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MICROBIAL BIOSENSOR OF PHENOLIC COMPOUNDS DETECTION USING CHITOSAN-Fe_3O_4 BIONANOCOMPOSITE

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By

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APPROVAL PAGE

This is to certify that I have read this thesis and that in my opinion it is fully adequate, in scope and quality, as a thesis for the degree of Master of Science.

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January 2014

MICROBIAL BIOSENSOR OF PHENOLIC COMPOUNDS DETECTION USING CHITOSAN-Fe3O4 BIONANO COMPOSITES

RabiuGarba AHMAD

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ABSTARCT

The main aim of this work is to develope microbial biosensor based on chitosan-Fe_3O_4 magnetic nanoparticles as a support matrices for immobolization of *E-coli TG1 pBS (kan-) ToMO*haboring the plasmid of Toluene-o-xyelenemonoxegenase (ToMO) onto gold-plate working electrode to detect phenolic compounds by amperometric biosensor. The presence of large surface area by Fe_3O_4 magnetic nanoparticles and the possession of porous morhophology of chitosan caused a high loading of bacterial cells which entrapped, leading to the bacterial cells to retain its bio-activity covalently onto the electrode, which creates and enhanced a fast electron transfer by the bio-catalysis activity of the magnetic nanoparticles. After the bacterial cells were immobolized in this system, the detection of phenolic compounds were investigated by the response of an amperometric.Cyclic voltammetry was conducted to determined the characterestics of bionanocomposite of the sensor.Different parameters such as working potential,pH and temperature were studied and optimised, reuseabilty as well as storage stability were also investigated. Hence, the proposed biosensor can be used in many applications for the determination of these phenolic compounds.

Keywords; Microbial biosensor, Microorganisms, phenolic compounds, chitosan, Fe3O4 nanoparticles

KİTOSAN-Fe_3O_4 BİONANO KOMPOZİTLERİ KULLANAN MİKROBİYAL BİOSENSÖRLER İLE FENOLİK BİLEŞİMLERİN TESPİTİ

Rabiu Garba AHMAD

Yüksek Lisans Tezi Genetik ve Biyomühendislik

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ÖZ

Bu çalışmanın ana amacı, Toluene-o-xyelenemonoxegenase (ToMO) plazmidi içeren *E-coli TG1 pBS (kan-) ToMO*yu kitosan-Fe_3O_4 nanoparçacıklarınıimobilizasyon için destek olarak kullanan, altın kaplı elektrod aracılığı ile fenolikbileşikleri tespit edebilen mikrobiyal bir amperometrikbiosensörgeliştirmektir.Manyetik Fe_3O_4nanoparçacıklarının büyük yüzey alanı ve kitosanın gözenekli morfolojisi yüksek miktarda bakteri hücrelerinin hapsolmasını sağlamakta ve bu vesile ile elektrodla yüksek kovalentbiyoaktivitesi gösterip, manyetik nanoparçacıkların yüksek elektron transferioluşturmasıylageliştirdi.Bakteri hücrelerinin bu sistem ile immobilizasyonundan sonra, amperometrik tepkiye dayanarak fenolik bileşiklerin tayini yapıldı. Biyonanokompozitinkaraketeristiklerini belirlemek için voltametri ölçümleri yapıldı. Çalışma potansiyeli,pH ve sıcaklık dahil olmak üzere çeşitli parametreler incelendi ve en uygun hale getirildi.Yeniden kullanılabilirlik ve depolama stabilitesi de incelendi. Sonuç olarak, sunulan biosensörün birçok uygulamada fenolik bileşiklerin tespiti için uygun olduğu bulundu.

Anahtar Kelimeler; Mikrobiyalbiosensör, microorganizmalar, fenolik bileşimler, kitosan, Fe3O4 nanoparçacıkları

To my beloved family

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SYMBOL/ABBREVIATION

Ag	Silver
AgCl	Silver chloride
CHTSN	Chitosan
E-coli	*Escherechia coli*
Fe3O4	Iron oxide
mL	Millilitre
mM	Milli molar
PBS	Phospahte buffer solutions
Pt.	Platinum
ToMO	Toluene o-xylene monoxegenase
µA	Micro ampare
µL	Micro litre
µM	Micro molar

CHAPTER 1

INTRODUCTION

1.1 BACKGROUND

Phenols and their derivatives commonly present in the environment and these compounds are used as the components of dyes, polymers, drugs and other organic substances.The presence of these compounds in the ecosystems is also related with production and degradation of numerous pesticides and the generation of industrial and municipal sewages [1]. Phenolic compounds are among the environmental pollutants and many of them are very toxic. Phenols are known to provide adverse effects such as reduced growth, reduced resistance against diseases [2]. The concentrations of phenol in surface water are different. In natural waters its amounts are between 0.01 – 2.0 μg/L . Relative fast degradation of phenol causes its concentration in waters exposed to strong anthropogenic pollution may be comparable. Concentration of phenol in surface water of Netherlands were of 2.6 – 5.6 μg/L. River water polluted with sewage derived from petrol processing plants contained the concentration of phenol over 40 mg/L. Phenol was also found in domestic water supply in the USA at a level of 1 μg/L [1]. Therefore increase in the release of a diverse range of hazardous compounds into the environment has made their detection very importance. In particular, for a successful clean-up of pollution from the environment and timely elimination of the consequences or a reduction of the scale of hazardous release this is necessary [3]. Phenolic compounds are one of the major pollutants of many industrial waste waters and, at the same time, the compounds have high toxicity to the human health when present above certain concentration limits,as such this require rapid, easy to operate and low-cost toxicity screening procedures [3].Usually phenols are detected using various spectrophotometric and chromatographic methods [2].Detection of phenolic compounds

in the environment is of great importance due to their toxicity and persistency in the environment [4].

Phenols, cresols, guaiacols and catechol are among the polyphenolic compounds that have been identified in industrial effluents. Therefore, there are interests in its determination and is very important due to their toxicity and persistency in the environment [5]. Phenols are important raw materials and byproducts in large-scale chemical industry, some of which are highly toxic. Many of the phenolic compounds are resistant to biotic and abiotic degradation. As a result, their wide existence in the environment causes an undesirable ecological effects[6]. Phenolic compounds are one of the most widely products present in biological degradation processes. When phenols are presence in food, for example, is an indication of lack of freshness.Mining, paint, plastic or pharmaceutical and some others industries, produce phenols that can be found in their wastewater. Some phenolic compounds are priority-considered pollutants in water due to their toxicity, even at low concentration levels, for humans and aquatic organisms. In the other way round, some phenols are used in pharmacopoeia, as for example, 4-acetamidophenol, widely used under the name of Paracetamol, present in many drug formulations.Common determinations, as the spectrophotometric reaction with 4-aminoantipirine, give rised a total phenol value, not permitting the identification of different phenols. Phenolic compounds make their separate quantification a hard problem to be solved due to similarity in reactivity and properties. As a result of this particular problem, there is a need of a heavy laboratory set-ups, such as HPLC or GC-MS which are useful for the separate quantification of phenolic compounds [7].

Phenolic compounds, are the polluting chemicals, that are widely distributed throughout the environment as by-products contaminant from various ground and surface water sources. High toxicity and persistence of these chemicals in the environment are a potential hazard to health in living organism of animals including human if absorbed. There-fore, the there is interests of selective and sensitive detection of phenolic compounds which have become important. In the detections of phenolic compounds, various standard procedures exist to monitor the chemicals, such as gas chromato-graphic, liquid chromatographic (HPLC), and spectrophotometric analysis. However, these techniques are expensive, non-portable, time consuming, need trained experts and and also require sample pre-treatment [8].

A biosensor is an analytical device that combines a biological sensing element wih a transducer to produce a signal proportional to the analyte concentration [9]. So also a microbial biosensor is an analytical device which integrates microorganism(s) with a physical transducer to generate a measurable signal proportional to the concentration of analytes. In recent years, a large number of microbial biosensors have been developed for environmental, food, and biomedical applications [10]. The development of sensors, which are easy and highly selective to handle opens the door to the many problems of analysis. Biosensors have found a promising applications in various fields, which include biotechnology, food and agriculture products processing, health care, medicine, and pollution monitoring [11].

Biosensors make use of a variety of transducers such as electrochemical, optical, acoustic and electronic. The function of a biosensor depends on the biochemical specificity of the biologically active material. In biosensor the choice of the biological material will depend on a number of factors which include the specificity, storage, operational and environmental stability [12].The selection also depends on the analyteto be detected such as chemical compounds, antigens, microbes, hormones, nucleic acids or any subjective parameters like smell and taste. The biological sensing elements which have been used in biosensor are enzymes, antibodies, DNA, receptors, organelles and microorganisms as well as animal and plant cells or tissues [12]. The use of microbial biosensors to determine the concentrations of substances is based on the presence of specific enzyme systems in microorganisms which transform certain chemical compounds. The transformation processes can be accompanied by the appearance of electrochemically active products or utilization of reaction co-substrates, which enable the use of standard electrochemical techniques such as amperometryor potentiometry [3]. In biosensor, the important point to improve the microbial biosensor is to increase the sensitivity towards the toxin. This can be achieved by using the suitable bacterial strains which are sensitive to toxin. Recently, the direct electrocatalysis towards glucose with E. coli as a biocatalyst after a MFC-evolved process has been reported [13].

Microbial sensors are similar to enzymebased sensors but are less selective by their sensitivity, time of response and stability of signals.This may be due to the complexity of the elements of the enzyme apparatus of cells. The use of microbial

sensors are preferable in some cases compared to enzyme sensors due to insignificant amount of biomass as well as high stability. This is especially true in the detection of a pool of toxic compounds showing similar composition, or the assessment of comprehensive indices of the condition of the environment as, for instance, biological oxygen demand (BOD) [11].

Recent studies demonstrated that, nanoparticles enhance the electrochemical reactivity of important biomolecules and can promote the transfer of electron in the reactions of proteins [14]. The use of superparamagnetic nanoparticles have been widely used in many applications in the fields of biology and medicine for different purposes, such as protein and enzyme immobilization, bio-separation, immunoassays, hyperthermia, drug delivery, magnetically improved transfection, tissue engineering and MRI. Iron oxides, such a smagnetite (Fe3O4), are commonly used for the synthesis of magnetic nanoparticles.They have stable magnetic response, which include biodegradable and biocompatible, and also have superparamagnetic effects on magnetic response imaging (MRI) [15]. Magnetic nanoparticles also have been widely used for biocatalysis, bio-labelingand separation or purification of biomacromolecules [9].

Microbial cells have been immobilized to used as a promising approach in constructing biosensors as the basis of the recognition elements. Some of the advantages in using microbial cells as biosensors includes: there is no need of enzymes to be isolated, microbial cells are more tolerant to inhibition by solutes and sub-optimal pH or temperature changes. Furthermore, the durability of microbial electrodes are more higher than enzyme electrodes because the enzymes are more stable in their natural environment in the cells [14]. Additionally, microorganisms have been widely used in biotoxicity assay because of their high sensitivity, easy manipulation and low cost [16]. Many microorganisms can be able to mineralize a variety of aromatic compounds as sole sources of carbon and energy [17] and these aromatic compounds are converted into an intermediate of a major central metabolic pathway by a series of aromatic catabolic reactions in these organisms. The use of *Escherichia coli* to produce recombinant proteins is common due to its fast growh to high densities on inexpensive media and its genetics characterestics are well understood [18]. Toluene monoxegenases are capable in hydroxylations of many aromatic compounds. ToMO is endowed with a

broad spectrum of substrate specificity and ability to hydroxylate more than a single position of the aromatic ring in two consecutive monooxygenation reactions [19].

In this work, we constructed a biosensor using Bacterial cells E-coli TG1 pBS (kan) ToMOharbouring plasmids of Toluene-0-xylene monoxegenase on gold-plate electrode via chitosan and magnetic nanoparticles as immobolization matrices.

1.2 ADVANTAGES OF USING MICROORGANISMS AS BIOSENSING ELEMENTS

Over the last decade, bio-molecules or whole cells systems immobolization for construction of biosensor were frequently used in various domains such as bio-medicine, environmental toxicity, pharmacy, and food industries.The use of living cells or sensing elements afford the opportunity for developing a high sensitive and specific device for detection of wide range of biologically active substances that affects the cells response [20]. In biosensor, enzymes are the most widely used biological sensing element in the fabrication. Although purified enzymes have very high specificity for their substrates or inhibitors, their application in biosensors construction may be limited by the tedious, time-consuming and costly enzyme purification, requirement of multiple enzymes to generate the measurable product or need of cofactor/coenzyme.Therefore, microorganisms provide an ideal alternative to these bottle-necks [9]. Many microorganisms are capable of mineralizing a variety of aromatic compounds as sole carbon and energy sources. In these organisms, aromatic compounds are converted into an intermediate of a major central metabolic pathway by a series of aromatic catabolic reactions [17]. Insignificant amount of biomass as well as high stability makes the use of microbial sensors preferable in some cases compared to enzyme sensors. This is especially true in the detection of a pool of toxic compounds showing similar composition, or the assessment of comprehensive indices of the condition of the environment as, for instance, biological oxygen demand (BOD) [21] . Instead of purified enzymes, whole cells can be used.Cells anchoring and displaying the specific enzyme onthe cell surface offer the advantage of eliminating the diffusion mass transport resistance due to the cell walls enveloping the biocatalyst [22]. The many enzymes and co-factors that co-exist in the cells give the cells the ability to consume and hence detect large number of chemicals; however, this can compromise the

selectivity. Microorganisms can be easily manipulated and adapted to consume and degrade new substrate under certain cultivating conditions. In the fabrication of biosensors, microbes have many advantages as biological sensing materials. They are present ubiquitously and are able to metabolise a wide range of chemical compounds.

