect transistors (ENFETs) [7]. Enzyme membranes are coated on the ion-selective gates of these electronic devices and the biosensor responds to the electrical potential change via the current output. Glucose oxidase was immobilized on the surface of ISFET for determination of glucose concentration in blood serum [8]. Many affinity biosensors have been developed by immobilizing biomolecules on the surface of gates [41, 42].

2.1.1 DNA-based ISFET biosensor

DNA strands bind to the gate surface of ISFETs, changes in surface potential occur due to the negative charge of DNA, thereby allowing for excellent performance of ISFET in DNA sensing. As for methods of DNA detection, the most widely used techniques depend on enzymatic, fluorescent, and radiochemical tags. A label-free detection of DNA using a FET device with a real-time electrical readout system for rapid, cost-effective, and simple analysis of DNA samples has been proposed [44]. As an example of this, a detection platform based on an amorphous silicon-based ISFET for the label-free detection of covalent immobilization of DNA and subsequent hybridization of its complementary DNA was developed by Goncalves *et al* [45]. In this study, DNA binding behavior was monitored using an ISFET biosensor, which was observed as changes in the threshold voltage (V_{Th}). It was proposed that by using this ISFET system with the appropriate DNA probes, it is potentially feasible to detect single-base-pair mismatches, revealing the possibility of the sensitive detection of single nucleotide polymorphisms (SNPs), one of the most frequent genetic alterations in the human population [46]. Since SNPs are commonly believed to be associated with response to drug and disease outcome, one of the most valuable applications of SNPs would be a biomedical application such as disease diagnosis and therapeutics.

2.1.2 ISFET for Electro-Immunological Sensing

The pH sensitive ISFET is the most popular device as an immunosensor with a large range of insulators (SiO_2, Si_3N_4, Al_2O_3 and Ta_2O_5) [47]. Regarding bio-recognition elements, antibodies are the most commonly used capture agents, enabling the identification and quantification of individual analytes, due to the specificity of the antigen-antibody interaction. An immuno-ISFET is composed of an antibody recognizing antigen coated onto the gate, and can be applicable for clinical diagnosis, thereby being increasingly emphasized [48]. As an example of immuno-ISFET, Starodub *et al*. [49] developed an immunosensor based on ISFET for the determination of simazine, a s-triazine-type of herbicide.

2.1.3 Enzyme Based-ISFET biosensor

Enzyme FETs are based on the principle of pH-sensitive ISFETs in which the concentration of hydrogen ions during an enzymatic reaction is proportional to the level of substrate. So far, a variety of enzyme FETs have been developed for the detection of numerous analytes such as glucose, penicillin, urea, pesticides, phenolic compounds, steroidal glycoalkaloids, creatinine, etc. Application of ISFET as a penicillin-responsive device was first reported in 1980 [50]. Marrakchi *et al.* developed the ISFET-type trypsin biosensor for the determination of the substrate (BAEE, α-benzoyl-L-arginine ethyl ester hydrochloride) [51], thereby potentially showing the feasibility of the ISFET application to monitor small pentapeptides composed of five amino acids. As an additional example of enzyme-based ISFETs, an electrochemical bi-enzyme sensor for the quantitative protein determination has been proposed [52, 53]. Recently, Rebriiev *et al.* [54] described an ISFET based enzymatic biosensor for urea measurement, commonly referred to as ISFET urea sensor. In this work, a simple and rapid enzyme immobilization method on the ISFET gate surface was adopted. The developed ISFET urea sensor showed a considerable improvement in sensitivity, detection limit, and response time, as the linear response is in the range of 0.05–20 mM, and response time 5–10 min, suggesting that the developed ISFET urea sensor has potential for clinical application for the analysis of urea in blood samples. An enzyme-functionalized ISFET for ATP sensing was developed by Migita *et al.* [55]. The enzyme immobilized on a Ta_2O_5–modified-ISFET surface catalyzes the dephosphorylation of ATP, which is followed by the accumulation of protons at the gate because the enzymatic reaction produces H^+ as a by-product. The authors demonstrated that the ISFET response was highly proportional to the concentration of ATP in the given solution, suggesting that this ISFET ATP sensor might be effective enough to provide a real hygienic measurement. Several approaches such as the use of the additional charged polymeric membranes, and buffer solutions with low capacities have been attempted by Volotovsky *et al* [56].

