DEVELOPMENT OF OPTICAL BIOSENSOR AND ITS APPLICATION FOR THE DETECTION OF CADMIUM IONS IN MILK

Thesis Submitted for the Award of the Degree of

DOCTOR OF PHILOSOPHY

in

CHEMISTRY

By

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2023

DECLARATION

I, hereby declared that the presented work in the thesis entitled "**Development of Optical Biosensor and its application for the detection of Cadmium Ions in the Milk**" in fulfilment of degree of **Doctor of Philosophy (Ph. D.)** is outcome of research work carried out by me under the supervision **Dr Anil Kumar**, working as **Assistant Professor** in the Department of Chemistry of Lovely Professional University, Punjab, India. In keeping with general practice of reporting scientific observations, due acknowledgements have been made whenever work described here has been based on findings of other investigator. This work has not been submitted in part or full to any other University or Institute for the award of any degree.

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CERTIFICATE

This is to certify that the work reported in the Ph. D. thesis entitled " **Development of Optical Biosensor and its application for the detection of Cadmium Ions in Milk** " submitted in fulfillment of the requirement for the reward of degree of **Doctor of Philosophy (Ph.D.)** in the **Department of Chemistry** , is a research work carried out by **Varsha Shivaji Pawar, 41900058**, is bonafide record of her original work carried out under my supervision and that no part of thesis has been submitted for any other degree, diploma or equivalent course.

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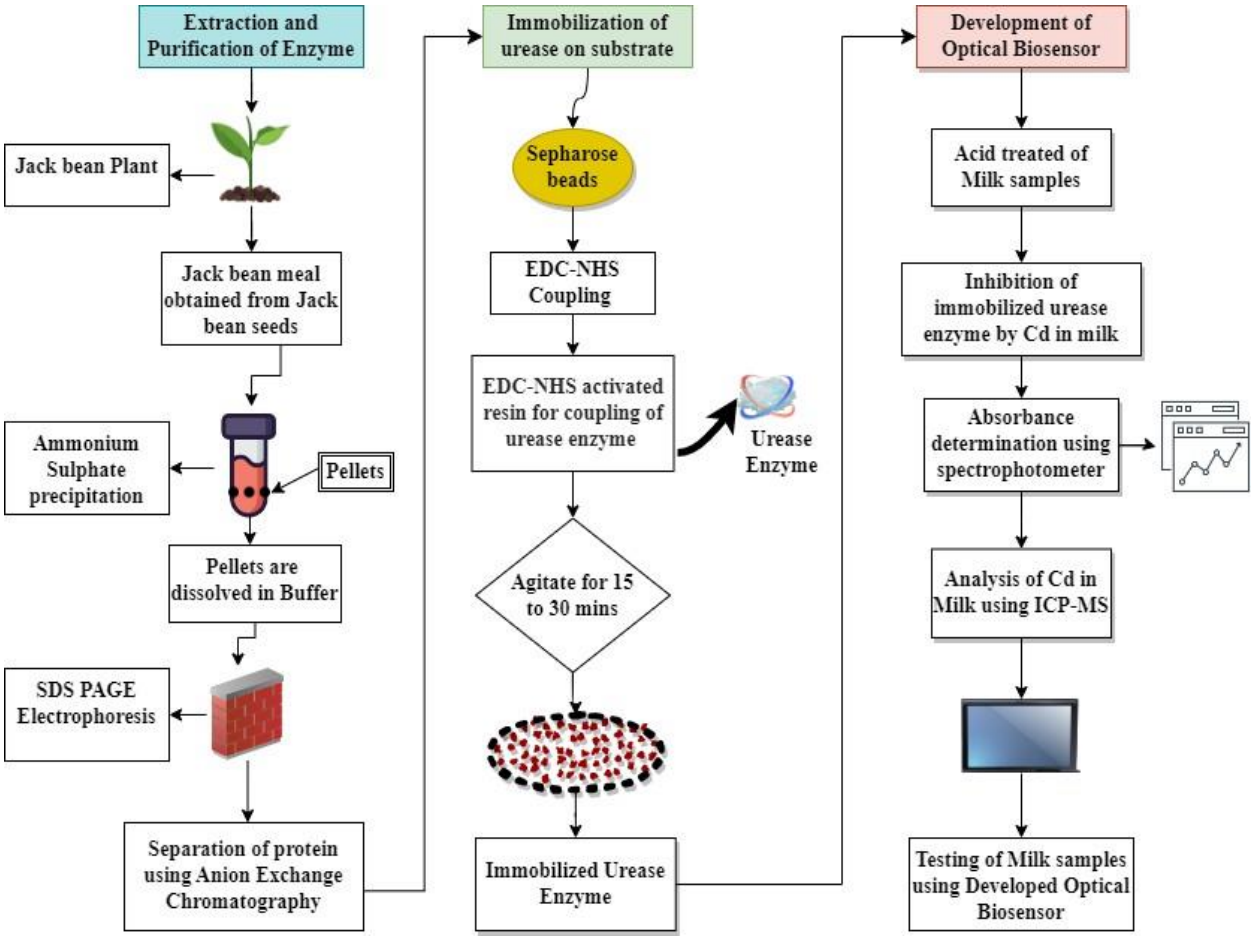
ABSTRACT

Food diseases and allergies are fundamental causes that majorly affect the world population's health. Milk and milk products as one of the primary requirements of nutrition but nowadays these products are critical types of packed foods consumed by the current global residents. Milk sources get polluted due to other sources like heavy metals, pesticide residues, and aflatoxin during cattle grazing, pre-processing, and inappropriate handling during the post-processing duration. Consequently, society requires a precise, fast response, stable and sound analytical instrument to detect milk contaminants in packed milk and milk by-products. Recent expansion in biosensors delivers renewed hope to gain the purpose of milk contaminants detection additional, precisely and accurately. The proposed research addresses the development of an optical biosensor for detecting toxic ions, i.e., cadmium in dairy and raw milk. The proposed research methodology get classified into three section: 1. Purification of urease enzyme 2. Immobilization of urease enzyme and 3. Development and validation of proposed optical biosensor for cadmium detection in the milk. Purification is essential to isolate the specific enzyme from a crude extract containing many other unwanted components. It is a vital process for separating protein under interest for its characterization and determining its structure. Thus, in the proposed research, purification of urease enzyme from jack bean meal was carried out using ammonium sulphate and acetone precipitation method. This proposed research observed purified urease at 90 kDa with specific activity (173.67 units /mg) and % purification (99.71%). The purified urease is further immobilized with the effectiveness of 1-Ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (Hydroxyethyl-piperazine ethane-sulfonic acid) (EDC-NHS) crosslinking approach for the functionalization of urease enzyme, extracted from jack bean. Such modified urease (EC.3.5.1.5) was immobilized onto sepharose beads to optimize stability and reactivity. The proposed coupling reaction was studied at different pH levels (7.0, 7.2, and 7.5) and at different coupling times, i.e., 15 mins and 30 mins. The effective immobilization was observed when the coupling conditions were slightly alkaline, i.e., pH 7.5 and longer coupling duration, i.e., 30 min. After this reaction time, a urease activity assay was performed for immobilized urease at pH 7.5. The confirmation and progression of functionalization and immobilization were evaluated using spectroscopic and imaging approaches. Further, the optimized urease was stable (67% in seven days and 66% for the next three

weeks) due to excellent crosslinking during functionalization and immobilization. The novelty of this optimized research is to establish a first-time novel approach of coupling urease enzymes on these surfaces as a function of time and pH, where the enzyme retains functionality and longer shelf life.