Microorganisms have a great capacity to adapt to adverse conditions and to develop the ability to degrade new molecules due to their high sensitivity with time, and also serve as an economical source of intracellular enzymes, this is because they are amenable for genetic modifications through mutation or through recombinant DNA technology [12, 20]. Application of whole microbial cells can be very attractive when oxidations are involved since cofactor and recycle systems form part of the cells' metabolism. Whole cell microbial sensors have received recent attention; because enzyme purification is unnecessary, whole microbial sensors are simple and inexpensive systems to construct andenzymes are usually more stable in their natural environment in the cell [23]. To date, over 90% of the enzymes known are intracellular and in this respect, the utilisation of whole cells as a source of intracellular enzymes has been shown to be a better alternative to purified enzymes in various industrial processes [12].

Additionally, the progress in molecular biology/recombinant DNA technologies has opened endless possibilities of tailoring the microorganisms to enhance the activity of an existing enzyme or express foreign enzyme/protein in host cell. All of these make microbes excellentbiosensing elements [9].

1.3 IMMOBOLIZATION OF MICROORGANISMS

The close contact between microorganisms and the transducer is the basic of a microbial biosensor . Thus, fabrication of a microbial biosensor requires immobilization on transducers with a close proximity. Immobilization technology plays a very vital role, since microbial biosensor response, operational stability and long-term use are, to some extent, a function of the immobilization strategy used, and the choice of immobilization technique is critical [9]. Additionally, the basic requirement of a biosensor is that the biological material should bring the physico-chemical changes in close proximity of a transducer. In this direction, immobilisation technology has played a major role [12].Immobilisation not only helps in forming the required close proximity

between the biomaterial and the transducer, but also helps in stabilising it for reuse. The biological material has been immobilised either directly on the transducer or in most cases, in membranes, which can subsequently be mounted on the transducer. Microorganisms can be immobilized on transducer or support matrices by either chemical or physical methods [9].

1.3.1 Chemical method of immobolization

In chemical methods of microbe immobilization, this can be a covalent binding or cross-linking. Covalent binding methods involves the formation of a stable covalent bond between functional groups of the microorganisms's cell wall components such as amine, carboxylic or sulphydryl and the transducer such as amine, carboxylic, epoxy and so forth. This goal can be achieve, when whole cells are exposed to harmful chemicals and harsh reaction condition, which may damage the cell membrane and decrease the biological activity. How to overcome this drawback is still a challenge for immobilization through covalent binding. But, this method has therefore not been successful for immobilization of viable microbial cells [9]. Cross-linking using bifunctional reagents like glutaraldehyde has been successfully used for the immobilisation of cells in various supports. Of these, proteinic supports such as gelatine, albumin and hen egg white have been extensively used. Even though this technique obviates some of the limitations of covalent binding, the chemical cross-linking reagents used often affect the cell viability. Therefore, in obtaining immobilised non-viable cell preparations containing active intracellular enzymes, cross-linking technique will be very useful [12].

1.3.2 Physical method of immobolization

In physical method, adsorption and entrapment are the two widely used for microbial immobilization. These methods are preferred when viable cells are required because the methods do not involve covalent bond formation with microbes and provide relatively small perturbation of microorganism native structure and function [9].

Physical adsorption is the simplest method for microbe immobilization. In this method, a microbial suspension is incubated with the electrode or an immobilization matrix, such as alumina and glass bead , followed by rinsing with buffer to remove un adsorbed cells. The microbes are immobilized due to adsorptive interactions such as

ionic, polar or hydrogen bonding and hydrophobic interaction. However, generally this method leads to poor long-term stability because of desorption of microbes, when using adsorption immobolization alone. Entrapment method of immobilization of microorganisms can be achieved by the either retention of the cells in close proximity of the transducer surface using dialysis or filter membrane or in chemical/biological polymers/gels such as (alginate, carrageenan, agarose, chitosan, collagen, polyacrylamide, polyvinylachohol, poly(ethylene glycol), polyurethane, etc [9].One of the most disadvantage of entrapment immobilization is the additional diffusion resistance offered by the entrapment material, which will result in lower sensitivity and detection limit.

Chitosan as a biopolymer and because of its biodegradability, nontoxicity, and biocompatibility make it a promising matrix for enzyme immobolization. The presence of amino and two free hydroxyl groups for each carbon six (carbon-6) building unit facilitate covalent binding of enzyme [24]. Additionally, amino groups make chitosan a cationic polyelectrolyte (p*K*a≈ 6.5), one of the few found in nature. This basicity gives chitosan singular properties: chitosan is soluble in aqueous acidic media at pH <6.5 and when dissolved possesses high positive charge on –NH3 + groups, it adheres to negatively charged surfaces, it aggregates with polyanionic compounds, and chelates heavy metal ions [25]. Both the solubility in acidic solutions and aggregation with polyanions impart chitosan with excellent gel-forming properties.

Chitosan is an ideal support for enzyme immobilization due to its hydrophilicity, biocompatibility, biodegradability and antibacterial properties. This biopolymer can be physically modified to give different forms (e.g., powder, nanoparticles, gel, film, and beads), crosslinked with different substances (e.g., glutaraldehyde, car-bodiimide, epichlorohydrin, and tripolyphosphate) and used in various fields of application including the construction ofbiosensors [26]. Chitosan (CHIT), the primary derivative of chitin, is obtained by N-deacetylationto a varying extent that is characterized by the degree of deacetylation, and is consequently a copolymer of N-acetyl glucosamine and glucosamine. Increasingly over the last decade, chitin- and chitosan based materials have been examined and a number of potential products have been developed for areas such as wastewater treatment, the food industry, agriculture, pulp and paper industry, cosmetics and toiletries, medicine and biotechnology, in biosensors as an

immobilization platform [23].Magnetic fields have been utilized in support systems to study enzyme immobilization. Several magnetic particles and magnetic supports such as microspheres of various biomaterials encapsulating the magnetic particles and copolymers with magnetic particles have been used with good results [27].

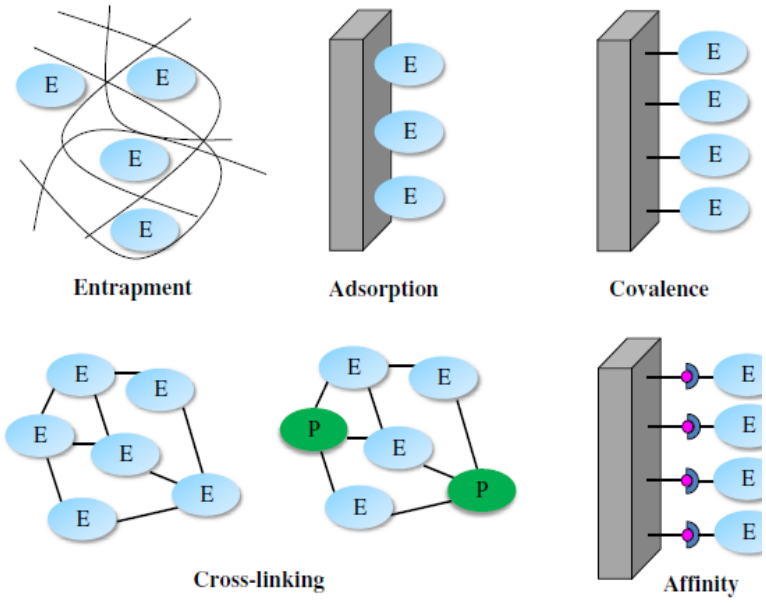


Figure 1.1 A Schematic Diagram of Immobolization Methods

1.4 APPLICATIONS OF MICROBIAL BIOSENSORS

1.4.1 Clinical diagnosis

The application of biosensor in clinical diagnosis made a great progress in recent years, although their application in clinical diagnosis is not very common, except for glucose biosensors representing about 90 % of the global biosensor market. Interferences with undesired molecules during measureements with real samples and also high selectivity and accuracy are still serious issue. This is very important, since treatment is often dependent on individual levels of clinical markers. Amperometric techniques based biosensors are the most described biosensors on what may indicate trends in biosensors development [28].Concentration of glucose is one of the most monitored indicators in many diseases, such as diabetes and other endocrine metabolic

disorders. Blood glucose is also the most common analyte measured after electrolytes and blood gases.

1.4.2 Environmental Application

Microbial biosensors plays a vital role in the environmental monitoring. BOD assays in microbial biosensors have been developed, a value related to total content of organic materials in wastewater. BOD sensors take advantage of the high reaction rates of microorganisms interfaced to electrodes to measure the oxygen depletion rates. BOD assays requires 15 min in biosensor-based analysis compared to standard BOD assay which requires 5 days.

A Japanese company Nisshin Electric in 1983 produced the first commercial BOD sensor and a number of other commercial BOD biosensors based on viable microbial cells are being marketed by Aucoteam**,** GmbH, Berlin; Prufgeratewrk, Medingen GmbH, Dresden; and Dr Lange, GmbH, Berlin. Another BOD sensor based on a soil bacterium *Pseudomonas putida* capable of determining low BOD levels in river water and secondary effluents and exhibiting negligible response to interference by chloride and heavy metals has been reported. The instrument is commercially available through Central Kagaku Corp,Tokyo. The use of these devices has been incorporated in to industrial standard methods in Japan [12]. In monitoring environmental pollution, chemical analysis by itself may not provide sufficient information to assess the ecological risk of polluted waters and wastewaters. In the European Union, along with more strict demands for water treatment (Council Directive 91/271/EEC), before the effluent can be discharged into the environment from industrial and urban wastewater effluents have to conform certain limits of toxicity .As a results, lot of bioassays and biosensors for toxicity evaluation were developed in recent years. For example, the toxicity assays MicrotoxR (Azure, Bucks, UK), is based on the use of luminescent bacteria, *Vibrio fischeri*, to measure toxicity from environmental samples. Other example is the CellsenseR, which is an amperometric sensor that incorporates *Escherichia coli* bacterial cells for rapid ecotoxicity analysis [28].

1.4.3 Application of MicrobialBiosensor in Food, Fermentation, and Allied products

Microbial biosensors contributes greatly in food industry and biotechnology fields where biosensor applications are not as common as in the field of medical diagnostics. This is because in the medical area the main matrices are blood, serum or urine, while in the food industry sector there are more types of samples with very variable composition. This makes the process of biosensor design, unification and optimization of measurement conditions more difficult. Company Biorealis Ltd together with Department of Nutrition and Food Assesment at Faculty of Chemical and Food Technology and with Institute of Measurement Science, Slovak Academyof Sciences developed the portable analytical device *Omnilab*utilizing biosensors [28].

The demand for quick and specific analytical tools for food and fermentation analysis has increased and is still expanding in recent years. in the quality assurance of food materials, both industry and government health agencies require a wide array of different analytical methods. There is needed for analysis in monitoring nutritional parameters, food additives, food contaminants,microbial counts, shelf life assessment and other olfactory characteristics like smell and odour. A variety of sensors based on enzymes and antibodies as well as electronic noses have been reported. Microbial biosensors have also shown potential in food analysis.The quality of milk monitoring is an important parameter because present methods involve bulk collection and prolonged storage [12].

CHAPTER 2

LITREATURE REVIEW

2.1 BIOSENSORS

A biosensor is an analytical device that combines a biological sensing elements with a transducer to produce a signal proportional to the analyte concentration [9].Therefore, a microbial biosensor is an analytical device which integrates microorganism(s) with a physical transducer to generate a measurable signal proportional to the concentration of analytes [10]. The signal can result from a change in protons concentration, release or uptake of gases, light emission, absorption and so forth, brought about by the metabolism of the target compound by the biological recognition element. In biosensor, the transducer converts this biological signal into a measurable response such as current, potential or absorption of light through electrochemical or optical means, which can be further amplified, processed and stored for later analysis [9].In the other hand, biosensor is also a device which composed of two intimately associated elements, [29] these are; a bioreceptor, that is an immobilized sensitive biological element (e.g. enzyme, DNA probe, antibody) recognizing the analyte (e.g .enzyme substrate, complementary DNA, antigen).

Enzymes are by far the most commonly used biosensing elements in biosensors although antibodies and oligonucleotides are widely employed. And a transducer, that is used to convert the (bio) chemical signal resulting from the interaction of the analyte with the bioreceptor into an electronic one. The intensity of signal produced is directly or inversely proportional to the concentration of analyte. Electrochemical transducers are often used to develop biosensors. There are some advantages offer in these system such as low cost, simple design or small dimensions.

Biosensors can also be based on gravimetric, calorimetric or optical detection [29]. Clark and Lyons in 1962 described the first biosensor [29,30] who immobilized glucose oxidase (GOD) on the surface of an amperometric oxygen electrode through a semi permeable dialysis membrane in order to directly quantify the concentration of glucose in a sample. Since then, various types of enzyme sensors have been developed for determining many different substances such as glucose, cholesterol or lactic acid in biological fluids (blood, serum, urine) , for toxicity analysis in environmental monitoring and in the biomedical and drug sensing arena.