2.1.4 ISFET for Monitoring Living Cell Responses

Living cells are utilized as their bio-contents, and the activity of living cells can be electrochemically monitored in a cell-based biosensor system. With these benefits, cell-based sensor systems have been considered as a promising technology for biomedical and pharmacological applications. Cell-based ISFET applications include some cellular phenomena such as cellular respiration and acidification which have been monitored simultaneously. Lehmann *et al*. reported pH-dependency in changes of the extracellular acidification and respiration rates under the same cell culture fluid [57]. ISFET operates as a pH sensor with a linear operating range of 2.5 V, threshold voltage of −1.5 V and a sensitivity of 46mV/pH. For the use of cells in a biosensor device, it is essential to localize the cells in the active area of the sensor, which can be achieved by different techniques such as dielectrophoresis (DEP) etc. The sensor consists of integrated DEP electrodes for cell positioning, ISFETs, and reference electrode [58].

2.1.5 Whole cell-based ISFET biosensor

A new whole cell *Aspergillus niger* amperometric biosensor for determination of glucose with enhanced upper linearity limit which was achieved by the presence of H_2O_2, was constructed [62]. The cells of *Saccharomyces* and *Pseudomonas* strains and of *Bacillus subtilis* were used for the construction of glucose amperometric biosensor [60-62]. Whole cells of *Gluconobacter oxydans* were employed in microbial sensor for xylose determinations using Clark-type electrodes. Bacterial cells were immobilised on chromatographic paper by simple physical adsorption method and attached to surface of electrodes. Physiological buffers showed little effect on biosensor function and responses were highly reproducible [63]. A conductimetric biosensor for the detection of acrylonitrile in solution was designed and characterised using whole cells of *Rhodococcus ruber*, which were immobilised into a disc of dimethyl silicone sponge. The biosensor was capable of the detection and quantification of acrylonitrile in aqueous solution, having a linear response to concentrations between 2 and 50 mM acrylonitrile [63]. A novel biosensor based on immobilised whole cell *Chlorella vulgaris* microalgae as a bioreceptor and interdigitated conductometric electrodes as a transducer has been developed and tested for alkaline phosphatase activity (APA) analysis. These sensors were also used for the detection of toxic compounds, namely cadmium ions, in aquatic habitats. Algae were immobilised inside bovine serum albumin (BSA) membranes cross-linked with glutaraldehyde vapours. The inhibition of *C. vulgaris* microalgae alkaline phosphatase activities in presence of cadmium ions was measured. The detection limit for the experiment was 1 ppb of Cd^{2+} [65]. For the sensitive determination of ethanol a new amperometric biosensor based on *Candida tropicalis* cells, which contains alcohol oxidase enzyme, immobilized in gelatin by using glutaraldehyde was developed. Ethanol determination is based on the assay of the differences on the respiration activity of the cells on the oxygenmeter in the absence and the presence of ethanol [66].

2.2 Biosensors based on sensing techniques

Toxic nature of heavy metals adversely affects ecology and living organisms, so there is a need to determine heavy metals at trace levels. Conventional methods such as atomic absorption spectrometry, X-ray absorption spectroscopy suffer disadvantage of high cost, need for trained personnel and laboratory bound tests i.e. cannot be used for field monitoring, complicated sample preparation and long measurement time period. Biosensors are now being utilized for toxicological measurement of various heavy metal ions. Based on sensing techniques, biosensors can be classified into two major groups: electrochemical and optical biosensors.

2.2.1 Electrochemical biosensors

2.2.1.1 Amperometric whole cell biosensors

Amperometry is the most widely used technique in electrochemical whole cell biosensors. Amperometric microbial biosensors have been extensively exploited for environmental applications [67-69]. A whole-cell amperometric sensing system for Cu^{2+} using FIA was developed based on the recombinant *S. cerevisiae* and an oxygen electrode [70]. *Acidithiobacillus ferrooxidans*, which is able to directly oxidize Fe^{2+} and $S_2O_3^{2-}$ during its aerobic metabolism,was immobilized on an oxygen electrode to construct an amperometric biosensor for the determination of Fe^{2+} and $S_2O_3^{2-}$ [71]. Several biosensors for glucose detection have been fabricated based on the oxygen consumption of the respiratory activity in the microbes. The microbial strains serving as the sensing element includes *Pseudomonas fluorescens* [72-76] and *Gluconobacter oxydans* [75, 77, 78]. All the transducers applied here were modified by either CNTs to enhance the amperometric response of the device. A whole cell based amperometric biosensor was fabricated using *Chlorella sp.* entrapped on the surface of platinum for detection of heavy metal ions such as Zn^{2+}, Cu^{2+}, Cd^{2+}, Co^{2+}, Ni^{2+} . The order of selectivity was $Zn^{2+} > Cu^{2+} > Cd^{2+} > Co^{2+} > Ni^{2+}$. The detection range was $10^{-9} – 10^{-11}$ M.