Immobilized urease is used as a bio component for identifying cadmium (Cd) metal in the milk. The milk sample is treated with nitric acid to remove significant impurities such as lead ions. An inhibition study of immobilized urease enzyme is performed with urea and phenol red indicator assay. The absorption variation due to the presence of cadmium in the milk sample was determined using the proposed optical biosensor. The proposed optical biosensor is a transducer that works on the bioassay principle based on the inhibition of urease activity. The obtained results of optical biosensors are validated with UV/Visible spectrophotometer and ICP-MS. The proposed optical biosensor is successfully applied to acid-extracted milk samples. The lowest Cd detection limit achieved was 4.8 µg/L. The linear range of detection of Cd concentration lies in the range of 4.8µg–10 µg/L. The biosensor stored at 4o C can detect Cd even after 35 days indicating its excellent sensing stability, which is a bottleneck for proposed biosensor. A good correlation of results was obtained with spiked milk samples also. The proposed research achieves a lower detection limit and more extended storage stability. But still, specific modification is required to reduce the proposed biosensor's lower detection limit to be suitable for the on-field detection of cadmium ions in milk samples.

Graphical Abstract



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Ms. Varsha. S. Pawar

Research Scholar

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LIST OF ABBREVIATIONS

FAAS	Flame atomic absorption spectrometry
EDTA	Ethylene Diamine Tetra acetic acid
PEG	Polyethylene Glycol
JBU	Jack bean urease
TCA	Trichloroacetic acid
PVC	Polyvinyl chloride
SAM	Self-assembled monolayer
GA	Glutaraldehyde
HRP	Horseradish peroxidase
AFM	Atomic Force Microscopy
SPM	Scanning Probe Microscope
XPS	X-ray photoelectron spectroscopy
TEM	Transmission electron microscopy
QCT	Quercetin
SDS-PAGE	Sodium dodecyl-sulfate polyacrylamide gel electrophoresis
CBB	Coomassie brilliant blue
EDC	1-Ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride
NHS	N-Hydroxy succinimide
HEPES	Hydroxyethyl-piperazine ethane-sulfonic acid
PBS	Phosphate buffer saline
FTIR	Fourier transform infrared
FESEM	Field Emission Scanning Electron Microscope
EDX	Energy Dispersive X-Ray Spectroscopy
ICPMS	Inductively coupled plasma mass spectrometry
AEX	Anion Exchange Chromatography
DEAE	Diethylamino ethane
SEM	Scanning Electron Microscope

CHAPTER 1

Introduction

1.1 Overview

The most frequent and important factor affecting the health of the global population is the increase in environmental pollution, as well as the pre- and post-processing methods used on milk by various machinery. Due to these methods, there are chances of contamination of milk with heavy metals [1]. Heavy metals have a harmful effect on human health, contributing to conditions like “blood cancer, kidney failure, osteoporosis, bone damage, gastrointestinal problems, and hormonal imbalances”.

The most hazardous heavy metal that can be discovered in milk is cadmium [2]. There are a variety of ways for detecting heavy metals in milk, however there are still problems with cost, size, sensitivity, and durability. To sense toxic metals in milk, new, precise, robust, and sensitive biosensors are therefore needed [3]. The new biosensor that is being proposed will make an effort to raise the detection thresholds, durability, and sensitivity for the finding of Cd ions in milk that can contribute to new knowledge in discipline of chemistry.

1.2 Significance of Milk in human life

Milk and milk product consumption is often considered essential in a strong and composed diet. It is considered to be the firstly nutrition for animals which can provide all nutrients to ensure good growing and development which is important for jawbone mass development [4]. It consists of nutrient contents like calcium, phosphorus, and potassium. It is an excellent source of vitamins, mostly B and D. Daily milk intake prevents diseases like osteoporosis and bone fractures, and it is also helpful to maintain a healthy weight. In addition, under nutrition in children’s can lead to a significant deterioration in linear growth, also can lead to hyperglycemia, hypertension, high blood lipids, and, in adulthood, hyperglycemia, hypertension, high blood lipids, obesity [5]. Also, many studies have confirmed the nutritive status of milk in the social food and provision the likely role of its feeding in avoiding numerous chronic circumstances like “cardiovascular diseases (CVDs)”,

approximately formulae of “cancer, obesity, and diabetes”.

Hence it is one of the most profitable businesses all over the world. Due to the environmental pollution, pre-processing and post-processing techniques of milk by different machinery, milk contamination due to heavy metals increases daily. Supply of good quality and hygiene milk is a significant issue in all developing countries; hence, contamination of milk due to the occurrence of heavy metals is one of the serious concerns to the world.

1.3 Causes of toxic metals in the milk

Toxic metals are persistent, non-biodegradable and gather in the food chain. Due to rapid industrial growth, pollution of the environment increases day by day, which can cause the transfer of heavy metals from nature into the food chain. Thereare many reasons behind the transfer of heavy metals into the milk, which are listedbelow: [6]

1. Use of effluents from industry in agricultural sector for farming and use of untreated sewage water.
2. Cattles during grazing can result in the consumption of contaminated grass as food.
3. Milk can also get contaminated due to the use of machinery and types of equipment during pre-processing as well as post-processing.
4. Heavy metals contamination in milk from the soil.
5. Also, environmental pollution is another crucial cause of milk contamination.

1.4 Consequences of heavy metals in human health

The elements having metallic properties at room temperature, which consists of high atomic weight from the periodic table, are considered heavy metals with a specific gravity of 5 grams per cubic centimeter. These elements accumulate in mammals and cause severe issues to human health [7]. Contamination from heavyweight metals in the human food chain occurs in different ways, such as garbage, dust, wastewater and sewage from factories. Out of all heavy metals, Mercury, Cadmium, and Lead have been reported as not having any benefit for humanity.

Heavy metals contain Lead, mercury, copper, cadmium, zinc, arsenic, chromium and iron as shown in Figure 1.1

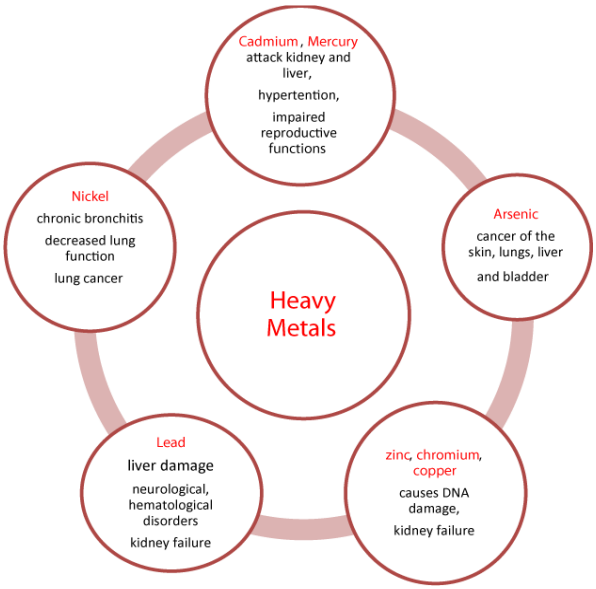


Figure 1.1 Effects of heavy metals on Human Health such as Lead, Nickel, Cadmium which can lead to health disorders related to heart, nervous system and development of different types of cancer.