In the past few years, the interest in biosensor for assessing toxicity has emerged as the most promising alternative for direct monitoring. In biosensor, the amplitude of the signal (current) generated is proportional to the level of metabolic activity of the biocatalyst (enzyme, microbe and organism). The toxic substrate will influencethe activity of the biocatalyst, and the toxicity can be detected according to the biosensor signal change. A preferred indirect electrochemical biosensing route based on the inhibition of bacteria has been developed by different groups [13]. There are various mediators which are used to shuttle the electrons between the microbe and electrode, which called mediated electron transfer (MET). The majority of microbial sensors utilize MET-type bioelectrocatalysis, since it can be applied to most microbes. However, high concentration of the mediator can be harmful to the cell and easily physical absorption on electrode surface.As result, the direct electron transfer (DET) of microbe towards electrode is quite attractive in electrochemical biosensor, which is sensitive to the changes in the metabolic status of the cellular biocatalyst and simplifiesthe construction procedure [13].

2.1.1 Types of Microbial Biosensors

Microbial biosensor can be classified based on the transducers into electrochemical, optical and others [9].

2.1.2 Electrochemical Microbial Biosensor

Electrochemical biosensors combine the analytical power of electrochemical techniques with the specificity of biological recognition processes. The main aim is to biologically generate an electrical signal that relates to the analyteconcentration.The most commonly employed technology is the sophisticated and state-of-the-art to

produce easy-to-use and inexpensive devices [26].The chemical reactions between immobilized biomolecule and target analyte produce or consume ions or electrons, which affects measurable electrical properties of the solution, such an electric current or potential which are the basic principle in this class of biosensors [28]. Electrochemical approaches are widely used in the developmentof microbial biosensors. Electrochemical techniques can be divided into amperometry, potentiometry, conductometry, voltammetry, and microbial fuel cell according to the detection principle (MFC) [10].

Amperometric microbial biosensor; operates at fixed potential with respect to a reference electrode and involves the detection of the current produced by the oxidation or reduction of species at the surface of the electrode. Amperometric microbial biosensors have been widely developed for the determination of biochemical oxygen demand (BOD) for the measurement of biodegradable organic pollutants in aqueous samples.

Conventional potentiometric microbial biosensors**;** consist of an ion-selective electrode (pH, ammonium, chloride and so on) or a gas-sensing electrode (*p*CO2 and *p*NH3) coated with an immobilized microbe layer. Microbe consuming analyte generates a change in potential resulting from ion accumulation or depletion. Potentiometric transducers measure the difference between a working electrode and a reference electrode, and the signal is correlated to the concentration of analyte [9].

Conductometry microbial biosensor; Many microbe-catalyzed reactions involve a change in ionic species. Associated with this change is a net change in the conductivity of the reaction solution. Even though the detection of solution conductance is non-specific, conductance measurements are extremely sensitive. Recently, A single-use conductivity and microbial sensor were developed to investigate the effect of both species and concentration/osmolarity of anions on the metabolic activity of *E.coli*recently.Inthis hybrid sensing system combines physico-chemical and biological sensing and greatly .increases the ease with which comparative data could be assimilated [9].

Voltammetry; is the most versatile technique in electrochemical analysis. In this type of technique, 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. This technique has a low noise which can endow the biosensor with higher sensitivity which is the major advantage of voltammetry biosensor. 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 [10].

Micrbial fuel cells (MFC) in this method, chemical energy is converted into electrical energy by means of the metabolic activity of microorganisms. MFC can be applied as a microbial biosensor for in situ analysis and for monitoring target chemicals since the consumption of target compounds by microbes or the inhibition of the metabolic pathway(s) by toxic compounds can potentially alter the production of electricity [10].

2.1.3 Typesof Electrode In Electrochemical Biosensors

There are three types of electrodes in electrochemical biosensors, these are as follows;

-Working electrode
-Reference electrode
-Auxilary or counter electrode

Reference electrode is a kind of standard hydrogen electrode. Hydrogen is potentially explosive and is not very suitable using an electrode with hydrogen gas for routine measurements. So there are two common use and commercially available reference electrode types:

Ag/AgCl Electrode: Ths is a Ag wire that coated with AgCl and dipped into NaCl solution.

Saturated-Calomel Electrode: Calomel is the other name of mercurous chloride (Hg2Cl2).

Working electrode is the most important component of an electrochemical cell and it is the electrode on which the reaction occur in an electrochemical system. The most commonly used working electrode materials are *platinum*, *gold*, *carbon*, and *mercury*.

Gold electrode are very useful, for the preparation of *modified electrodes* containing surface structures known as *self-assembled monolayers (SAMs)*.It behave similarly to platinum, but have limited usefulness in the positive potential range due to the oxidation of its surface.

- Auxilary or counter electrode functions as a cathode whenever the working electrode is operating as an anode and vice versa.

Carbon electrodes allow scans to more negative potentials than platinum or gold, as well as good anodic potential windows. The most common form of carbon electrode is *glassy carbon*, which is relatively expensive and difficult to machine. *Carbon paste electrodes* are also useful in many applications.

Platinum is likely the favorite, demonstrating good electrochemical inertness and ease of fabrication into many forms. The biggest disadvantage to the use of platinum, other than its high cost, is that the presence of even small amounts of water or acid in the electrolyte leads to the reduction of hydrogen ion to form hydrogen gas (hydrogen evolution) at fairly modest negative potentials (E = -0.059 x pH). This reduction obscures any useful analytical signal.

Mercuryhas historically been a widely used electrode material, primarily as a spherical drop formed at the end of a glass capillary through which the liquid metal is allowed to flow. It displays an excellent potential window in the cathodic direction, but is severely limited in the anodic direction by its ease of oxidation.

2.1.4 Optical Microbial Biosensors

Optics is another commonly used technique in microbial biosensors. 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.

Genetically engineered microorganisms have been widely applied in optical whole-cell biosensors. A reporter gene is fused with an inducible gene. In the presence of a target analyte, the inducible gene is activated and consequently activates (“turn on”) or represses (“turn off”) the expression of a reporter gene which is responsible for the production of measurable optical signal [10]. Optical based biosensors offer advantages of compactness, flexibility, resistance to electrical noise, and a small probe size [9].

Fluorescent; Based on the detection mode, fluorescent microbial biosensors can be divided into two categories: in vivo and in vitro. In vivo fluorescent microbial biosensor makes use of genetically engineered microorganisms with transcriptional fusion between an inducible promoter and a reporter gene encoding fluorescent protein. Green fluorescent protein (GFP), encoded by gfp gene, is among the most popular tools due to its attractive stability and sensitivity, and the fluorescence emitted by GFP can be conveniently detected by modern optical equipments with little or no damage to the host system [10]

Bioluminescence; thi**s** is associated with the emission of light by living microorganisms and it plays a very vital role in the monitoring of realtimeprocess.The bacterial luminescence *lux* gene has been widely applied as a reporter either in an inducible or constitutive manner. In the inducible manner, the reporter *lux* gene is fused to a promoter regulated by the concentration of a compound of interest. As a result, the concentration of the compound can be quantitatively analyzed by detecting the bioluminescence intensity [9].

Colorimetric microbial biosensors involve the production of colored compound which can be measured and correlated with the analytes concentration. Recently, a whole-cell colorimetric biosensor was constructed for the detection of arsenite with high sensitivity. Briefly, photosynthetic bacterium, Rhodovulumsulfidophilum, synthesizes carotenoids through the spheroiden (SE) pathway where the yellowish SE is catalyzed by SE monooxygenase (CrtA) to form reddish spheroidenone which is the predominant carotenoid under semi-aerobic conditions. In this biosensor, a genetically engineered photosynthetic bacterium, crtA-deleted R. sulfidophilum was used as the host strain which accumulated SE and hence displayed yellow color [10].

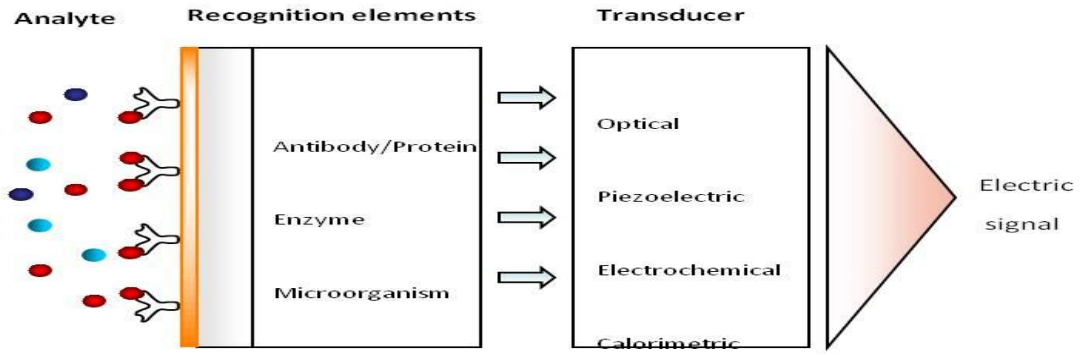


Figure 2.1 Schemeticdiagramofprinciple of biosensors.

2.2 MICROORGANISMS

Many microorganisms can be able to mineralize a variety of aromatic compounds as sole sources of carbon and energy. These microorganisms converts aromatic compounds intoan intermediate of a major central metabolic pathway by a series of aromatic catabolic reactions. The proteins responsible for the expression of aromatic catabolic enzymes are mostly transcriptional activators that interact with a target DNA through a helix-turn-helix DNA-binding motif [17]. These activators induce transcription initiation by interacting with the aromatic substrates, or with structural analogs, which serve as inducer molecules and provide regulatory specificity. *Escherichia coli* which is commonly abbreviated as (*E. coli*) is a Gram-negative, rod-shaped bacterium commonly found in the lower intestine of warm-blooded organisms (endotherms). Most of the starins of *E. coli* are harmless, but some serotypes are capable of causing serious food poisoning in humans. The harmless strains are part of the normal flora of the gut, and can benefit their hosts by producing vitamin K2, and by preventing the establishment of pathogenic bacteria within the intestine. *E. coli* and related bacteria constitute about 0.1% of gut flora, and fecal-oral transmission is the major route through which pathogenic strains of the bacterium cause disease [31] .Some strains of *E. coli* are the pathogenic in nature and are dangerous to human health that can cause human diseases such as traveler'sdiarrhea, infant diarrhea and infections of the urinary tract [32].

*Escherechia coli*ist the common micro-organism used in the production of various recombinant compounds suc as heterologous proteins.It offers the advantage of growing at predetermined growth rates at high cell densities by simple fed-batch techniques using defined media with inexpensive carbon sources such as glucose and glycerol [33]. *E-coli*serves as the most common used host cells herboring hybrid plasmids to achieve overproduction of heterologous proteins [34]. Depending on culture conditions, growth yields on these carbon substrates differ considerably, reflecting the variable efficiency with which aerobic-growing *E-coli* generates energy via substrate-level and oxidative phosphorylation and subsequently utilizes this energy for biosynthetic purposes. *E-coli*has been the working organism for production of recombinant proteins because of its fast groth, well-known genetic characterestics and availability of various tools for genetcs expression.

2.2.1 Toluenemonoxegenases and ToMO

After discovering that toluene 4-monooxygenase (T4MO) of *Pseudomonas mendocinaKR1* which is capable of oxidizing nitrobenzene to 4-nitrocatechol, albeit at a very low rate, this reaction was later improved using directed evolution and saturation mutagenesis. Screening of 550 colonies from a random mutagenesis library generated by error-prone PCR of tmoAB using *Escherichia coli TG1/pBS(Kan)*T4MO on agar plates containing nitrobenzene led to the discovery of nitrocatechol-producing mutants [35] which is a derivatives of phenolic compounds.

Toluene 4 monoxegenase of *Pseudomonas mendocina* KR1, toluene 3 monoxegenase (T4MO) of *Ralstoniapickettii* PKO1, toluene/o-xylene monoxegenase (ToMO) of *Pseudomonas stutzeri* OX1, and toluene-orthomonoxegenase (TOM) of *Burkholderiacepacia* G4 belongs to the family if diironmonoxegenases.TOM of *B. cepacia* oxidizes toluene to o-cresol and then to 3 methylcatechol while appearing to transform benzene to phenol and then to catechol atlhough this has been challenged. ToMO of *P. stutzeri* OX1 oxidizes toluene to 3 and 4-methylcatechol and convert o-xylene to 3,4-dimethylcatechol but is reported to transfom benzene only to phenol.T4MO of P. mendocina KR1 and T3MO of *R.pickettii* PKO1 has been reported to convert benzene to phenol and toluene to p-cresol and m-cresol respectively, but have not been shown previously to perform successive hydroxylation on aromatics.T4MO

and T3MO are four-compenent enzymes consisting of an $(\alpha\beta\gamma)_2$ hydroxylase (from *tmo ABE* and *tbu A1A2U* respectively), a NADH oxidoreductase (from *tmo F and tbu C* respectively), a rieske-type (2Fe-2S) ferrodoxin (from *tmoC and tbuB*respectively), and an effector protein (from *tmoD and tbuV* respectively), while TOM consist of an $(\alpha\beta\gamma)_2$ hydroxylase (from *tomA1A3A4*) a NADH oxidoreductase (from *tomA5)* an effector prtein (from *tomA5*), and a relatively unknown subunit effector protein (from *tomA0*). The T3MO tbu locus is most similar (>60%) to T4MO tmo genes in the DNA sequence, but both T3MO and T4MO are distantly related to TOM and substantially different enzymes (as evidence by different toluene hydroxylations) since the hydroxylase alpha fragments of T3MO and T4MO share only 48% DNA homology and only 23% protein identity with TOM [35]. Toluene monoxegenases are capable in hydroxylations of many aromatic compounds. Toluene-o-xylene monoxegenase (ToMO) is endowed with a broad spectrum of substrate specificity and ability to hydroxylate more than a single position of the aromatic ring in two consecutive monooxygenation reactions [19].