2.2.1.2 Conductometric whole cell biosensor

The conductometric microbial biosensor is attractive and appealing owing to its fast and sensitive response to the analytes. A conductometric biosensor using *C. vulgaris* microalgae as the bioreceptor was constructed to detect heavy metal ions and pesticides in water sample [79]. *C. vulgaris* was immobilized on a bovine serum albumin membrane deposited on a platinum interdigitated electrode (IDE). The developed biosensors were sensitive to Cd^{2+} and Zn^{2+} with a limit of detection of 10 ppb for both ions after a 30min exposure. A similar conductometric biosensor using *C. vulgaris* as the sensing element was fabricated to detect Cd^{2+} and the detection limit was as low as 1 ppb Cd^{2+} [80]. Another conductometric biosensor was constructed by entrapping lyophilized *Brevibacterium ammoniagenes* in the pH sensitive polyaniline on a Pt twin wire electrode to detect urea [81]. Hassan *et al* [99] developed biosensor for the detection of Cd^{2+}, Zn^{2+}, Hg^{2+}, Pb^{2+} and CN^{-} based on *Acidithiobacillus caldus* a sulphur oxidising bacteria. Toxicity was monitored by measuring changes in electrical conductivity and pH change. The sensor detects CN^{-} in range of 100-1000 ppb and 50 ppb for the metals. Similarly, Oh *et al* [100] fabricated SOB based biosensor for the detection of Cr^{6+} with detection limit of 5 ppb.

2.2.1.3 Potentiometric whole cell biosensor

Potentiometric whole cell biosensors detect the amount of analytes by measuring the potential difference between the working electrode and the reference electrode separated by a selective membrane. A potentiometric whole-cell biosensor based on ion-sensitive field effect transistor (ISFET) pH electrode has been successfully applied to the detection of *E. coli* K12 bacterial activity using a three-dimensional agarose as the immobilization matrix [82]. An NH_4^+ selective electrode in conjugation with potentiometry based biosensor was developed for monitoring Ni^{2+} using *Bacillus sphaerius* NTCC 5100 yielding urease enzyme as bioreceptor. The detection range reported was 0.002-0.04 ppb. Potentiometric biosensor for detection of Zn^{2+} ion was reported by Datta *et al* [101], *Pseudomonas striata* was used as biosensing element and cells were entrapped in the polyvinyl chloride matrix.

2.2.1.4 Voltammetric whole cell biosensors

Voltammetry such as cyclic voltammetry (CV) is another commonly used technique in electrochemical analysis. A voltammetric microbial biosensor for the determination of Cu^{2+} was reported [83]. The biosensor was based on *Circinella sp*. modified carbon paste electrode. Cu^{2+} was preconcentrated on the electrode and measured by CV, which was then interpreted as peak current to determine the concentration of the target analyte. Furthermore, a modified stripping voltammetry was proposed by taking advantage of both bacterial adsorption on a mercury surface and metal fixation capacity on A. *ferrooxidans* [84]. Whole cell based biosensor was prepared by immobilising *Chlorella sp*. microbes over glassy carbon electrode for detection of Hg^{2+} in the presence of Ag^{+} and the detection limit reported was 10^{-14}M [102].

2.2.2 Optical biosensors

2.2.2.1 Fluorescent whole cell biosensors

Chlorophyll has been employed as a fluorescent compound for anti-PSII herbicide detection [85]. Vegetal whole cells *C. vulgaris* were immobilized in an inorganic translucent sol-gel matrix. Chlorophyll could be metabolized by photosystem II (PSII) activity in the microbes. The presence of anti-PSII herbicide (e.g. diuron) would inhibit the electron transfer in the PSII and result in an increase of chlorophyll fluorescence emission. Another fluorescent biosensor [86] was based on the catabolism of contaminant compounds by specific bacterium and concomitantly reducing the redox indicator (e.g. resazurin) to provide the fluorescent signal in a whole-cell biosensor. The formation of reducing equivalents was enhanced in the assay when a recognized analyte entered the catabolic pathway, resulting in an increased fluorescent intensity. On the basis of this concept, toluene-degrading bacteria along with redox indicator resazurin were employed as the sensing element to detect toluene pollutant and a detection limit of 0.2mM was achieved.