It can cause serious health problems; therefore, accurately determining their residues is a concern which can result into illness such as "respiratory disorders, nervousness", and different types of "cancers, infertility problems, system disorders, weakening of immune system, renal failure, genetic mutations and cardiovascular diseases". Also, lead can result into health disorders related to immune system, anemia, and damage to the kidneys, liver, the digestive tract, heart and blood vessels, central nervous system disorders and development of different types of cancers.[8] Due to Lead, there are possibilities of two types of poisoning: chronic and acute. Chronic poisoning is related to "antisocial behavior, blindness, kidney malfunctioning and loss of sexual desires". While in the second case, there may be nervous behavioral problems, convulsion, absent-mindedness and nausea. Heavy metal such as cadmium can lead to diseases which can affect "heart, liver, bones, kidneys, lungs, and blood vessel". It gathers in tissues like the liver and kidneys, resulting in increased blood pressure. It is poisonous, particularly in the prostate and lungs which causes the tumors to develop. Food contamination due to "cadmium causes bone demineralization, hypertension in pregnant women, disturbed calcium metabolism, and hypercalciuria". Nickel sources numerous "blood, brain and bone cancers and local infections". Due to the presence of mercury, there are chances of neurological

disorders in terms of decreased memory, IQ loss, autism and eye-tongue-related problems. [9]. After considering the different complications, it becomes substantial to emolument courtesy for the incidence of these metals in milk as it has extraordinary ingestion group of all ages which is the main objective of research. In short, heavy metals harm the world population's health in terms of diseases like hormonal disorders, kidney failure, osteoporosis, blood cancer, skeletal damage, and gastrointestinal [10]. In our research proposal, we are planned to detect cadmium ions contamination in milk with the help of the optical biosensor in a particular region of India.

1.5. Regulatory limits

Toxicity of metal can be defined as toxicity having destructive effects due to the consumption of metal beyond certain limits. It is meticulously related to age, sex, direct route, input level, solubility, metal oxidation condition, retention rate, and duration of orientation, the commonness of information, absorption rate and agencies/efficiency of excretion [11].

Metallic poisonousness from milk is a serious matter associated to additional food sources due to the high intake of milk in infants and old age people. The exact figure of toxicity is an unresolved issue as there are no standard calculations regarding health impacts. Nickel, Cobalt, and Copper are stated to have some health benefits, but intense more than allowable limits might generate fitness dangers. Lead, Cadmium, and Mercury have no assistances noted concerning humanoid fitness. Thus, milk is beneath strong regulations due to its high consumption and susceptibility to heavy metals. Owing to cadmium and lead harmfulness to human health their levels in milk are prohibited or restricted by global international regulations and national regulations in different countries. Many regulatory boards have recognized permissible limits for Cadmium in milk due to their substantial toxicity. Different countries havedifferent acceptable limits, thus the permissible limit in India is 0.0028 to 0.0035 µg/ ml and the world health organization (WHO) regulatory board is 0.003 µg/ml. This limit is taken from joint FAO/WHO food standards programmer codex committee on contamination in food report from Fifth edition 2011.

1.6 Cadmium ions detection methods

To detect cadmium in milk, two possible approaches are used by different researchers: Analytical chemical techniques and Biosensor techniques, as shown in Figure 1.2

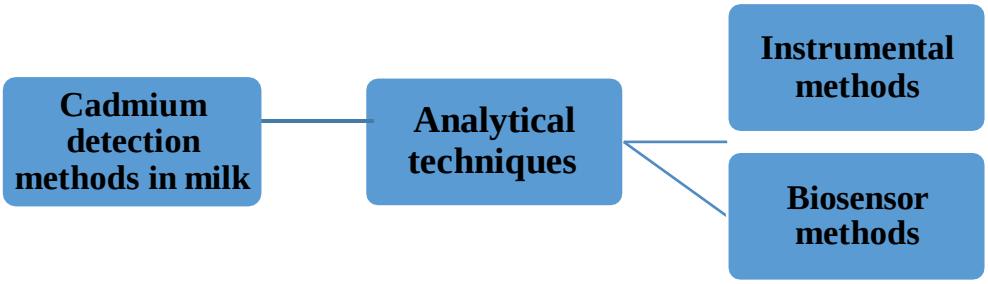


Figure 1.2 Different analytical techniques available for Cadmium detection in milk which are classified into instrumental and biosensor methods

1.6.1 Instrumental methods

Subsequently the level of trace elements of contaminants of Cd is low, it is essential to find analytical methods with adequate sensitivity [12]. In the traditional analytical way, many researchers used different techniques such as Emission of X-rays, Mid-infrared spectrometry, Electro thermal atomic absorption spectrometry (FAAS), differential pulse anodic stripping voltammetry, Complex metric titration, Capillary zone electrophoresis, flame absorption spectroscopy, atomic absorption spectroscopy, graphite furnace atomic spectrometer and inductively coupled plasma mass spectrometer to detect cadmium in milk.

Merits:

Instantaneously detection of multiple metal ions from the milk samples.

1. High precision and sensitivity.
2. Reasonable detection limits and dynamic linear range.
3. High sample throughout
4. Large amount of sample required for analysis.

Demerits:

1. Analysis of only solutions can be carried out.
2. Instruments are larger in size.
3. Requires skilled operators for handling.

4. Expensive
5. Unsuitable for online and in-person heavy metal detection from milk.

1.6.2 Biosensor Technique

Biosensors are gaining more popularity in recent years because it is small in size, fast in response and low in cost. Due to the on-field detection ability, they are primarily suitable for online applications such as environmental pollution control, healthcare, food quality control etc. In heavy metals detection, many researchers have proposed and designed different biosensors like Electrochemical, Optical, and Enzyme based to detect cadmium in milk.

The term biosensor is an analytically sensitive tool that can convert biological signals from an analyte into electrical signals. The biosensor comprises of four dissimilar components, as shown in Figure 1.3. The initial biosensor was established by Leland C. Clark Jr. (1918-2005), who is the Forefather of biosensors, recognized as the Clark conductor for dimension of oxygen in the blood, water and additional liquids in 1956 [13].

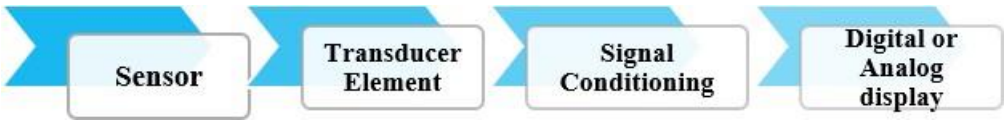


Figure 1.3 Various components of Biosensor which measures signals resulting from the interface of analyte into electric indications which are measured by Analog or digital display.

Out of four components, the sensor is one of the essential components in the improvement of biosensors which is made up of sensitive organic resources such as tissue, microorganisms, organelles, enzymes, receptors, antibodies etc. In comparison, transducer elements use different principles such as physicochemical, optical, piezoelectric and electrochemical to convert signals resulting from the interaction of analyte into user-friendly electrical signals. Signals obtained from the transducer element are further modified in terms of amplification and noise reduction with the help of signal conditioning components. The further readout is made by either analogue or digital display [14]. Biosensors are in large applications in various fields due to the following merits and demerits:

Merits

1. Wide range of detection limit
2. High stability
3. Inexpensive
4. Smaller in size
5. Fast in response
6. High selectivity towards targeted ions
7. The system is portable.