2.3 CHITOSAN

The history of chitosan dates back to the 19th century, when Rouget discussed the deacetylated forms of the parent chitin natural polymer in 1859. A substantial amount of work has been reported on chitosan and its potential use in various bioapplications during the past 20 years. Chitosan is derived from naturally occurring sources, which is the exoskeleton of insects, crustaceans and fungi that has been shown to possessed some characterestics such as biocompatible and biodegradable. The polymers of chitosan are semi-synthetically derived aminopolysaccharides that have unique structures, multidimensional properties, highly sophisticated functionality and a wide range of applications in biomedical and other industrial areas [36].Chitosan (CHIT), the primary derivative of chitin, is obtained by Ndeacetylation to a varying extent that is characterized by the degree of deacetylation, and is consequently a copolymer of N-acetylglucosamine and glucosamine [23]. Chitosan can be defined as the chitin sufficiently deacetylated to form soluble amine salts, the degree of deacetylation necessary to obtain a solubleproduct being 80–85% or higher [25]. Commercially, chitin and chitosan are obtained at a relatively low cost from shells of shellfish (mainly crabs, shrimps, lobsters and krills), wastes of the seafood processing industry.

[25].Increasingly over the last decade, chitin-and chitosan based materials have been examined and a number of potential products have been developed for areas in biosensors as a flatform for immobolization as well as in wastewater treatment, the food industry, agriculture, pulp and paper industry, cosmetics and toiletries, medicine and biotechnology. The biocompatible composite nature of CHIT–CNT has been applied as matrix for the immobilization of biological materials [23].

The functional material of the biopolymer chitosan offers excellent characteristics such as biocompatibility, film forming ability, nontoxicity, physiological inertness, antibacteriaproperties and high mechanical strength. As such, it has been extensively used for the immobilization of enzymes and the construction of amperometric biosensors [4].Chitosan possesses distinct chemical and biological properties. Due to its linear polyglucosamine chains of high molecular weight, chitosan has reactive amino and hydroxylgroups, amenable to chemical modifications. Chitosan as a biopolymer and due to its biodegradability, nontoxicity, and biocompatibility characterestics make it a promising matrix for immobolization of enzymes.The presence of amino and two free hydroxyl groups for each carbon six (carbon-6) building unit facilitate covalent binding of enzyme [24]. Additionally, amino groups make chitosan a cationic polyelectrolyte (p*K*a≈ 6.5), one of the few found in nature. The basicity of chitosan give it singular properties: chitosan is soluble in aqueous acidic media at pH <6.5 and when dissolved possesses high positive charge on –NH3 + groups, it adheres to negatively charged surfaces, it aggregates with polyanionic compounds, and chelates heavy metal ions [25]. Both the solubility in acidic solutions and aggregation with polyanions impart chitosan with excellent gel-forming properties.

The possession of chitosan characterestics, such as hydrophilicity, biocompatibility, biodegradability and antibacterial properties make chitosan an ideal support for immobilization of enzymes. This biopolymer can be physically modified to give different forms (e.g., powder, nanoparticles, gel, film, and beads), crosslinked with different substances (e.g., glutaraldehyde, car-bodiimide, epichlorohydrin, and tripolyphosphate) and used in various fields of application including the construction ofbiosensors [26]. Chitosan (CHIT), the primary derivative of chitin, is obtained by N-deacetylationto a varying extent that is characterized by the degree of deacetylation, and is consequently a copolymer of N-acetyl glucosamine and glucosamine. Over the last

decade,it has been increasingly that, chitin- and chitosan based materials have been examined and a number of potential products have been developed for areas such as wastewater treatment, the food industry, agriculture, pulp and paper industry, cosmetics and toiletries, medicine and biotechnology, in biosensors as an immobilization platform [23]. Magnetic fields have been utilized in support systems to study enzyme immobilization. Several magnetic particles and magnetic supports such as microspheres of various biomaterials encapsulating the magnetic particles and copolymers with magnetic particles have been used with good outcomes [27].

2.3.1 Manufacture and Properties

Chitosan is commercially produced by deacetylation of chitin, which is the structural element in the exoskeleton of crustaceans (such as crabs and shrimp) and cell walls of fungi. The degree of deacetylation (%DD) can be determined by NMR spectroccopy, and the %DD in commercial chitosans ranges from 60 to 100%. On average, the molecular weight of commercially produced chitosan is between 3800 and 20,000 Daltons. A common method for the synthesis of chitosan is the deacetylation of chitin using sodium hydroxide in excess as a reagent and water as a solvent. This reaction pathway, when allowed to go to completion (complete deacetylation) yields up to 98% product. The amino group in chitosan has a pKa value of ~6.5, which leads to a protonation in acidic to neutral solution with a charge density dependent on pH and the %DA-value. This makes chitosan water soluble and a bioadhesive which readily binds to negatively charged surfaces such as mucosal membranes.

Chitosan improves the transport of polar drugs across epithelial surfaces, and is biocompitable and biodegradable. It is not approved by FDA for drug delivery though. Purified quantities of chitosans are available for biomedical applications. Chitosan and its derivatives, such as trimethylchitosan (where the amino group has been trimethylated), have been used in nonviral gene delivery. Trimethylchitosan, or quaternised chitosan, has been shown to transfect breast cancer cells, with increased degree of trimethylation increasing the cytotoxicity; at approximately 50% trimethylation, the derivative is the most efficient at gene delivery. Oligomeric derivatives (3-6 kDa) are relatively nontoxic and have good gene delivery properties [37].

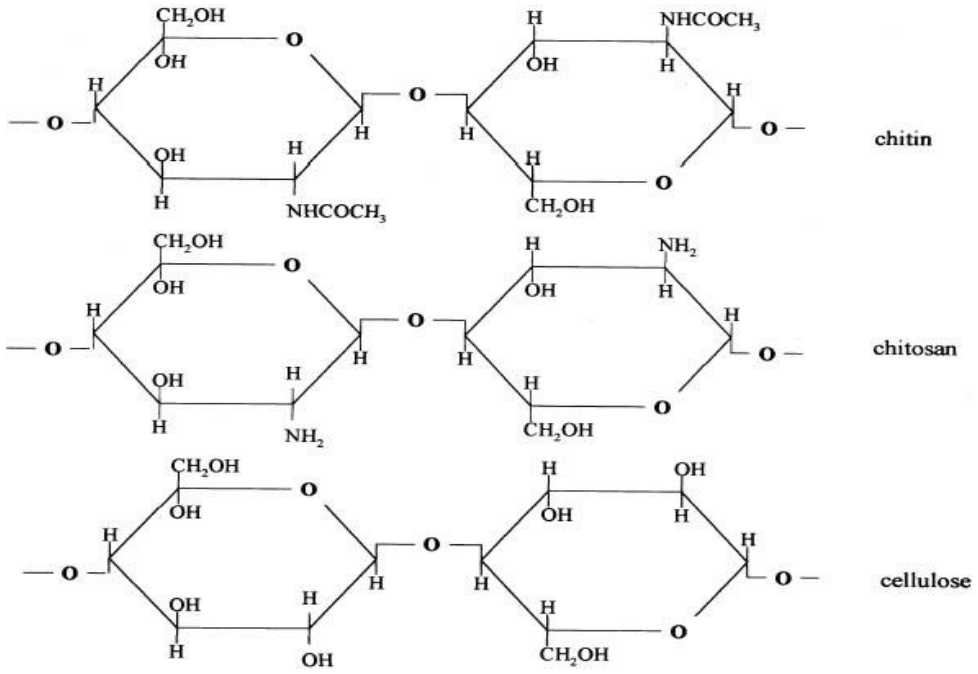


Figure 2.2 SchemeticStructure of chitin, chitosan and cellulose.

2.3.2 Properties of Chitosan

Majority of naturally occurring polysacchrides, eg. Cellulose, dextrin, pectin, alginic acid, agar, agarose, and carragenal are natural and acidic in nature, where as chitin and chitosan are examples of highly basic polysacchrides. The properties chitosan includes, solubility in various media, solution, viscosity, polyelectrolyte behavior, ploy oxysalt formation, ability to form films ,metal chelations, optical and structural characterestics [38].

2.3.3 Chemical Properties of Chitosan

The chemical properties of chitosan are pointed out as follows;

- Linear polymine,
- Reactive amino groups,
- Reactive hydroxyl group available,
- Chelates many transitional metal ions.

2.3.4 Biological Properties of Chitosan

Among the biological properties of chitosan includes;

- Biocompatible
  -Natural polymer,
  -Biodegradable to normal body constituents,
  -Safe and non-toxic, (The research in chitinase is noteworthy in this respect)
  -Bind to mammalian and microbial cells aggressively,

* Regenerative effects on connective gum tissues,
* Accelerates the formation of osteoblast responsible for bone formation
* Homostatic,
* Fungistatic,
* Spermacidal,
* Antitumor,
* Anticholesteremic,
* Accelerates bone formation,
* Central nervour system depressant,
* Immunoadjuvant.

2.4 FUNCTIONS OF NANOPARTICLES IN BIOSENSORS

Many kinds of nanoparticles, including metal nanoparticles, oxide nanoparticles, semiconductor nanoparticles, and even composite nanoparticles, have been widely used in electrochemical sensors and biosensors [39]. Although these nanoparticles play different roles in different electrochemical sensing systems based on their unique properties, the basic functions of nanoparticles can be mainly classified as follows;

1) immobilization of biomolecules
2) catalysis of electrochemical reactions
3) labeling biomolecules and
4) acting as reactant
5) promote the transfer of electron in the reactions of proteins.

2.5 PHENOL

Phenol — also known as **carbolic acid** - with the molecular formula C_6H_5OH. It is a volatile and white crystalline solid. The molecule consists of a phenyl group ($-C_6H_5$) bonded to a hydroxyl group (-OH). It is mildly acidic, but requires careful handling due to its propensity to cause burns. Phenol was first extracted from coal tar, but today is produced on a large scale (about 7 billion kg/year) from petroleum. It is an important industrial commodity as a precursor to many materials and useful compounds. Its major uses involve its conversion to plastics or related materials. Phenol and its chemical derivatives serves as key for building polycarbonates, Bakelite, nylon, detergents, herbicides, such as phenoxyherbicodes, and numerous pharmaceutical drugs. Although similar to alcohols, phenols have unique distinguishing properties. Unlike in alcohols where the hydroxyl group is bound to a saturated carbon atom, in phenols the hydroxyl group is attached to an unsaturated ring such as benzene or other arene ring. Consequently, phenols have greater acidity than alcohols due to stabilization of the conjugate base through resonance in the aromatic ring [40].

2.5.1 Properties of Phenol

Phenol is appreciably soluble in water, with about 8.3 g dissolving in 100 mL (0.88M). Homogeneous mixtures of phenol and water at phenol to water mass ratios of ~2.6 and higher are also possible.

The more water soluble salts of phenol is the sodium salt of phenol, which is sodium phenoxide.

Acidity:

Phenol is weakly acidic and at high pH's gives the phenolate anion $C_6H_5O^-$ (also called phenoxide)The chemical equation is as follws:

$$PhOH \rightleftharpoons PhO^- + H^+ \qquad (K = 10^{-10})$$

Compared to aliphatic alcohols, phenol is about 1 million times more acidic, although it is still considered a weak acid. It reacts completely withaqueous NaOH to lose H^+, whereas most alcohols react only partially. Phenols are less acidic than carboxylic acids,and even carbonic acid.One explanation for the increased acidity over alcohols is

resonance stabilizationof the phenoxide anion by the aromatic ring. In this way, the negative charge on oxygen is delocalized on to the ortho and para carbon atoms. In another word, increased acidity is the result of orbital overlap between the oxygen's lone pairs and the aromatic system. In a third, the dominant effect is the induction from the sp^2 hybridised carbons; the comparatively more powerful inductive withdrawal of electron density that is provided by the sp^2system compared to an sp^3 system allows for great stabilization of the oxyanion.The PKa of the enol of acetone is 10.9, comparable to that for phenol. The acidities of phenol and acetone enol diverge in the gas phase owing to the effects of solvation. About 1/3 of the increased acidity of phenol is attributable to inductive effects, with resonance accounting for the remaining difference.

Phenoxide anion:

The phenoxide anion has a similar nucleophilicity to free amines, with the further advantage that its conjugate acid (neutral phenol) does not become entirely deactivated as a nucleophile even in moderately acidic conditions. Phenols are sometimes used in peptide synthesis to "activate" carboxylic acids or esters to form activated esters. Phenolate esters are more stable toward hydrolysis than acid anhydrides and acyl halides but are sufficiently reactive under mild conditions to facilitate the formation of amide bonds.Phenol exhibitsketo-enoltautomerism with its unstable keto tautomer cyclohexadienone, but only a tiny fraction of phenol exists as the keto form. The equilibrium constant for enolisation is approximately 10^{-13}, meaning that only one in every ten trillion molecules is in the keto form at any moment. The small amount of stabilisation gained by exchanging a C=C bond for a C=O bond is more than offset by the large destabilisation resulting from the loss of aromaticity. Phenol therefore exists entirely in the enolformPhenoxidesareenolates stabilised by aromacity.Under normal circumstances, phenoxide is more reactive at the oxygen position, but the oxygen position is a "hard" nucleophile whereas the alpha-carbon positions tend to be "soft".The table below shows a summary of the properties.