2.2.2.2 Bioluminescent whole cell biosensors

A bioluminescent microbial biosensor measures the luminescence change emitted by living microorganisms. The luminescence change is, in fact, caused by lux gene-coded luciferase responding to the target analyte in a dose-dependent manner. Bioluminescent microbial biosensor based on luxCDABE marked *Acinetobacter sp*. bacterium was used to assay the toxicity of wastewater contaminated by heavy metals such as Zn^{2+}, Cd^{2+}, Fe^{2+}, Ni^{2+}, Co^{2+}, $Cr^{6+}$and Cu^{2+} [87]. The bacterial cells, *E.coli* and *Shigella sonnei* were used for the preparation of bioluminescence based biosensor for the detection of Cd^{2+}, Pb^{2+}, As^{5+} and Hg^{+}. The trends of sensitivity for the detection of heavy metals in case of *E.coli* was $As^{5+} > Hg^{+} > Pb^{2+} > Cd^{2+}$ and for *Shigella sonnei* it was $Hg^{+} > Cd^{2+} > Pb^{2+} > As^{5+}$. Roda *et al* [103] developed luminescence based Hg^{2+} based biosensor by utilizing transgenic *E.coli* with a detection limit of $1.67*10^{-13}$M.

2.2.2.3 Colorimetric whole cell biosensors

Colorimetric microbial biosensors involve the generation of colored compound which can be measured and correlated with the concentration of analytes. A whole-cell colorimetric biosensor was constructed for the detection of arsenite with high sensitivity [88]. Whole cells of *Flavobacterium sp.* were entrapped in glass fiber filter and applied for the detection of methyl parathion pesticide [89]. This disposable colorimetric biosensor was based on the OPH activity of *F. sp.*, which is able to hydrolyze methyl parathion into PNP. PNP was then quantified by measuring its absorbance at wavelength = 400 nm. The limit of detection was 0.3 μM methyl parathion.

2.2.2.4 Optical fibre based biosensors

Durrieu *et al* [104] developed optical fibre based biosensor for determination of Cd^{2+} and Pb^{2+} that employ alkaline phosphatase present on the membrane of *Chlorella vulgaris.* The sensor detected Cd^{2+} and Pb^{2+} in range of 0.01-1.0 mg/L [90]. Monk *et al* [91] and Marazuela *et al* [92] described various types of optical fibre based biosensors in different reviews. They classified optical biosensors on the basis of biorecognition element used for sensing.

3 GAP IN THE STUDIES

A lot of work has been done in the field of biosensors based on enzyme using various techniques such as amperometry, voltammetry, conductometry, optical devices and potentiometry. Comparatively less work is done whole cell biosensors based on ISFET. Our present work is based on whole cell biosensor based on ISFET using *Chlorella* sp. for the study of effect of heavy metal ion on the activity of ascorbic acid phosphatase enzyme bound on the surface of algae. Whole cell offer many advantages over enzymatic bioreceptor which includes low cost, easy handling, optimum stability of enzyme which is not possible in case of purified enzymes which are comparatively costly to obtain. Although, whole cells have low selectivity but they recognize a wide range of analytes, sometimes, multiple analytes at one time.

We have carried out study of effect of heavy metal on both free and immobilised (Ca-alginate beads and on gate surface of ISFET) whole cells. The enzyme ascorbic acid phosphatase acts on substrate 2-Phospho-L-ascorbic acid trisodium salt generating ascorbic acid. The generation of ascorbic acid is studied using ISFET as pH sensor and a reference electrode and voltage change is measured.

4 EXPERIMENTAL

4.1 Material

2-Phospho-L-ascorbic acid trisodium salt, $MgCl_2$, $CaCl_2$ were purchased from Sigma-Aldrich (India) and used without further purification. Sodium alginate was purchased from Loba Chemie (India). Hydrated salts like $Cd(NO_3)_2$, $Zn(NO_3)_2$, $Co(NO_3)_2$, $Ni(NO_3)_2$, $Cu(NO_3)_2$, $Hg(NO_3)_2$, $Pb(NO_3)_2$ and $Ag(NO_3)_2$ (analytical grade) were purchased from Sigma-Aldrich (India) and used as such.

4.2 Algae Culture

Algae *Chlorella sp.* was identified, cultured and sub-cultured in BG-11[93] media every three weeks. Algae was harvested by centrifugation and starved by suspending in phosphate free BG-11 media to induce maximum alkaline phosphatase activity.

4.3 Cell Immobilization and biosensor preparation

Cells of algae were immobilized on Ca alginate beads. Grown algae cells (1mL) were suspended in 5% (w/v) solution of sodium alginate. The mixture was pumped drop-wise into 2% (w/v) $CaCl_2$ solution and beads were obtained. Grown algae cells (5 μL)-Bovine Serum Albumin (BSA) were immobilized on the gate surface of ISFET after rubbing slightly the nearby area of gate and making it rough. ISFET was then placed in the environment of glutaraldehyde vapors [94] (used as a cross-linker) for 2 hours followed by 20 minutes of drying in air at room temperature (25^oC). The glutaraldehyde would react with amines provided by the BSA and shall not be available to react with microbes [95]. ISFET biosensor thus formed was then ready to use.