Demerits

1. Performance of biosensor sensitivity is influenced by pH and temperature
2. Complicated measurement conditions
3. Necessity for sample preparation [15]

The presence of cadmium ions in milk is a global problem of research as milk contamination due to cadmium has been detected in many nations in the last ten years. Thus, biosensors have played a significant role in detecting different heavy metals in milk. Milk contamination is a severe issue for both developed and developing countries. But in a developed country, there is strict control over the permissible limit. Still, in developing countries, all heavy metals are reported because of unhealthy situations of dispensation, muted food and water used for natures, negligence, and unawareness of personnel. Hence, there is a strong need for research in the development of miniature, fast in response, low in cost and susceptible devices for regulation of cadmium contamination in milk.

1.6.3 Mechanism of Interaction of Cadmium with enzyme

The naturally occurring enzyme urea amide hydrolase (EC 3.5.1.5), which is widely distributed in fungi, "bacteria, algae, plants, and other species," is entirely dependent on the metal ion nickel. [16,17] Furthermore, carbamic acid and ammonia are produced when urea is broken down by urease enzyme [18,19]. The heteromeric molecules are further divided into three types: α, β, and γ. The subunit has two Ni^{2+} (Ni1 and Ni2) centres and the active urease site is located there. [20]. Furthermore, the general catalytic mechanism of urease depends on these two nickel ions. The binding and activation of urea are carried out by one of the nickel ions, and the other

nickel ion carries out the binding and activation of nucleophilic water molecules. As seen in Figure 1.4, the nitrogen atom of the amino in the urea interacts with Ni2, which encourages the release of NH_3 and carbamate.

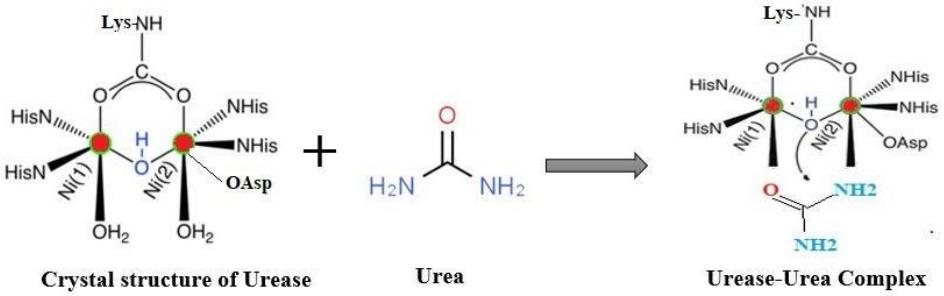


Figure 1.4 The crystal structure of the urease -urea complex, indicates chelate complex of urea to the 'two nickel ions in the active site" showing the primary step of the catalytic mechanism of urea hydrolysis.

The existence of carbon-based materials, heavy metals, and substrate equivalents alters the activity of enzymes. Many studies have shown that the activity of several enzymes is impacted by "heavy metals such as Cd , Cr , Cu, Fe, Hg, Ni , and Ag". The active site or enzyme may interact with cadmium, obstructing it or altering its structure, which prevents substrate attachment and the formation of products as shown in Figure1.5. This stops the enzyme from working properly.[21]

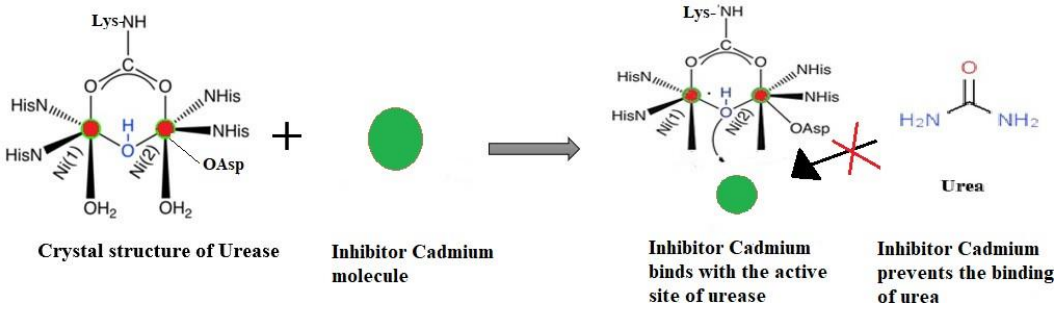


Figure 1.5 Inhibition of urease activity by Cadmium molecule which prevents the binding of urea substrate to the urease enzyme.

As a result, the cadmium molecule attaches to an enzyme's active site and blocks urea from attaching to the urease enzyme. Consequently, urease activity is inhibited, which prevents the hydrolysis of urea.

1.7 Motivation

The increase in environmental pollution, pre-processing and post-processing techniques of milk by different machinery leads to the rise in the chances of milk contamination day by day, and it is the most common and leading cause which affects the health of the world population. Detection of milk contamination due to heavy metals is an attractive growth area of research as it is strongly associated with the risk to human health. The health of the global population is negatively impacted by heavy metals in terms of conditions like "blood cancer, kidney failure, osteoporosis and bone damage, gastrointestinal and hormonal issues." Cadmium is one of the maximum poisonous heavy metals originate in milk. Hereafter, the investigation undertaken in the proposed work addresses the societal problem of predicting the likelihood of cadmium in milk and conceptual enrich the discipline. Even though there are numerous ways for screening heavy metals in milk, there are still problems with cost, size, durability, and sensitivity. As a result, there is no widely accepted biosensor that is dependable, economical, small, robust, and highly sensitive. Hence there is a demand for developing new,accurate, stable and sensitive biosensors for heavy metals detection in milk. The proposed new biosensor will attempt to increase the detection limits, durability and sensitivity for the detection of cadmium ions in milk which will help to enhance newknowledge in the discipline of chemistry.

This proposed research covers the problem of international-level milk contamination due to cadmium with the help of a novel biosensor which will be helpful for the welfare of society and enrich the healthcare solutions, which is the primary need of every nation.

1.8 Outlines of thesis

Chapter 1 "Introduction" This Chapter is the opening chapter which highlights the aspects concerning the intended field of study.

Chapter 2 is entitled- **"Literature survey"** on diverse state-of-art techniques for cadmium detection techniques. This survey consists of the contribution of various researchers toward the analytical methods for cadmium detection and also focuses on the history and background of the intended area of research. The primary focus of this chapter is to provide extensive literature survey for intended area of research to the readers.

Chapter 3 “Materials and Methods ", presents the details about the purification of the urease enzyme. This chapter provides a comprehensive and comparative precipitation method for urease extraction and “SDS-Page electrophoresis” to specify the mol wt. of the desired protein. Further, it explains the anion exchange chromatography for separating protein under study. It also enlightens the novel method for maximum % purification and activity of urease enzyme. This chapter also discuss the research gap and objectives of the proposed study. It also describes the methodological measures in detail to accomplish the desired goals

Chapter. 4 "Results and Discussion" explains the result outcomes of the novel method for extraction, purification and immobilization of urease enzyme using sol-gel techniques. This chapter further illustrates the evaluation of the activity of the immobilized urease enzyme for cadmium detection in milk.