Table 2.1 Properties of Phenols.

PROPERTIES	
Molecular formula	C_6H_6O
Molar mass	94.11 g mol^{-1}
Appearence	Transparent crystalline solid
Odor	Sweet and tarry Cont.
Density	1.07 g/cm^3
Melting point	40.5 °C, 314 K, 105 °F
Boiling point	181.7 °C, 455 K, 359 °F
Watre solubility	8.3 g/100 mL (20 °C)
Acidity (pKa)	9.95 (in water), 29.1 (in acetonitrile)
Dipole moment	1.7 D

2.5.2 Reactions of Phenol

Phenol undergoes various reactions which include the following;

Reaction type: Electrophilic Aromatic Substitution

Summary of phenol reactivity

- *Phenols* are potentially very reactive towards eletrophillic aromatic substitution
- This is because the hydroxy group, -OH, is a strongly activating, *ortho*- / *para*-directing substituent
- Substitution typically occurs *para* to the hydroxyl group unless the *para* position is blocked, then *ortho* substitution occurs.
- The strong activation often means that milder reaction conditions than those used for benzene itself can be used (see table below for a comparison)

- Phenols are so activated that polysubstitution can be a problem (similar problems occur with anilines) [41].

Reaction	**Phenol**	**Benzene**
Nitration	dil. HNO_3 in H_2O or CH_3CO_2H	HNO_3 / H_2SO_4
Sulfonation	conc. H_2SO_4	H_2SO_4 or SO_3 / H_2SO_4
Halogenation	X_2	X_2 / Fe or FeX_3
Alkylation	ROH / H^+ or RCl / $AlCl_3$	RCl / $AlCl_3$
Acylation	RCOCl / $AlCl_3$	RCOCl / $AlCl_3$
Nitrosation	aq. $NaNO_2$ / H^+	

2.5.3 Sources/Uses of Phenol

Phenol is obtained by fractional distillation of coal tar and by organic synthesis. By far, its largest single use is in manufacture of phenolic resins and plastics. Other uses includemanufacture of explosives, fertilizers, paints, rubber, textiles, adhesives, drugs, paper, soap, wood preservatives, and photographic developers. When mixed with slaked lime and other reagents, phenol is an effective disinfectant for toilets, stables, cesspools, floors, and drains.

Phenol was once an important antiseptic and is still used as a preservative in injectables. It also is used as an antipruritic, a cauterizing agent, a topical anesthetic, and as a chemical skin-peeler (chemexfoliant). It can be found in low concentrations in many over-the-counter products including preparations for treatment of localized skin disorders (Castellani's paint, PRID salve, CamphoPhenique lotion), in topical preparations (Sting-Eze), and in throat sprays and lozenges (Chloraseptic, Ambesol, Cepastat, Cheracol) [42].

2.5.4 Toxicity of Phenol

Phenol and its vapors are corrosive to the eyes, the skin, and the respiratory tract. Repeated or prolonged skin contact with phenol may cause dermatitis,dermatitis, or even second and third-degree burns. Inhalation of phenol vapor may cause lung

edema. The substance may cause harmful effects on the central nervous system and heart, resulting in dysrhythmia, seizures, and coma. The kidneys may be affected as well. Long-term or repeated exposure of the substance may have harmful effects on the liver and kidneys. There is no evidence that phenol causes cancer in humans. Besides itshydrophobic effects, another mechanism for the toxicity of phenol may be the formation of phenoxyl radicals. Chemical burns from skin exposures can be decontaminated by washing with polyethylene glycol isopropyl alcohol or perhaps even copious amounts of water. Removal of contaminated clothing is required, as well as immediate hospital treatment for large splashes. This is particularly important if the phenol is mixed with chloroform (a commonly-used mixture in moleculabiologyfor DNA and RNApurification) [43]. Phenol (carbolic acid) is also one of the oldest antiseptic agents. Currently it is used as a disinfectant, chemical intermediate and nail cauterizer.

Phenol is a general protoplasmic poison (denatured protein) with corrosive local effects. Phenol derivates are less toxic than pure phenol.The lethal dose is between 3 to 30 g, but may be as little as 1 g. Phenol is well absorbed by inhalation, dermal application, and ingestion. MANIFESTATIONS OF ACUTE POISONING: Local manifestations. Skin exposure produces lesions which are at the begining painless white patches and later turn erythematous and finally brown. Phenol produces mucosal burns and coagulum. They cause eye irritation and corneal damage. When phenol is ingested, it causes extensive local corrosions, pain, nausea, vomiting, sweating, and diarrhea. Severe gastrointestinal burns are uncommon and strictures are rare. Inhalation produces respiratory tract irritation and pneumonia. Systemic manifestations develop after 5 to 30 minutes postingestion or post dermal application, and may produce nausea, vomiting, lethargy or coma, hypotension, tachycardia or bradycardia, dysrhythmias, seizures, acidosis, hemolysis, methemoglobinemia, and shock [42].

Routes of Exposure:

Inhalation:

Phenol is absorbed rapidly from the lungs. However, because of its low volatility, inhalation hazard is limited. The odor threshold of phenol is about 100 times lower than the OSHA PEL;As such, it provides adequate warning of hazardous concentrations.

Phenol vapor is heavier than air. Children exposed to the same levels of phenol vapor as adults may receive larger doses because they have greater lung surface area: body weight ratios and increased minute volumes: weight ratios. Children may be more vulnerable to corrosive agents than adults because of the relatively smaller diameter of their airways. In addition, they may be exposed to higher levels than adults in the same location because of their short stature and the higher levels of phenol vapor found nearer to the ground.

Skin/Eye Contact

All forms of phenol cause irritation, and acute toxic effects of phenol most often occur by skin contact. Even dilute solutions (1% to 2%) may cause severe burns if contact is prolonged. Systemic toxicity can result from skin or eye exposures. Phenol vapor and liquid penetrate the skin with an absorption efficiency approximately equal to the absorption efficiency by inhalation. In one case, death occurred within 30 minutes after skin contact.Children are more vulnerable to toxicants absorbed through the skin because of their relatively larger surface area:body weight ratio.

Ingestion:

Accidental and intentional ingestions of phenol have been reported. As little as 50 to 500 mg has been fatal in infants. Deaths in adults have resulted after ingestions of 1 to 32 g.

2.5.5 Management of Phenol Poisoning

Phenol poisoning requires immediate medical evaluation, in cases of significant phenol ingestion (more than 1 g for adults or 50 mg for infants) or symptomatic intoxication. It is necessary to establish and maintain vital functions and establish vascular access. Treatment includes the following: shock (fluids and dopamine), arrhythmias (lidocaine) and convulsions (diazepam). Health personnel should use gowns and rubber gloves. Inhalation of 100% oxygen is recommended. Intubate and assisted ventilation might be necessary. Metabolic acidosis should be managed by 1 to 2 mEq/kg of sodium bicarbonate. Methemoglobinemia should be treated if greater than 30%, or in cases of respiratory distress, with methylene blue 1 to 2 mg/kg of 1%

solution, slowly i.v. If phenol is ingested, avoid emesis, alcohol and oral mineral oil and dilution, because they may increase absorption. Gastric lavage is usually not recommended. Immediate administration of olive oil and activated charcoal by small bore nasogastric tube is necessary[44].

2.6 CATECHOL

Catechol, also known as **pyrocatechol** or **1,2-dihydroxybenzene**, is an organic compound with the molecular formula $C_6H_4(OH)_2$. It is the *ortho*isomer of the three isomeric benzodiols . This colorless compound occurs naturally in trace amounts. Catechol was first discovered by destructive distillation of the plant extract catecin. About 20 million kg are now synthetically produced annually as a basic organic chemical, mainly as a precursor to pesticides, flavors, and fragrances.Catechol occurs as feathery white crystals that are very readily soluble in water.

2.6.1 Isolation and Synthesis

In 1839, catechol was first isolated by H. Reinsch when distilling it from the solid tannic preparation catechin, which is the residuum of catechu , the boiled or concentrated juice of *Mimosa catechu* (Acacia cattechuL.f). Upon heating catechin above its decomposition point, a substance Reinsch first named "pyrocatechol" distilled and condensed as a white solid ("pyro" referring to heat). This was a thermal decomposition product of the flavanols in catechin. "Pyrocatechol" is now simply referred to as catechol. Catechol has since been shown to occur in free-form naturally in kino and in beechwood tar. Its sulfonic acid has been detected in the urine of horses and humans [45]. Catechol is produced industrially by the hydroxylation of phenol using hydrogen peroxide:

$$C_6H_5OH + H_2O_2 \rightarrow C_6H_4(OH)_2 + H_2O$$

Previously, catechol was produced by hydrolysis of 2-substituted phenols, especially 2-chlorophenol, with hot aqueous solutions containing alkali metal hydroxides. Its methyl ether derivative, guaiacol, converts to catechol via hydrolysis of the CH_3-O bond as promoted by hydriodic acid. Thee diagrams below shows the structures of catechol, resorcinol, and hydroquinone respectively [46].

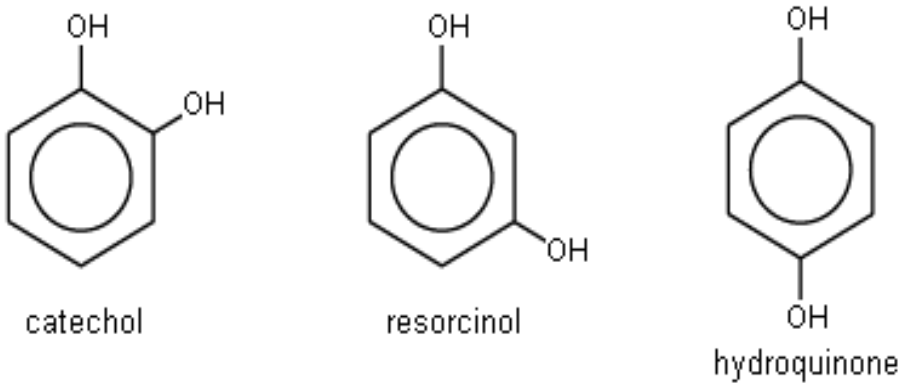


Figure 2.4 Structure of Catechol, Resorcinol and Hydroquinone.

2.6.2 Properties of Catechol

The following table shows the various properties of catechol.

Table 2.2Properties of Catechol.

PROPERTIES	
Molecular formula	$C_6H_6O_2$
Molar mass	110.1 g/mol
Appearence	white to brown feathery crystals
Odor	faint, phenolic odor
Density	1.344 g/cm³, solid
Melting point	105 °C, 378 K, 221 °F
Boiling point	245.5 °C, 519 K, 474 °F (sublimes)
Solubility in water	43 g/100 Ml

Table 2.2(Cont.)	
Solubility	Cont. very soluble inpyridine soluble in chloroform,benzene,CCl4,ether,acetate
Log P	0.88
Vapour pressure	20 Pa (20 °C)
Acidity (pKa)	9.48
Refractive index (n_D)	1.604

2.6.3 Uses of Catechol

Approximately 50% of synthetic catechol is consumed in the production of pesticides, the remainder being used as a precursor to fine chemicals such as perfumes and pharmaceuticals.

It is a common building block in organic synthesis. Several industrially significant flavors and fragrances are prepared starting from catechol.Guaiacol is prepared by methylation of catechol and is then converted tovanillin on a scale of about 10M kg per year (1990). The related monoethyl ether of catechol, guethol, is converted toehtylvanillin, a component of chocolate confectioneries. 3-*Trans*-Isocamphylcyclohexanol,widely used as a replacement for sandalwood, is prepared from catechol via guaiacol and camphor. Piperonal, a flowery scent, is prepared from the methylene diether of catechol followed by condensation withglyoxal and decarboxylation. Catechol is used as a black-and-white photographic developer, but, except for some special purpose applications, its use until recently was largely historical. Modern catechol developing was pioneered by noted photographer Sandy King. His "PyroCat" formulation enjoys widespread popularity among modern black-and-white film photographer [47].

Sources and Potential Exposure of catechol

Catechol may be released into the environment during its manufacture and use.

- The major routes of human exposure are consumption of contaminated drinking water and ingestion of contaminated food.
- Catechol occurs naturally in fruits and vegetables.
- It has been detected in cigarette smoke.

2.6.5 Health Hazard Information of catechol

Acute Effects:

- Skin contact causes eczematous dermatitis in humans.
- In humans, absorption through the skin results in an illness resembling that induced by phenol, except convulsions are more pronounced.
- Large doses of catechol can cause depression of the CNS and a prolonged rise of blood pressure in animals. The rise of blood pressure appears to be due to peripheral vasoconstriction.
- Due to the lack of information regarding the duration of exposure in the above studies, it is not clear whether these health effects were observed following acute or chronic exposure.
- Acute animal tests in rats, mice, guinea pigs, and rabbits have demonstrated catechol to have high acute toxicity by oral or dermal exposure.

Chronic Effects (Noncancer):

- No information is available on the chronic effects of catechol in humans or animals.
- EPA has not established a Reference Concentration (RfC) or a Reference Dose (RfD) for catechol [48].

Reproductive/Developmental Effects:

- No information is available on the reproductive or developmental effects of catechol in humans or animals.

Cancer Risk:

- No information is available on the carcinogenic effects of catechol in humans.

- In orally exposed rats, adenocarcinomas in the glandular stomach were reported.
- Catechol increased the carcinogenic effects of benzo[*a*]pyrene on the skin in mice when applied together dermally.