4.4 Instruments

All experiments were performed on ISFET pH sensor model no.3330, Agilent U8001A Single Output DC Power Supply (0-30 V, 3A) and Agilent U1233A True RMS Multimeter.

4.5 ISFET study

Experiment was done by adding *Chlorella* sp. (3% in case of free/immobilized on Ca alginate beads and 0.05% in case of immobilized cells on ISFET) in a solution containing 1mM $MgCl_2$ (enzyme activator). No Tris-HCl was required as buffer. 2-Phospho-L-ascorbic acid trisodium salt was added as a substrate with constant stirring. Ascorbic acid was generated as a product and voltage (V) was measured.

4.6 Heavy metal testing

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

5 RESULTS AND DISCUSSION

5.1 Principle of detection of heavy metal ions

Alkaline phosphatase enzyme present in the cell membrane is commonly used as a tool for the detection of heavy metal ions [96]. Even a trace amount of heavy metal ions would inhibit the activity of enzyme because enzyme has a tendency to complex with heavy metal ions to favor the formation of a stable metal enzyme complex [97]. The greater degree of inhibition by a metal ion species is due to greater stability of the metal enzyme complex. Mercury having nd^{10} $(n-1)$ f^{14} electronic configuration forms a stronger complex with enzyme than that of other heavy metal ions. This is also due to greater tendency of mercury to form back bonding with nitrogen containing ligands (present in enzymes) as compared to various other heavy metal ions (being located quite lower in group 12/II B, its nucleus experiences greater shielding and more polarizability of the outermost electrons). The selectivity for mercury is far more than other elements due to its tendency for greater covalency as compared to other similar elements [98]. The decreased activity would in turn lead to lesser reduction of the phosphate substrate resulting in lesser production of ascorbic acid. APA causes the production of electroactive ascorbic acid from electroinactive 2-Phospho-L-ascorbic acid trisodium salt which gets oxidized at electrode, leading to the flow of current. The decrease in magnitude of the current generated for a given concentration of microbial cells can be related to the concentration of heavy metal ions.

5.2 Optimization of incubation time

Enzyme activity measurement experiments were carried out at different durations of incubation time of the mixture solution. Samples were incubated from 0 to 72 hours to know the most stable incubation time. Results are shown in Figure 1. It can be seen that after 8 hours of incubation there is no further change in voltage of the solution on increasing the incubation time beyond 8 hours. All subsequent experiments were run with 8 hours incubation period. Experiments carried out with different samples showed same trend of stable output voltage.

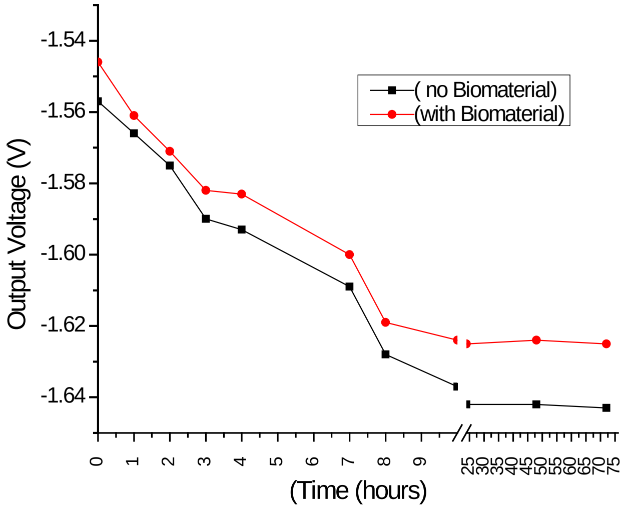

Figure 1: Voltage vs. incubation time plots of ascorbic acid at optimized concentration of *Chlorella* sp.

5.3 Optimization of biomaterial concentration

Different amounts of the biomaterial were taken in the solution and total volume was made up to 10.0 mL with water and the output voltage was measured with the help of ISFET, for each amount of the biomaterial. Results are given in Figure 2 and it is seen that voltage varies with increase in amount of biomaterial up to 0.3 mL/10.0 mL of the mixture solution. Beyond this concentration and up to 1.0 mL/10.0 mL, the voltage stays almost constant. Hence, 0.3mL can be taken as optimized concentration of biomaterial.

5.4 Optimization of substrate concentration

To understand the effect of different substrate concentrations on the enzyme release activity of the algae, experiments were conducted by taking different substrate concentrations in the range of 0.1 mg/ 10 mL to 1.0 mg/10 mL. From the results in Figure 3, it can be seen that minimum concentration of 0.3 mg/10 mL is required to get stable output voltage.