Chapter 5 The **"Summary and Conclusion”** summarizes the proposed research work with its findings and elaborates on the future scope of research which could help the future scientist in the research area.

CHAPTER 2

Literature Survey

2.1 Overview

The study of the available literature determines the future research that should pursue in light of the collection of existing knowledge. The initial phase of the proposed research includes a comprehensive review of the literature to gain an inclusive understanding of the intended field of the research area. Any literature review aims to amalgamate and interpret the ideas and factual information across previous research in a relevant area. This current chapter is further classified into four subsections: the initial section will address the background and historical literature review of the proposed research area. The following sections include an extended literature review on urease extraction, purification, and immobilization utilizing sol-gel techniques and, in conclusion, the study of the optical biosensor methods for cadmium detection in milk and milk products.

2.2 History and Background intended area of research

Due to the increase in industrial activity, the pollution from heavy metals has grown widely throughout the world since the late 19th and early 20th centuries. Heavy metals cause serious health problems therefore accurately determining their residues is concerned seriously. Heavy metals in the body may cause side effects such as nervous system disorders, renal failure, genetic mutations [4], types of cancers, neurological disorders, respiratory disorders, and cardiovascular, immune system weakening and infertility. Hence in this literature review, various research innovations for detecting cadmium from milk and milk products with different sensors are studied in detail to understand their implementation strategy, merits, demerits, future scope, etc., to get better clarity on the proposed research topic literature survey is divided into three subsections

1. Urease Enzyme Extraction and Purification
2. Enzyme immobilization
3. Detection of cadmium using biosensors and conventional methods

The subsections are as shown in Figure. 2.1

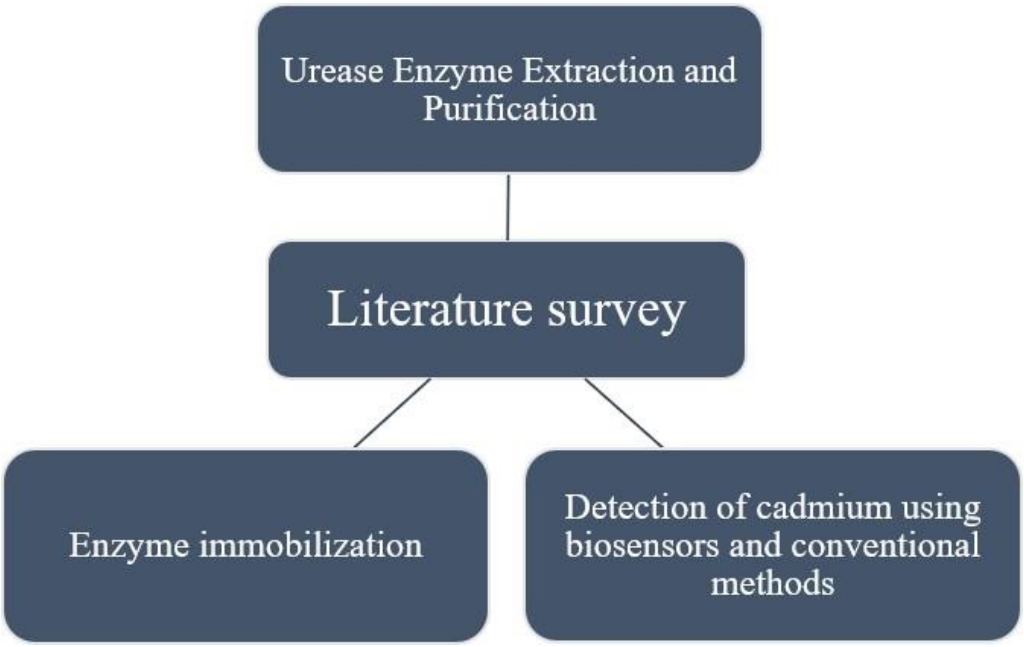


Figure 2.1 Literature review strategies for various enzyme immobilization techniques and different biosensors and conventional methods for the detection of cadmium

2.2.1 Urease Enzyme Extraction and Purification.

The immediate source of urease extraction is plants and beasts. The principal challenge researcher's face is the extraction of urease enzymes urea amidohydrolase from plants and animals as it is endangered by a stiff cell partition made of inquisitive materials (i.e., proteases). Furthermore, age and the type of plant part (seed, leaf, or fruit) are used to impact the extraction outcomes [22]**.** Proteins are incredibly complex macromolecules with a wide range of structural and functional variations. The interpretation in a native setting has an impact on protein extraction as well. Constructing an environment using a buffer and conducting removal at the proper temp is required to accomplish good-quality removal of enzyme and % yield. [23]. It is also crucial to utilize the appropriate balance of additives and reducing representatives to promote the extraction process. Proteins are enzymes that serve as biological catalysts by accelerating specific chemical reactions. They are also exact while using their substrates transferases, lyases, isomerases, ligases, etc., which are the six major enzyme types [24, 25]. Phosphotriesterases and Amidohydrolases are groups of metalloenzymes with considerable molecular weight, including urease.

In 1975, Kappaun *et al.* [26] identified urease's physical function, which is suitable for a nickel, an enzyme that all plants, and several soil-dwelling bacteria, fungi, and algae, use

to hydrolyze urea, producing ammonia and carbon dioxide [27]. The findings state that urease is directly related to plants; urease extracted from the jack is the most accurately described of all the plant sources. Various commercial uses for the urease enzyme have been reported [28], including the dimensions of urea in intemperate drinks, noticing heavy metal ions by biosensors in seeing environmental monitoring techniques and industrial effluents [29]. Unfortunately, the primary constraint of enzyme removal is that little enzyme is separated, which costs a considerable share. The central goal of this analysis paper is to deliver a particular enzyme removal method that will yield a large amount of output at a low extraction and purification expense.

Additionally, urease enzymes are toxic, multifunctional proteins that are becoming more significant in animal and human diseases and crop protection. Therefore, there is a social need for an effective urease enzyme extraction and purification technology. The methods used by various researchers to pull urease from jack bean seeds vary. Since urease extraction is costly and challenging, there is a shortage of information on this subject, which is why it is featured in this section. An innovative method to pull natural urease from the source of jack beans was suggested by Khodadadi *et al*. [30]. The removal of enzyme from the JB meal is represented in Figure 2.2.