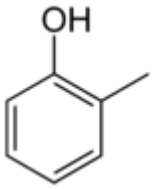
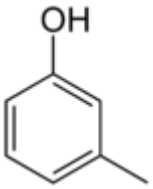
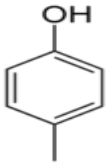
2.7 CRESOL

Cresols are organic compounds which are methylphenols . They are a widely occurring natural and manufactured group of aromatic organic compounds, which are categorized as phenols (sometimes called *phenolics*). Depending on the temperature, cresols can be solid or liquid because they havemelting points not far from room temperature. Like other types of phenols, they are slowlyoxidized by long exposure to air and the impurities often give cresols a yellowish to brownish red tint. Cresols have anodor characteristic to that of other simple phenols, reminiscent to some of a “coal tar" smell. The name creosol reflects their structure, being phenols, and their traditional source,creosote.

2.7.1 Structure and production of cresol

In itschemical structure, a molecule of cresol has a methyl group substituted onto the ring of phenol. There are three forms (isomers) of cresol: *ortho*-cresol (o-cresol), *meta*-cresol (m-cresol), and*para*-cresol (p-cresol). These forms occur separately or as a mixture, which can also be called cresol or more specifically, **tricresol**. About half of the world's supply of cresols are extracted from coal tar. The rest is produced synthetically, by methylation of phenol or hydrolysis ofchlorotoluenes. Table 2.3 summarised the isomers of cresol.

Table 2.3 Isomers of Cresol.

ISOMERS OF CRESOL			
Skeletal formula			
General			
Common name	***o*-cresol**	*Cont.**m*-cresol**	***p*-cresol**
Systemic names	2-methylphenol	Cont. 3-methylphenol	4-methylphenol
Other names	*ortho*-cresol	*meta*-cresol	*para*-cresol
Molecular formula	C_7H_8O		
SIMILES	Oc1c(C)cccc1	Oc1cc(C)ccc1	Oc1ccc(C)cc1
Molar mass	108.14 g/mol		

2.7.2 Properties of cresol

The table below shows various properties of cresol.

Table 2.4 Properties of Cresol

PROPERTIES			
Density and phase	1.05 g/cm^3, solid	1.03 g/cm^3, liquid	1.02 g/cm^3, liquid
Solubility in pure water at 20−25 °C	2.5 g/100 ml	2.4 g/100 ml	1.9 g/100 ml
soluble in strongly alkaline water			
Melting point	29.8 °C (303.0 K)	11.8 °C (285.0 K)	35.5 °C (309.7 K)
Boiling point	191.0 °C (464.2 K)	202.0 °C (475.2 K)	201.9 °C (475.1 K)
Acidity (pK_a)	10.26	10.09	10.26
Viscosity	solid at 25 °C	Cont. ? cP at 25 °C	solid at 25 °C

2.7.3 Toxicity of cresol

Information about the effects of cresols in humans is derived mainly from case reports of accidental or intentional ingestion of cresol solutions or from accidental contact of cresol with the skin. Cresols produce corrosive damage at sites of contact; therefore, the skin and mucosal membranes are targets for cresols toxicity. In a single study of controlled exposures in volunteers, brief exposures to 6 mg/m3 o-cresol caused 8 out of 10 subjects to complain of respiratory irritation. Fatalities due to ingestion and dermal exposure to cresols have been described. Other effects reported in these acute high oral and/or dermal exposure scenarios include respiratory failure, tachycardia and ventricular fibrillation, abdominal pain, vomiting, and corrosive lesions of the

gastrointestinal tract, methemoglobinemia, leukocytosis and hemolysis, hepatocellular injury, renal alterations, skin damage, metabolic acidosis, and unconsciousness. Many of these effects may not have been caused directly by cresols, but may be a result of secondary reactions to shock caused by external and internal burns.Inhalation or dermal exposure of animals to cresols has produced irritation and corrosion at the site of contact.

Animals exposed acutely to cresol vapors and aerosols showed signs of respiratory irritation, although the levels associated with irritation have not been reliably documented. Inflammation and irritation of the upper respiratory tract, pulmonary edema, and hemorrhage and perivascular sclerosis in the lungs were seen in a variety of animal species exposed intermittently to 9–50 mg/m3 of o-cresol for ≥1 month; other isomers were not tested. White mice exposed acutely to commercial mixtures of cresol isomers exhibited irritation and inflammation of the eyes and nose. Also noticed in these inhalation studies were effects on the nervous system (excitation, fatigue, convulsions). Animals that died had fatty degeneration and necrosis of the liver, degeneration of the tubular epithelium in the kidneys, bronchitis, pulmonary hemorrhage, and dystrophic changes in the heart and in nerve cells and glia in the brain [49]. The nervous system also appears to be a sensitive target of cresols toxicity in oral studies, although this seems to be limited to oral gavage studies. Rodents administered cresols by oral gavage for acute or intermediate durations showed neurological signs such as hypoactivity, excessive salivation, labored respiration, and tremors, in addition to decreased body weight gain. Some neurological signs were observed in rats dosed by gavage with as low as 50 mg/kg/day of cresol isomers. None of these effects have been seen in dietary studies, or if seen, they have occurred at much higher dose levels than oral gavage studies. The reason for this difference is unknown, but it probably is related to toxicokinetic differences between the two modes of oral dosing.

Dietary exposure to higher doses of cresols, generally >240 mg/kg/day, caused increases in liver weight; thresholds for these changes in liver weight were comparable among cresol isomers. Kidney weight was only increased in rats dosed with ≥861 mg/kg/day o-cresol for 28 days. Clinical chemistry tests gave no indication of altered function in these organs and no gross and microscopic alterations were seen, even at the highest doses administered (>1,000 mg/kg/day). Other systemic effects observed in rats

and mice treated with relatively high doses of cresols (>1,000 mg/kg/day) in the diet for 13 weeks included decreased weight gain (all isomers). A 2-year study also provided evidence of kidney toxicity in rats (720 mg/kg/day m/p-cresol) and thyroid gland toxicity in mice (≥100 mg/kg/day m/p-cresol [49].

CHAPTER 3

EXPERIMENTAL PART

3.1 MATERIALS AND METHODS

3.1.1 Materilas

E-coli TG1 pBS (kan) and *E-coli* TG1 pBS (kan) ToMO were obtained from Assist. Prof.Dr.Gonul SHARAH in the Proteins Engineering laboratory,Department of Genetics and Bioengineering, Fatih University Istanbul, Turkey. Acetic acid was purchased from (Reidel-de Haen) Sigma-Aldrich, Phenol (EG Nr.:7664-41-7), Catechol (EG Nr.:204-427-5), Pyrogallol (EG-Nr:.201-762-9) and p-Cresol were purchased from Alfa Aesar. LB broth, LB agar and kanamycin were all purchased from MERCK. Chitosan and acetone were obtained from Sigma- Aldrich. All chemicals used were of analytical grade and were all used without further pretreatment. All solutions were made in milli-Q-water.

3.1.2 Apparatus

Cyclic voltammetric and amperomertic measurements were performed using a CHI model 842B electrochemical analyzer where the whole cells modified gold-plate was used as working electrode. An Ag/AgCl-saturated KCl reference electrode and platinum (Pt) wire as counter electrode of a conventional three-electrode electrochemical cell system obtained from CH instruments. All measurements were Ag/AgCl aq. reference electrode. Cleaning of the working electrode was performed using an Ultrasonic water bath. Allegra^{TM} X-22R centrifuge (BECKMAN COULTER$_{TM}$) and an incubator shaker series (Innova$^{(R)}$ 44 New Brunsnick Scientific) were used in growing the bacterial cells.

3.1.3 Methods

3.1.3.1 Growing of the bacteria

Cultures of *E. coli TG1pBS (Kan)* and *E. coli TG1 pBS (Kan)ToMO* were started by streaking -80 ^{O}C glycerol stocks on to the plates containing LB (100) kan agar [50, 55] and incubated overnight at 37^{o}C. After incubation, the obtained coloniese were transfered in to LB (kanamycin) liquid culture media and also incubated overnight in incubator shaker series (Innova$^{(R)}$ 44 New Brunsnick Scientific) at 37^{o}C.The cells were grown in flask containing LB (kan) in a shaker till its $OD_{600:1}$ was obtained. The grown cells were centrifuged using Allegra TM X-22R centrifuge machine for 5 minutes at 6500 rpm RT. The centrifuged cells were then washed with TrisHNO3 buffer pH 7.0 and also centrifuged again with the same buffer and pH respectively. The pellets were also dissolved in TrisHNO3 buffer.

2.1.3.2 Preparation of Fe3O4 nanoparticles

Fe3O4 nanoparticles was perfomed by first preparation of 0.2 M aqueous solution of ferrous chloride (FeCl2.4H2O) in deionised water and then to precipitate a metals as hydroxide gel, 2 M aqueous solution of NH4OH was added drop-by-drop. The hydrated iron gel was completely washed and then transferd to a flask fitted with water condenser. To avoid the anion contaminants of Cl- and NO3- by formation of soluble salts with Fe2+ that might impede the reaction. The gel was stired under reflux for 4 h at 100 ^{o}C under N2 flow to ensure atmospheric inertness.During the reflux process, the continous influx of the solvent breaks the gel network into more energetically favourable small crystalline ironoxide regions and then the solid product was filtered and dried in an oven after refluxing [51].

3.1.3.3 Preparation of 1% chitosan solution

A 1.0g of chitosan powder was weighed using a weighing balance and added in beaker containing 100mL 1% acetic acid and allowed for 3 h to dissolved the chitosan completely [52] .High molecular weight chitosan was used due to posession of many hydroxyl and amino group which play a vital role to facilitate in an enzymes binding. The prepared chitosan solution was stored at 4 ^{O}C in refrigerator when not in used.

3.1.3.4 Modification of the electrode

Prior to the modification of the gold plate working electrode, the electrode was first cleaned by washing with acetone and rinsed with distilled water and also sonicated for 2min in an ultrasonic water bath. The Fe3O4 magnetic nanoparticles suspension (1.7mgmL−1) was prepared by dispersing Fe3O4 nanoparticles in distilled water with ultrasonication for about 2 h. To get the best amperometric responses of the biosensor, the composition of the whole cells–Fe3O4 nanoparticle–chitosan was used. Typically,the Fe3O4 nanoparticles, chitosan, and bacteria cells (Cells density of $6.0x10^4$) with a volume ratio of 1:1:2 were mixed thoroughly, and 10 µL of this mixture was dropped on the surface of the cleaned Gold-plate working electrode [53] and spreads evenly. Then the electrode was dried at 4 ^{O}C for 45 minutes in regeregerator. Before measurements, the biosensor was immersed in 50 mM PBS (pH 7.4) to wash out the non immobilized components from the electrode surface.In control experiment same procedure was used using *E-coli TG1 pBS (kan)* without ToMOplasmid.The biosensor was preserved at 4 ◦C when not in used. Daily microbial electrodes preparation with fresh cells were used in all the experinments.

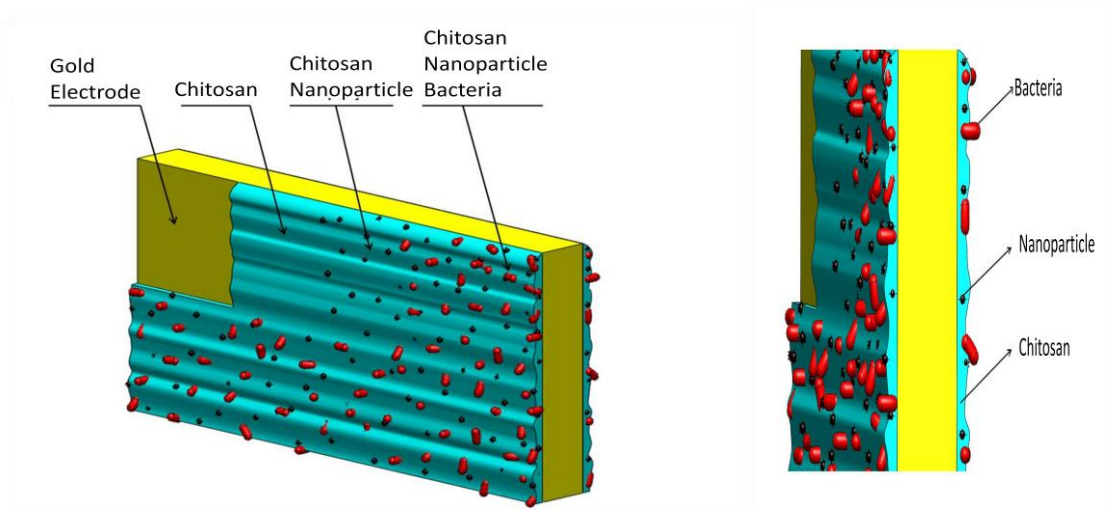


Figure.3.1 Immobolization system of bionanocomposite.

3.1.3.5 Electrochemical measurements

A cyclic voltammetric measurements were performed using a conventional three-electrode system in the reaction cell in an unstirred PBS conditions at an applied desired scans rate. An amperometric measurements were carried out at room temeperature using CH model 842B electrochemical analyzer which was purchased from CH instruments under continous and constant maganeticstiring. The reaction cell contained a three conventional electrode system with a platinum (Pt) wire as counter electrode, an Ag/AgCl-saturated KCl reference electrode as well as gold plate working electrode. The working electrode, reference and a counter electrodes were all immersed in 15 mL phosphate buffer (pH 7.4, 50mM) which was the working buffer. The desired operation potential was applied. After reaching a steady-state, a known amount of the substrates were injected individually into the reaction cell at regular intervals of time and the responses as well as the changed of the currents after additions were observed and recorded respectively.