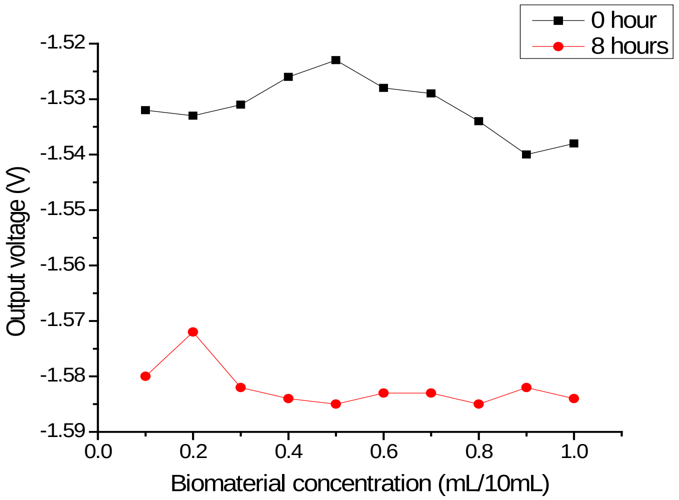

Figure 2: Voltage vs. concentration plots of ascorbic acid at different concentrations of *Chlorella sp.* after 0 hour and 8 hours incubation

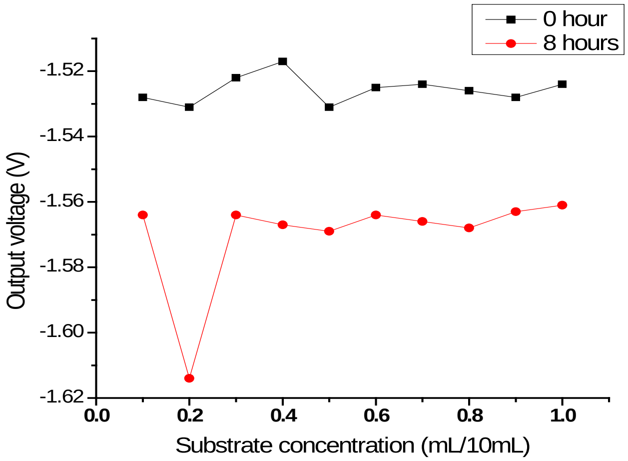

Figure 3: Plots of voltage as a function of substrate concentration for ascorbic acid phosphatase system using optimsed amounts of the *Chlorella* sp. at incubation time of 8 hours

5.5 Effect of heavy metals on the enzyme activity

It is well established that presence of heavy metals like transition elements- Zn, Co, Cd, Hg, Ni, Cu influence the enzyme activity of the biomaterial. But different metals affect the enzyme activity to different extents, probably due to difference in the stability of their respective complexes with the enzyme (as explained in section 5.1). Experiments were done in the presence of different heavy metal ions like Co^{2+}, Ni^{2+}, Cu^{2+}, Cd^{2+}, Co^{2+}, Hg^{2+}, Pb^{2+}, Ag^{+} and Zn^{2+}. Voltages for the ascorbic acid system were measured in the presence of each metal ion taken at the concentration of 10^{-1}M. Although trends of inhibition by different metal ions were not in regular pattern yet their toxicity towards the *Chlorella* sp. can be predicted. On the basis of measurements carried out with different metal ions at concentration of 10^{-1} M these can be arranged in decreasing order of their toxicity as given in Figures 4 and 5.

Order of microbial inhibition after 8 hours of incubation:

$$Hg^{2+} > Ag^{+} > Zn^{2+} > Cu^{2+} > Pb^{2+} > Cd^{2+} > Ni^{2+} \sim Co^{2+}$$

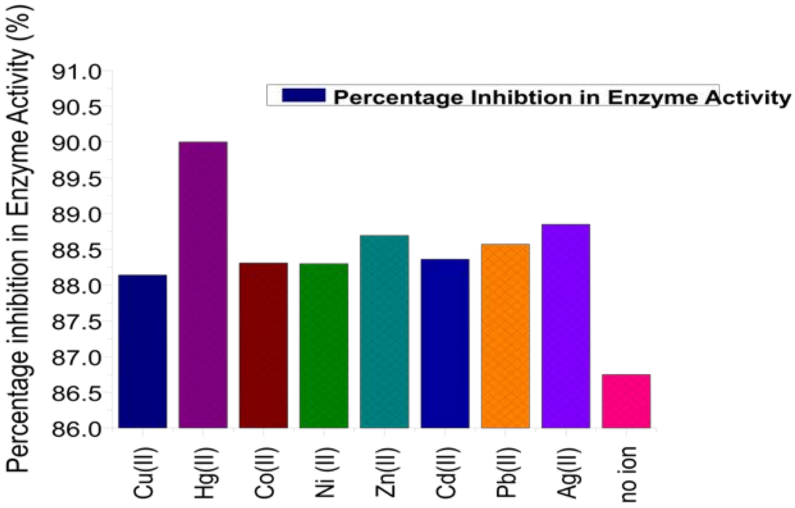

Figure 4: Extent of inhibition of enzyme activity by different heavy metals at a concentration of 10^{-1}M after 8 hours of incubation