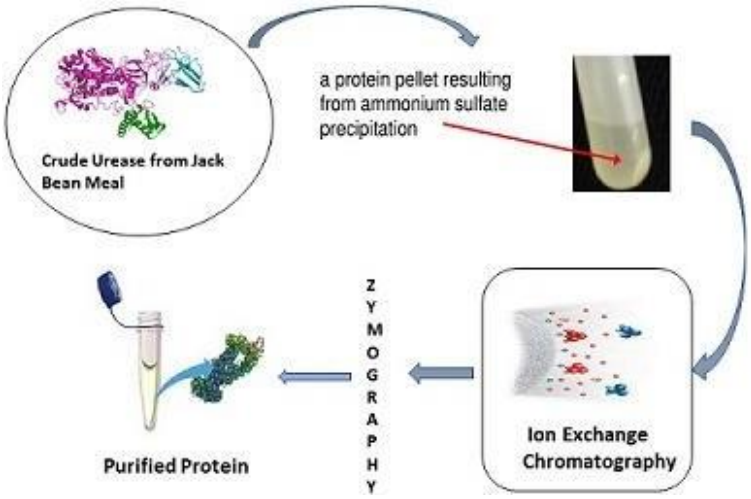


Figure 2.2 Pictorial representation of extraction of urease from JB meal using $(NH_4)_2SO_4$ precipitation and Ion exchange chromatography techniques

The technique projected that; a 50 g input sample was dipped overnight in 200 milli liter of the extracted solution at 7.5 pH along with the temperature of 4-degree celcius. 2mM of ethylenediamine tetra acetic acid and 20 mM of phosphate buffer made up the extraction

solution (EDTA). The JBM was centrifuged in a similar way regulated and filtered out. The supernatant was then diluted with 40 percent volume of acetone which was pre-chilled. The combination was centrifuged for 15 minutes at 25,000g between 0-degree and 4-degree. "SDS-PAGE" was used to confirm the final outcomes for the appearance of urease protein and activity, which was discovered to be about 90 kilo Dalton urease protein having precise activity of arithmetical value 867 U/ml.

The planned route for the extraction of Jack bean gives a good quantity of urease protein and moderated particular activity. One of the most important considerations for getting a good purity of protein from a JBS is the good route for extraction and purification. The extraction and purification rate is an essential concern for gaining a good purified protein output from a JBS plant. Therefore, to increase the rapidity of the technique, "Weber *et al"*. [31] suggested the extraction which was further carried by a crystallization route to get urease from the JBS plant. The outcomes of proposed route shows that the 40 % extraction of the urease enzyme carried out in the existence of acetone solvent. The planned route by authors is the utilization of PEG has an outstanding removal result for the isolation and extraction of urease enzyme from the JBS plant. The suggested approach for Jack bean extract produced more urease and reduced precise activity for each of the 3 ladders of extraction. Therefore, Weber *et al.* [31] suggested the removal trailed by a crystallization approach to abstract urease from the JB source rapidly. According to the results of this approach, 40% of the urease was extracted while acetone was present. For the removal and segregation of urease from the JB meal, these authors' suggested method involves using "poly (ethylene glycol)", which has an outstanding isolation result. The achieved particle sizes in this separation procedure were less than 2 m. Using PEG 4000 at pH 6.0, the maximum precise activity was 162 Units/mg. PEG solutions wash-down urease precipitates and reported the finest selectivity compared with the remaining 2 abstraction mediums, $LiSO_4$ and NaCl, which is another innovative aspect of this method. Tekinar *et al*. [32] presented an "immobilized metal affinity chromatography method" for purifying jack bean urease (JBU) employing "poly [PHEMAH-$^{2+}$Cu] chelated with copper II (Cu^{2+})". In this study, the distillation of JB urease was skilful in one step with the yield of 88.80 percent and below 10 out of a hundred of column capacity loss after eight binding-elution cycles. With the use of MAH monomer, work as a metal chelating ligand. In this procedure, Cu

ion can be directly added to the PHEMAH cryogel underprivileged of experiencing any additional alteration. The JB urease monomer's MW was determined to be 90 kDa using common protein markers. With this route the detailed activity having value 8.83 Units/mg was restrained. An easy method for urease removal was presented by "Balasubramanian *et al".* [33], the authors proposed that jack bean meal and acetone were highly concentrated. On a 10% "SDS-PAGE", the purified urease appears as a single 90 kDa band. The primary restriction on this technique was that the results of the enzyme's exact activity were not available for the judgment with other report. A limited articles were discussed likewise for gaining urease from plants. Hefnawy *et al.* [34] used Pisum Sativum L. seeds to segregate urease with a produce of 40% and a distillation fold of 12.86. Because it makes a lot of enzymes, the microorganisms is used to monitor contaminations. The "acetone precipitation" method was utilised by Zusfahair *et al.* [35] to get urease enzyme from black eyed pea seed of plant. The precise activity achieved in FA 80 during the fractionation phase was 428.59 Units/milli gram having cleanliness of 2.2 times. Consequently, the enzyme's refined fold was 4.5 and its recovery rate ranged from 55% to 98%. It has been found that some adjustments and actions are necessary to obtain more ideal outcomes for the purification plus extraction of urease enzyme from the source of JB meal. In similar vein, this study describes how to abstract and disinfect the enzyme from JB. In the suggested procedure, salt precipitation can be a commanding instrument for protein purification through precipitation. According to published literature [36], the most common method is "acetone/TCA precipitation". By changing the medium pH with "trichloroacetic acid (TCA)", protein precipitation is accomplished using this approach. TCA is added to the mixture to greatly lower the electrostatic forces, revealing more of the protein's hydrophobic structure. For the reduction of solubility of the protein and lower the dielectric constant of an organic solvent, acetone is employed in conjunction with TCA [37]. This process has a lesser level of purity and is more expensive, which is its principal disadvantage.

2.2.2 Immobilization of enzyme

Enzymes are fastened to or contained by solid supports during the immobilization process, resulting in a heterogeneous immobilized enzyme system. Enzymes that are immobilized mimic the way they normally function in living cells, where they are typically attached to organelle, membrane, and cytoskeleton components.

However, they are not available to typical carbon-based chemistry techniques, which brands them particularly intriguing for biotechnology [38]. Enzymes are organic reactants that aid in the transformation of chemical species into biological systems. They are made up of thousands of atoms arranged in particular orders, and they can catalyze the various chemical reactions taking place in biological cells. Enzymes have a very high degree of substrate specificity and can catalyze reactions below moderate states, which prevents the buildup of byproducts. Numerous intricate chemical transformations between biological macromolecules are among the processes that are catalyzed [39] applications. Enzymes were capable of evaporation activities at the start of the twentieth century, and researchers began to question the makeup of these molecules.

Enzymes catalyze reactions in a variety of states, including surfaces, aggregation of other entities, and individual molecules in solution. The concept "restrained enzymes" denotes to enzymes that are contained or confined in a precisely distinct spatial area while retaining their catalytic actions and are able to be used recurrently and endlessly. In any event, the use of immobilized catalysts has substantially improved the economy and technical performance of automated operations [40]. As shown in Figure 2.3 several strategies, including adsorption, covalent bonding, cross-linking, and encapsulation, are described in the literature for immobilizing biomolecules.

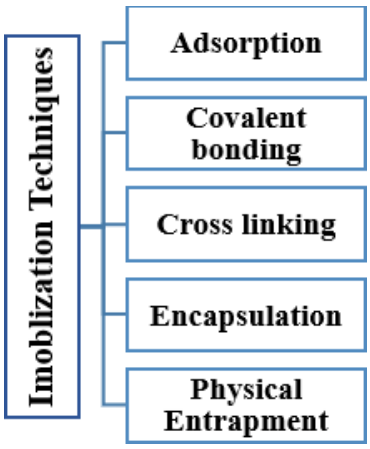


Figure 2.3 Various Enzyme/cell immobilization techniques to trap the enzyme on a solid matrix support to increase the enzymes stability and reusability

The process of trapping an enzyme with a specific support or matrix is known as immobilization. The substrates, effectors, particles can interchange between the backing

and matrix on which the enzymes are immobilised. Restrained enzymes' main advantages are increased functional effectiveness and the ability to reuse enzymes [41]. With a better version of stability, it also reduces reaction time. More importantly, it reduces the suggested application's capital expense. Despite these advantages, immobilization techniques have certain drawbacks, such as the high cost of enzyme separation, purification, and recovery [42]. Additionally, certain enzymes lose their catalytic characteristics after being immobilised and become unstable.