CHAPTER 4

RESULTS AND DISCUSSIONS

After performing all the experimental analysis, the following results were obtained and discussed accordingly.

4.1 CYCLIC VOLTAMMERTIC CHARACTERESTICS OF THE BIOSENSOR

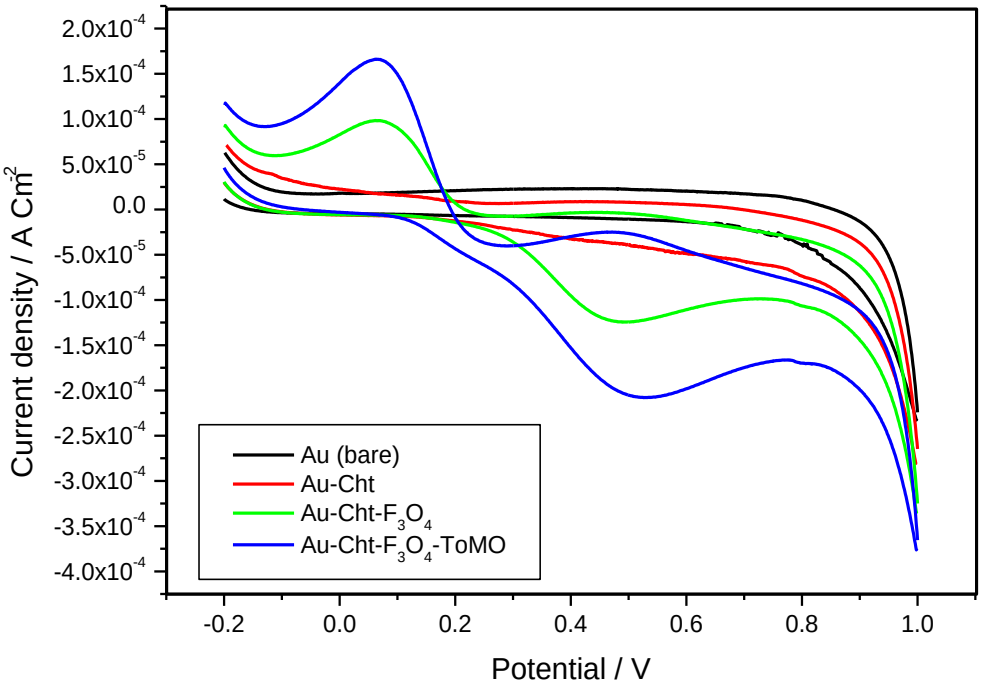


Figure 4.1 Electrochemical sensing characteristics of the whole cells–Fe3O4 nanoparticles–chitosan nanobiocomposite biosensor.

In this study, catechol was used as phenolic model compound to investigate the electrochemical sensing characteristics of the whole cells–Fe3O4 nanoparticles–chitosan nanobiocomposite biosensor.The resultsshows the typical cyclic voltammograms of catechol at bare Gold-plate electrode , chitosan–Gold plate electrode, Gold plate electrode–chitosan-Fe3O4and bacterial cells–Fe3O4 nanoparticles–chitosan respectively.It was found that, there is no redox peak observed at the bare Gold electrode and also not well-define at chitosan–Fe3O4–Gold electrode in the absence of bacterial cells even at a concentration of catechol up to 1 mM. A well defined redox peaks were observed at the chitosan–Fe3O4 nanoparticles-Gold electrode and bacterial cells–Fe3O4 nanoparticles–chitosan–Gold electrode. This clearly shows that, the observed redox peak was attributed to the bacterial enzyme-catalyzed reaction on the electrode surface [53] which comfirmed that, the gold electrode surface was modified.

4.2 APPLIED POTENTIAL

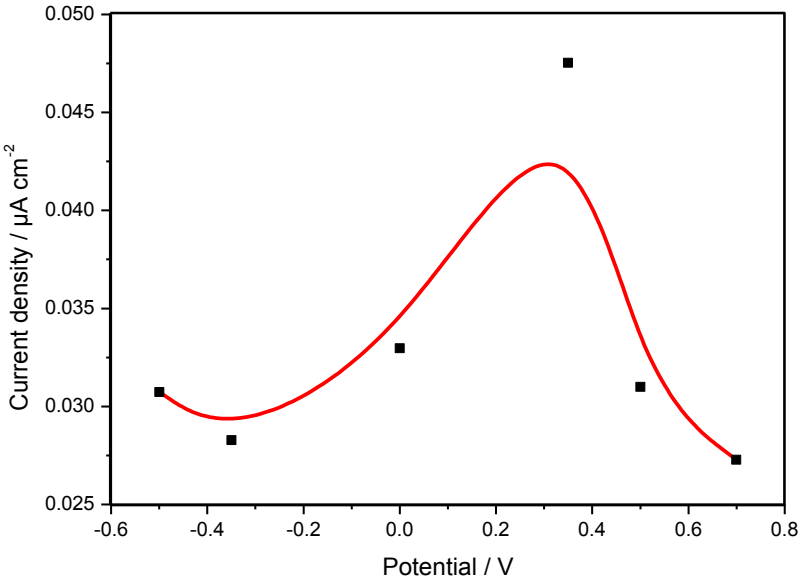


Figure 4.2 Response of the amperometric sensor at different potentials.

To investigate the optimum potential for the operation of biosensor, the working electrode was operated at a different potential (between −0.5 and 0V, and +0.5V potential step), and the transient currents were allowed to decay to a steady-state value.The above figure shows the effect of the working potential of biosensor on the amperometric response of 1 µM catechol and the background currents. The reduction current was initially observed at -0.5V, and it increased rapidly as the applied potential shifted from−0.5 V to 0V, and also from 0V to +0.35 V maximum and then decreased from +0.35 down to 0.5V. The highest ratio of signal-to-background current was obtained at +0.35 V.Therefore, +0.35 V was choosen as an optimum potential and all amperometric measurements were carried out at +0.35V and the proposed sensor still used low potential when compared with previous studies of Cell-based biosensor for measurement of phenol and nitrophenol toxicity [54] and is 0.04V greater than the potential used by[16]respectively. Additionally, lower potential can bring interferences from electroactive species [53].

4.3 EFFECTS OF pH

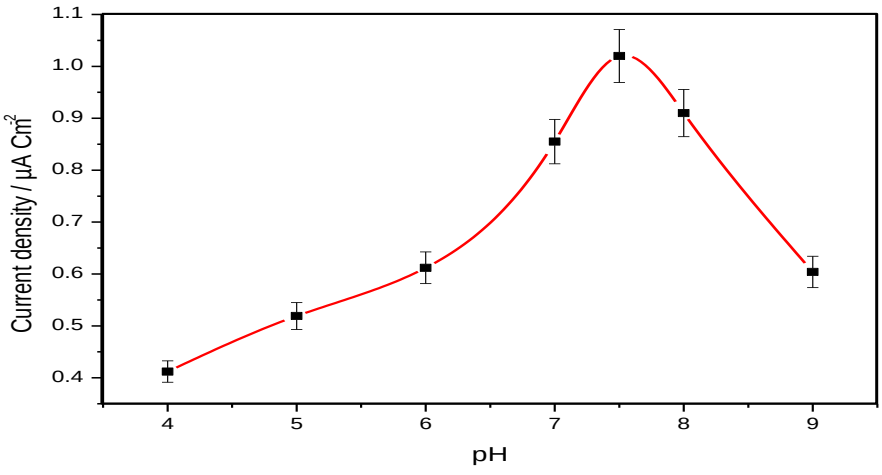


Figure 4.3 Effects of pH after addition of 1 µM catechol at a potential of 0.35 V.

In order to optimised the effects of pH, the optimization studies was investigated on the amperometric response using phosphate buffer system (50 mM) between pH 4 and 9 for 1 µM catechol in 15 mL PBS. The response of the amperometric bacterial electrode was significantly increased from pH 4 until it reached a maximum at pH 7.5 and also a sharp decreased was obatianed from maximum pH 7.5 down to pH 9. Therefore pH 7.5 was choosen as maximum pH which is 0.5 above the optimum pH obatained by [23] and this was nearly the optimum for the free enzyme, and this effect may be attributed either to the effect of the cell walls on which the enzyme is anchored and displayed or the influence of the chitosan matrix in which the cells were immobilized [23]. In daddtion, pH flactuations can adversely affect the normal metabolic activity of the bacteria [18]. Obviouly, the effcts of pH increased with increasing the pH from 4 to 7.5 and decreased from 7.5 to 9 with increasing pH.

4.4 EFFECTS OF TEMPERATURE

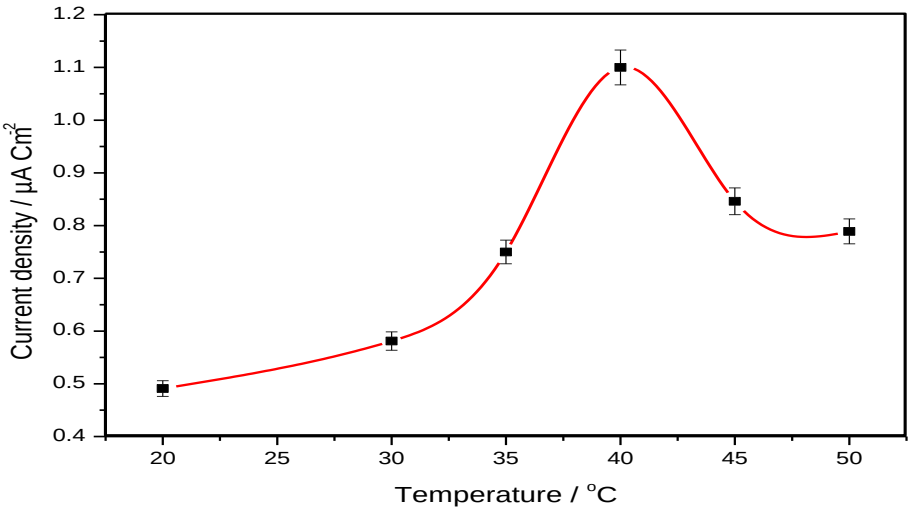


Figure 4.4 Effetcts of temperature after addition of 1 µM catechol at potential of 0.35V.

The effects of temperature based on the amperometric response of the working electrode was investigated between 20 ^{O}C to 50 ^{O}C, using a phosphate buffer system (50

Mm, pH 7.5) for 1 µM catechol. And it was shown that, there was an increased in response from 15 ^{0}C up to the maximum at 40 ^{O}C in which the best current value was observed, which was closed to the optimum growth temperature of the bactearia (37 ^{O}C,) and *E-coli starin* aerobically cultivated at 37 ^{0}C [13] and 40 ^{O}C was choosen as the optimum temperature obtained. A sharp decreased was also obtained at the optimum 40 ^{o}C down to 50 ^{O}C. This shows that the present biosensor can be affected by a temperature as shown in the above figure. However, room temperature was used for all other experinments.

4.5 STEADY-STATE RESPONSE OF THE AMPEROMETRIC BACETRIAL ELECTRODE

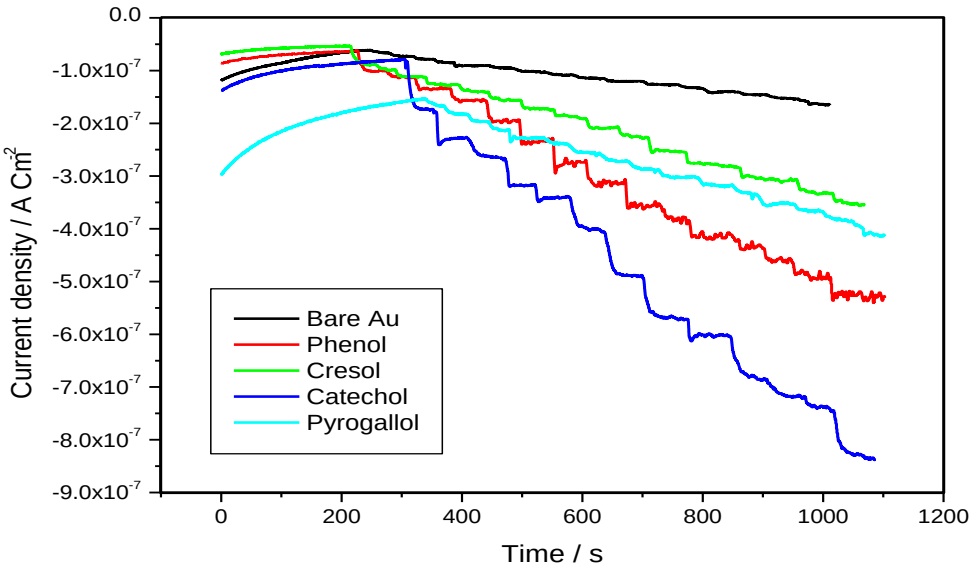


Figure 4.5 A steady-state responses of the amperometric bacterial electrode to different substrates using same amount of 1 µM at an applied potential of 0.35V.