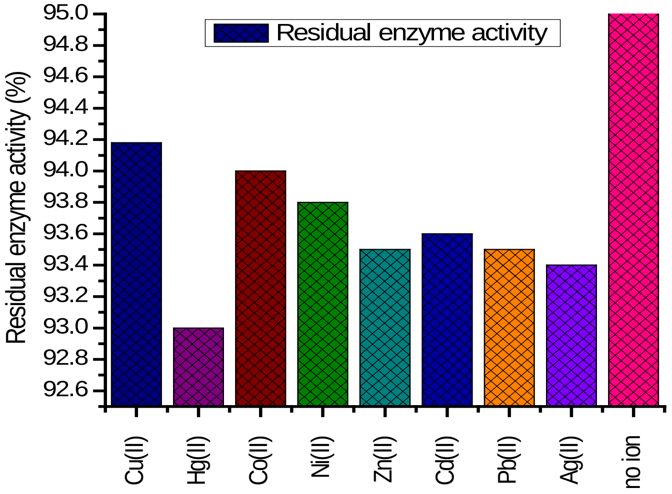

Figure 5: Extent of residual enzyme activity by different heavy metals at a concentration of 10^{-1}M after 8 hours of incubation.

Plots of voltage measurement were drawn against concentration of Hg^{2+} in the range of 10^{-8} to 10^{-4} M. Figure 7 shows trends of decrease in voltage with decreasing concentration of Hg^{2+}. The potential observed for the system containing no heavy metal ion was taken as 100% potential. All other potentials were measured with reference to this observed reading.

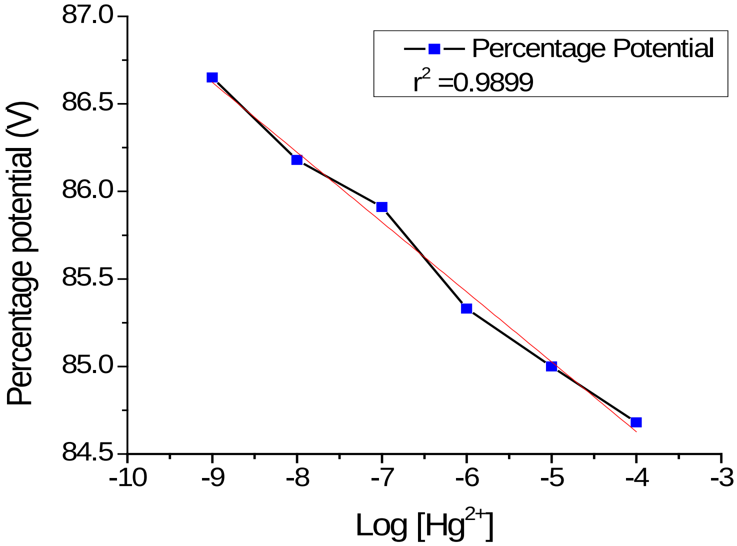

Figure 7: Plots of percentage potential vs. logarithm of Hg (II) ion concentration

5.6 Effect of heavy metals on enzyme activity in whole cell using Ca-alginate beads

Ca-alginate beads were generated by precipitation when sodium alginate mixed with biomaterial was added to a cold solution of $CaCl_2$. The Ca-alginate beads thus prepared contained biomaterial encapsulated in its core. Experiments were done in the presence of different heavy metal ions like Co^{2+}, Ni^{2+}, Cu^{2+}, Cd^{2+}, Co^{2+}, Hg^{2+}, Pb^{2+}, Ag^{+} and Zn^{2+}. Voltages (corresponding to ascorbic acid) were measured with each metal ion at the concentration of 10^{-1} M. Although trends of inhibition by different metal ions were not in regular pattern yet these can be arranged in the decreasing order of their toxicity towards the *Chlorella* sp. encapsulated in the core of Ca-alginate beads. On the basis of measurements carried out with different metal ions at concentration of 10^{-1} M these can be arranged in decreasing order of their toxicity as given in Figure 8.