2.2.2.1 Matrix or support used in immobilization technique

The technology for immobilization of cells and enzymes evolved steadily for the first 25 years of its existence but in recent years it has reached a plateau, if not a slight decline. However, the expansion of biotechnology, and the expected developments that will accrue from advances in genetic technology, has revitalized enthusiasm for immobilization of enzymes and cells. There are several different matrix carriers and supports that can be used for immobilisation. [43] These substances should be inexpensive, easily accessible, and require the least amount of time to react with the enzymeor other substances in the medium. There are three main categories of materials utilised for immobilisation: inorganic compounds, synthetic polymers, and natural polymers. Alginate, Chitosan and Chitin, Collagen, Carrageenan, and Cellulose are some of the natural polymers that are most frequently used. [44] Ion exchange resins or polymers that serve asan insoluble support with a porous surface are known as "synthesis polymers." The enzymes or entire cells can become trapped in their porous surface. For instance, “polyvinyl chloride (PVC), UV-activated polyethene glycol (PEG), diethylaminomethyl cellulose “(DEAE cellulose), ceramics”, silica glass activated carbon, and charcoal are examples of inorganic materials [45-46]. Limitations include decreased retention, stability, storage, and operational simplicity despite additional immobilization procedures.

This thesis is divided into several sections that each cover the most recent techniques for immobilising enzymes. Covalent bonding is covered in Section 2 whereas cross-linking processes are covered in Section 3. The adsorption is further discussed in Section 4, and the entrapment is discussed in Section 5. Finally, section6 provides a discussion, and Section 7 concludes the article.

2.2.2.2 Covalent bonding techniques

The most popular technique for immobilising enzymes involves the creation of covalent connections between chemical groups on a support or carrier. This approach uses an enzyme to swiftly establish covalent connections between hydroxyl and amino groups. The alpha carboxyl group at the enzyme's C terminal, the alpha-amino group at the enzyme's N terminal, and the phenol ring of tyrosine are a few of the crucial functional groups that help the enzyme create covalent bonds with support carriers [47]. Carrier or support materials employed in these methods include "inorganic carriers, protein carriers (collagen, gelatin), synthetic agents (polyacrylamide), and carbohydrates (cellulose, agarose) (Porous glass, silica)". The fundamental idea behind covalent bonding techniques is shown in Figure 2.4.

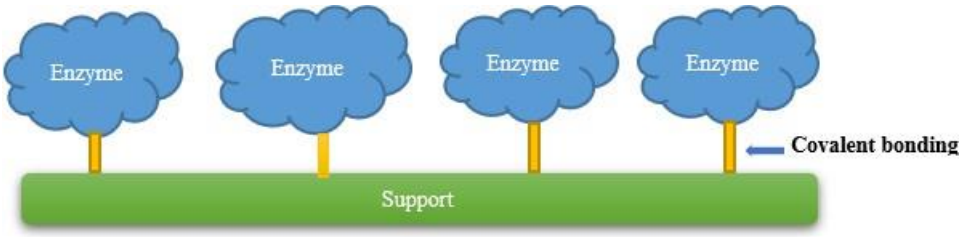


Figure 2.4 Covalent bonding immobilization technique refers to the formation of covalent bond between the enzyme and support matrix.

For the propose of immobilising enzymes, Nguyen *et al*. [48] proposed two phases of covalent bonding. In the initial step, the surface was activated by Linkers, Multipurpose reagents made of inorganic material, polymers, or membranes, either natural or manufactured. This resulted in a SAM. Enzyme covalent coupling was carried out to give enthusiastic support in the second stage. In the literature on this methodology, numerous advanced techniques are documented. Another investigation proposed by Chen *et al.* [49] used glutaraldehyde as a cross-linker to form covalent bonds between thionine-bovine and Nano-Au. Peroxidase Horseradish was immobilized by connecting nano-Auto and other free amino groups of thionine (HRP) with a revealing limit of 2.4 x 10^{-9} M and a Menten –Michaelis K- Mapp value of 0.025 mM, the biosensor reacted closely to H2O2 in a linear range in-between 4.9 x 10^{-8} to 1.7 x 10^{-4} M. This method involved doing amperometric and cyclic voltammetry studies as well as an AFM imaging of the film under a SPM. It involves a combination of electrochemical techniques (amperometry and cyclic voltammetry) and atomic force microscopy (AFM) to study a film or surface.

Measurements from XPS were made using a spectrometer. Using KBr discs and an infrared spectrophotometer with a Fourier transform, IR spectra were gathered. The dimensions of Au colloidal nanoparticles were determined using TEM. The created amperometric biosensor stood out for its storage stability, which demonstrated a longer lifetime when the electrode was suspended in 0.11 M PBS (pH 7.5) at 480 C in a fridge. After three days, two weeks, and one month, the existing reaction to 0.10 mM "H2O2 dropped by 3.7%, 9.8%, and 21%" of its initial value. As a result, the novel approach was used to encapsulate enzymes and produce reagent-free Amperometric biosensors. To create Th-BSA conjugate, thionine" was first covalently attached to the "BSA film. To create a "nano-Au/Th-BSA matrix "and immobilize HRP", nano-Au was adsorbed onto it. The resulting technology gives electrochemical devices a new bio-composite platform. Immobilized HRP maintains strong enzymatic activity and high affinity for H_2O_2, as evidenced by a low apparent Michaelis-Menten constant, a broad linear range, and a low detection limit for H_2O_2 measurement. Based on free-enzyme and free-substrate electrocatalytic pointer magnification using porphyrin-based covalently connected nanomaterial, Lio *et al*. [50] created a new electrochemical biosensor for the finding of "uracil-DNA glycosylase (UDG) (OAPS-Por)". It also had strong electrocatalytic activity that allowed it to diminish Thi deprived of by H_2O_2. Using an Au-S bond, the hairpin DNA hDNA with four uracil bases was immobilised on AuNPs/GCE. By means of DNA hybridization, the signal probes are then joined to the unfolded "DNA". Due to the active "electrocatalytic activities of OAPS-Por toward Thi", the Thi in signal probes might provide a unique electrochemical signal that could then be further amplified and output. As a result, the electrochemical biosensor as it was first built had an extensive direct range from 0.004 to 1.0 U mL^{-1}. A low finding limit of 7.97 x 10^{-4} U mL^{-1} was also established. Moreover, this biosensor has excessive probable for practise in biomedical investigation and scientific diagnostics because it can extent UDG reserve (UGI) and UDG activity in real materials HeLa cell lysates and humanoid blood serums. Gomes *et al*. [51] created a procedure in which (EDC/ NHS) were used to covalently encapsulate hodian MAMB-05 on "carboxymethyl Botryosphaeria". By means of a basic carbon dark adhesive rod as a transducer, this process was recycled to create an innovative "laccase-based biosensor" to electrochemically quantity quercetin (QCT). EIS was used to examine the recommended biosensor, and Nyquist graph were