An amperometric bacterial electrode to different substrates were carried out.This has been acheived by additions a known amount of the chemical into 15 mL PBS (50 mM, pH 7.5) reaction cell, of three-electrode system with Pt. as counter electrode, Ag/AgCl reference electrode as well as gold-plate as the working electrode, after a steady-state was reached, the changed of currents and responses were observed and

recorded respectively. The chemicals tested includes; phenol, p-cresol, catechol, and pyrogallol, and each of the chemical tested gave a significant responses while the negative control using bacterial cells without ToMO after additions of catechol, the response produced was insignificant compared to the bacterial cells with ToMO as shown in figure 4.5 above.

4.6 CULIBRATION CURVE GRAPHS

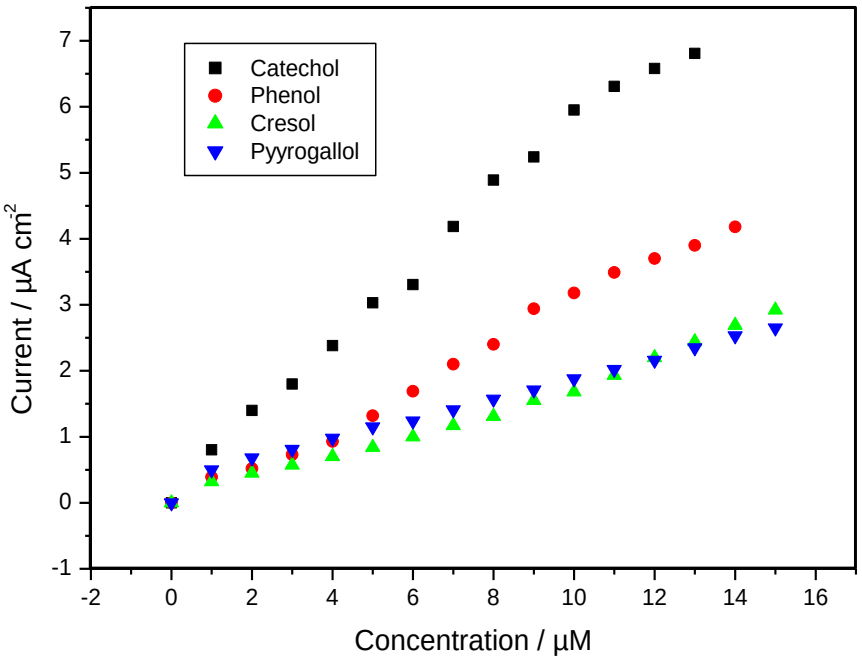


Figure 4.6The calibration curve graphs with different phenolic compounds at a potential of 0.35 V vs Ag/AgCl.

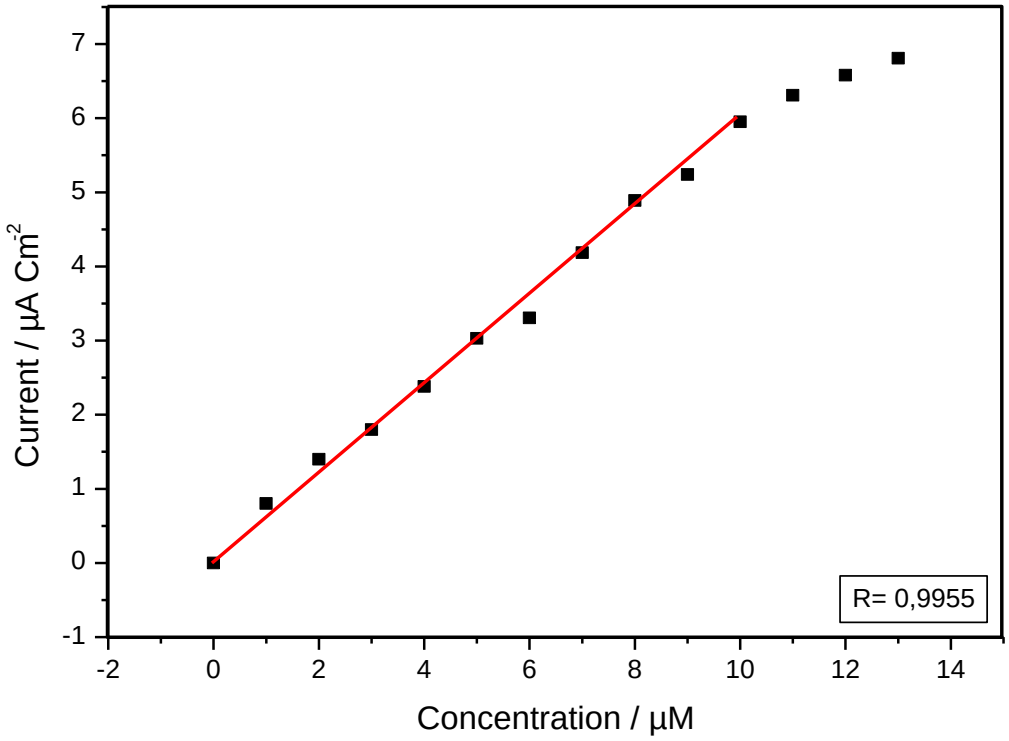


Figure 4.7 Calibration curve graph of catechol to the bacterial electrode at an applied potential of 0.35 V vs Ag/AgCl.

The above figures shows the calibration curve graphs of the bacterial electrode to different phenolic substrates, the responses rapidly increases with increasing the concentration of the substrates and then reached a plateau.As shown in figure 4.6 the best response was obtained from catechol within a shot response time of 3s followed by phenol than cresol and lastly pyrogallolrespectively.The linear range of catechol was found to be 1-10 µM, with a detection limit of 0.5 µM and sensitivity of 30 µA/µM respectively. From figure 4.7, it can be observed that, the calibration graph of the catechol gave a good linearity as the value of correlation coefficient (R) of 0.9955.

4.7 REUSEABILITY

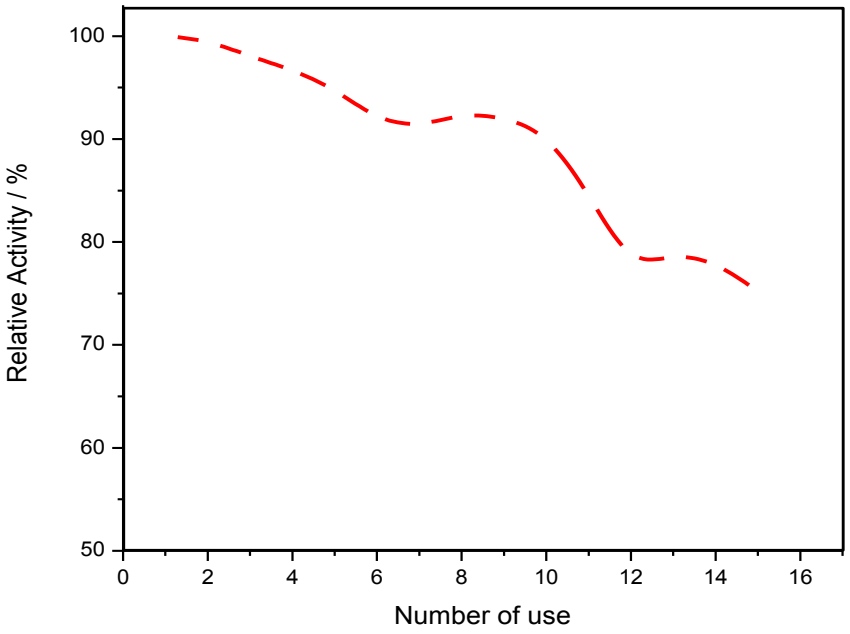


Figure.4.8 Reuseablityofamperometric response of the bacterial electrode to 1 µM catechol additions at an applied potential of 0.35 V.

The reuseatability of amperometric response of the bacterial electrode to 1 µM catechol was examined as shown in the figure 4.6 for about fifteen consecutive measurements with same working electrode. It was obseved that, from 1 to 9 measurements maintained about 90% of the bio-activity and then decreases drastically.Therefore, the proposed biosensor shows a good reuseabilitywhich retained about about 75% bio-activity after fifteen consecutive measurements. As such, the present biosensor can be used in a number of times using same working electrode without theworking electrode lost it total activity.

4.8 STORAGE STABILITY

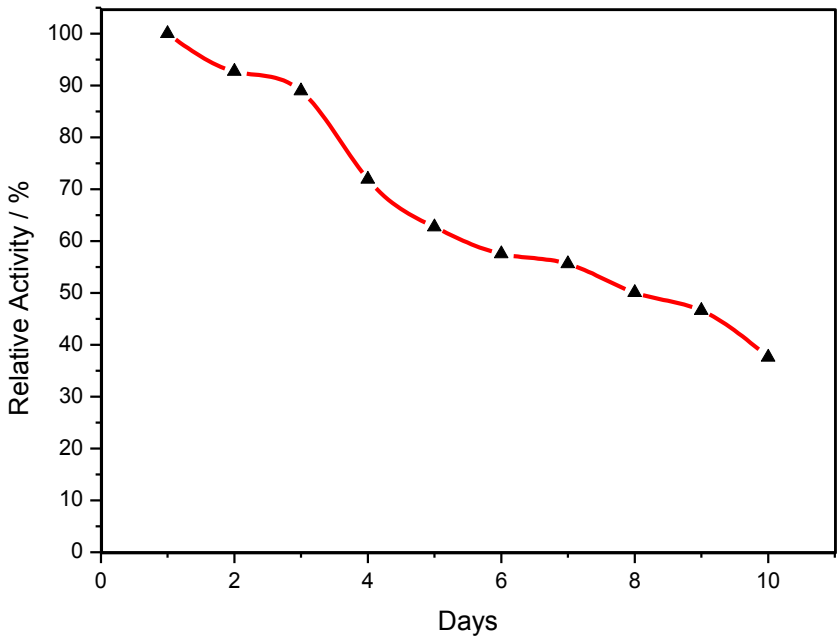


Figure 4.9 Storage stability response of the bacterial electrode to 1 µM addition catechol at an applied potential of 0.35 V.

The storage stability was investigated by addition of 1 µM catechol into the reaction cell. The electrode was measured at interval of time for ten days and the response of the microbial sensor was recorded. When the electrode is not in used, it was stored in a phosphate buffer at 4 ^{0}C in a referegerator. It was observed that,in the first two days the sensor retained its bioactivity for about 90%, and then started to decreases drastically and in the last ten days maintained about 35% bio-activity. After ten days, the sensor started to lost itsbio-activity and therefore, this sensor shows that,it can be used for some days without the electrode lost it total activity and it can be closely related when compared to the storage stability of amperometric bacterial phenol biosensor as reported by [2] which retained its bioactivity for about 25% after storage.

Table 4.1 Table of comparison of analysis for the performance of biosensors.

Electrode	RT(s))	LR(μM)	DL(μM)	Ref no
Fe3o4-Chitosan-bacteria	3	1-10 μM	0.5	Present work
Fe3O4-Si(GMA-co-Vf)-HRP	3	500-15000	500	[51]
Fe3O4NPs/cMWCNT/PANI/Au	3	0.1-10	0.03	[5]
ITO/Sol–AuNPs/Tyr	–	1-6.0	0.3	[8]
GCE-Sol-gel/ Tyr	18	1-60	0.2	[57]
Pt-Au-organosilica@chitosan	–	0.06–90.98	0.02	[58]
PANI/ Modified enzyme electrode	–	5–500	0.8	[59]

LR; linear range, DL; detection limit, RT;response time.

From the above Table 4.1shows a table of comparison of analysis for the performance of the biosensors with previous studies.The response time,linear range as well as detection limit of the present biosensor exhibit a good results when compared to [8], [51], and [59] respectively.

CHAPTER 5

CONCLUSSION

5.1 CONCLUSSION

Conclussively, a simple whole cells amperometric biosensor in combining with chitosan and Fe3O4 nanoparticles as immobolization matrices has been developed sucessufully for the determination of phenolic compounds. *E-coli TG1 pBS (kan) ToMO*was used as an experinmental working organisms and also an *E-coli TG1 pBS (kan)* was used in the experinmental control respectively.The Fe3O4 nanoparticles possessed a large surface area and the presence of porous morphology of the chitosan, leads to the high loading of the bacterial cells which entrapped, consequently leading to the retaining of the bio-activity of the bacterial cells covalently onto the working electrode which creates and enhanced a fast electron transfer by the bio-catalysis activities of the magnetic nanoparticles.

In optimisation studies, the effects of pH, temperature and optimum working potential were all investigated. Cyclic voltammetry was also performed to investigate the electrochemical sensing characteristics of the whole cells–Fe3O4 nanoparticles–chitosan nanobiocomposite of the biosensor. It was found that, the bionanocomposite of whole cells-Fe3O4 nanoparticles-chitosan showed a good sensing characterestics when compared to gold electrode-chitosan-Fe3O4, gold electrode-chitosan as well as bare gold electrode respectively. Storage stability was also studied and the results shows that, the proposed sensor can be stored for about ten days without the working electrode of the sensor lost its total bio-activity. Reuseability was performed for about fifteen consecutive measurements using same working electrode and results of the present biosensor shows that, the sensor can be used more than one time using same working electrode without thelost of total bio-activity of the sensor.

A steady state responses of different chemicals compounds were tested, among others includes phenol, p-cresol, catechol and pyrogallol.Calibration curve graphs of different phenolic compounds were presented.

Therefore, the proposed biosensor can be widely used successufully and unexpensively for the determinations of these phenolic compounds for many commercial applications because of the whole cells used which is simple and non expensive compared to enzyme biosensor which purification of the enzyme is very difficultsto be done. However, the improvement is needed in further investigations.

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