Order of inhibition after 8 hours incubation using Ca-alginate beads:

$$Hg^{2+} > Ag^{+} > Zn^{2+} > Cu^{2+} \sim Pb^{2+} > Cd^{2+} > Ni^{2+} \sim Co^{2+}$$

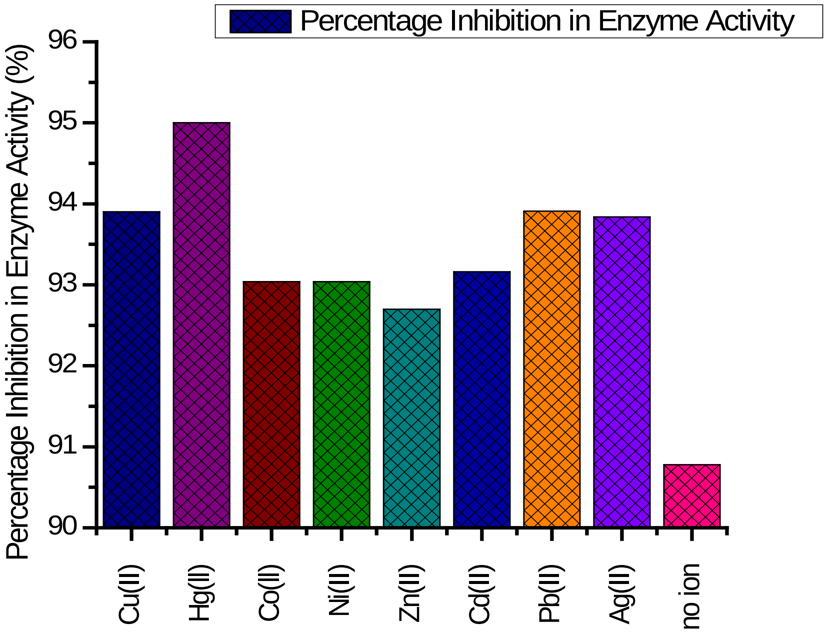

Figure 8: Extent of inhibition of enzyme activity by different heavy metals (10^{-1}M) using Ca-alginate beads after 8 hours of incubation

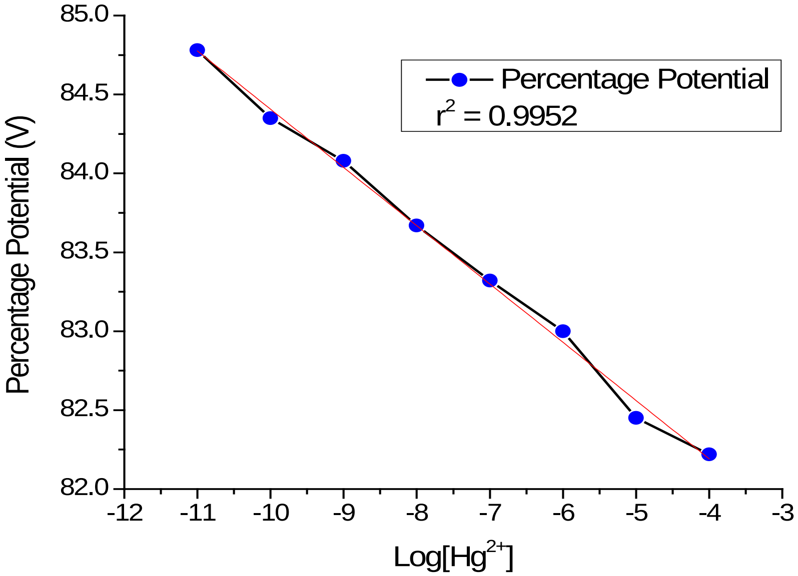

Figure 9: Plot of percentage potential vs. logarithm of Hg (II) ion concentration in the range of 10^{-11} to 10^{-4} M using Ca-alginate beads

Plots of voltage measurement were drawn against concentration of Hg^{2+} in the range of 10^{-11} to 10^{-4}M. Figure 9 shows trends of decrease in voltage with increasing concentration of Hg^{2+}. The potential observed for the system containing no heavy metal ion was taken as 100% potential. All other potentials were measured with reference to this observed reading.

6 CONCLUSIONS

Effect of heavy metals on the enzyme activity of whole cells (free and encapsulated biomaterial) was studied by using ISFET (Ion Sensitive Field Effective Transistor) as pH sensor. The parameters such as concentration of biomaterial, substrate and incubation time were optimized to get desired results of change in potential. In presence of heavy metal ions the activity of enzymes present in whole cell membrane was decreased caused the change in potential measured with ISFET. All metal ions showed the effect on enzymatic activity causing it to decrease, but the extent of inhibition was found different with different metal ions. Inhibition of enzymatic activity by different heavy metal ions have been observed as:

$$Hg^{2+} > Ag^{+} > Zn^{2+} > Cu^{2+} \sim Pb^{2+} > Cd^{2+} > Ni^{2+} \sim Co^{2+}$$

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