labouring to measure the enzyme's restriction. The investigational circumstances for "laccase immobilization, the pH" of the backup electrolyte, and the involved limitations of the electroanalytical technique were amongst the variables that were enhanced for defining QCT. With a hypothetical recognition boundary of 2.8 x 10^{-9} mol L^{-1}, a direct association among the cathodic current greatest and QCT attentiveness was originate in square-wave-voltammogram data. QCT was successfully determined using the suggested method in samples of drinks, medications, and biological material. The probable use of this biosensing stage in chemically compound explanations was established by the recommended biosensor device's robust selectivity in the occurrence of uric acid, dissimilar inorganic ions, and other phenolic substances. The laccase-operational biosensors and analytical stability was assessed, and respectable intra-day (SD = 1.24%), inter-day (SD = 2.33%), and prolonged storing repeatability (SD = 3.48%) are revealed. With quick reaction times, chemical selectivity, cost-effectiveness, and non-polluting recognition elements, the suggested biosensing platform described in this paper seems to have broad applicability in bio analytical findings. Murphy *et al.* [52] produced a unique approach to produce the TPP-HA [TFSI]) and characterize it with single-crystal X-ray diffraction and additional phantom methods. Completed a glossy carbon electrode covered with MWCNT, the manufactured TPP-HA [TFSI] was placed (GCE). In this technique, "TPP-HA [TFSI]/MWCNT/GCE" was shaped and cast-off as a host matrix for the covalent immobilisation of the water-soluble protein cytochrome c (Cyt c) through the EDC/NHS coupling reaction to create a stable amide bond between the free eCOOH groups.

When the electrode was electrochemically characterised by cyclic voltammetry, the cathodic and anodic peaks at 0.39 V and 0.27 V, respectively, were found to correspond to the heme active sites of "Cyt c (FeIII/FeII)". Additionally, the modified electrode performed superbly in the "electrochemical reduction of H2O2". An ampere meter was used to amount the stable catalytic reply at an working voltage of 0.45 V. H_2O_2 was slow and its straight range, sensitivity, and finding limit were found to be 20 to 894 M, 0.15 A M1 cm^2, and 6.3 M, correspondingly. The future modified electrode, in specific, showed unsettled selectivity and sensitivity for the discovery of H_2O_2, as well as amazing stability and reproducibility. In direction to amount in situ hydrogen peroxide, Bocanegra *et al.* [53] created a chemiluminescent biosensor built on the covalent immobilization of the horseradish peroxidase (HRP) enzyme on a polydimethylsiloxane (PDMS) substrate

(H_2O_2). H_2O_2 as the rusting agent has been stately using the chemiluminescent process using luminol as anoxidizable substrate and HRP as the catalyst.

Applications of the suggested biosensor's performance include assessing the distribution of H_2O_2 from denture cleanser medicines and determining the quantity of H_2O_2 unconfined by cells in a culture medium. With a discovery limit of 0.023 M and robust linearity over the variety of 0.061^{-10} M, the conclusions of both examines presented that the biosensor is sensibly valued, delicate, and discriminating. The correctness comparative normal deviance, % RSD 6 was similarly satisfactory. The ease, movability, and reusability of the strategies make this biosensing system robust; it can be cast-off up to 60 times while retentive 90% of its activity. The consequences established that the biosensor is a forthright and dependable device for calculating hydrogen peroxide by procurement a comprehensive linear variety, a low discovery limit, and a rapid response time. This method delivers in-situ capacities with a tall grade of correctness and has pertinent informations of value, such as great sensitivity. Moreover, the capacities can be rapidly composed (just 10 s). The biosensor encounters supplies for correctness, strength, and solidity. It must also be noted that the device is refillable, permitting it to be used more than sixty times. Due of hydrogen peroxide's role in frequent procedures, the biosensor has long-term applicability. By an outline with vigilant image, Murphy *et al*. [54] shaped a new electrochemical biosensor too rapidly and exactly amount hydrogen peroxide (H_2O_2). The HRP based biosensor was shaped by covalently assigning the enzyme to an ionic liquid with TBACOOH-IL. Furthermore, it was immobilised on a glossy carbon electrode with a multiwalled carbon nanotube deposit "(MWCNT/GCE)". The mortal – NH_2 of HRP and the COOH groups of "TBACOOH-IL" are used to establish a steady amide bond, and the pi-pi stacking securely attaches the "TBA-COOH-IL" to the MWCNT/GCE to form "HRP/TBA-COOHIL/MWCNT/GCE". As an outcome, the created "HRP/TBA-COOH-IL/MWCNT/GCE" established a sharp redox peak at a prescribed potential (E) of -0.32 V, which is consistent with the hidden "FeIII/FeII" redox centre of the immobilised HRP enzyme. The created biosensor was also used to measure "H_2O_2 electro catalytically" under stationary and dynamic environments, and it exhibited a broad linear variety from 0.02 to

4.30 mm with good sensitivity and a low discovery limit of 160.6 A mM^{-1} cm^{-2} and 6 M, respectively. The stable covalent attaching of an easily water-soluble enzyme on a newly advanced, highly leading, and biocompatible platform is accountable for the biosensor's excellent presentation. The created biosensor also presented good repeatability and prolonged long-term stability. By covalently immobilising HRP to the quaternary ammonium-based carboxyl functionalized ionic liquid modified "MWCNT/GCE", a simple biosensor for the discriminating discovery of H_2O_2 was created. The biosensor has a leakage-free rigid surface thanks to the covalent anchoring of "HRP on TBA-COOH-IL" via the creation of amide bonds, and it is stable even under dynamic circumstances. The "TBA-COOH-IL" that has remained synthesised has excessive biocompatibility, which assistances to continue the biological activity of the HRP. Trigonzyl substitution in "TBA-COOH-IL" and "MWCNT's" pi- desirability would have providing improved conductivity and stable immobilisation in the temporary. The afresh intended "TBA-COOH-IL/MWCNT/GCE" platform's superior conductivity made it easier to communicate electrically with the deeply buried redox centre of HRP, and as a result, the direct electrochemistry of HRP was effectively established. Additionally, the created biosensor displayed great stability and reproducibility along with a notable reduction in H_2O_2. This innovative multifunctional platform's fabrication of "HRP/TBA-COOHIL/MWCNT/magnetic GCE's characteristics" makes it a suitable biosensor for quick and accurate limit detection. Thenmozi *et al.* [55] created the enzyme "horseradish peroxidase", which covalently bound the water-soluble agent toluidine blue to the precursor of "3-aminopropyl trimethoxy silane". This modified silane was used to create a "stiff ceramic composite electrode" that was then used to create an amperometric H_2O_2 biosensor. "Cyclic voltammetry" was used to track the electrochemical performance of the adapted biosensor in the potential range of 0.2 V to 0.4 V versus SCE. The biosensor displayed a stable voltammogram with cathodic and anodic points at 0.232 and 0.172 volts, individually, and a formal possible of 0.203 volts. To advance the employed conditions, a figure of essentials influencing the biosensor's performance, including buffer solution, pH, high temperature, and energy, were looked at. With no barrier from possible interferents, the better-quality biosensor established decent catalytic performance for dropping H_2O_2 at a lesser potential of 0.27 V. With a detection limit of 0.172 mM, it was discovered that the

analytical working range for H_2O_2 was 0.430 M to 0.457 mM. In adding to its high sensitivity, rapid response, and benefit of surface renewability over forthright mechanical enhancing, the industrial biosensor is strong for long-term usage. Table 2.1 provides an illustration of a comparative analysis of covalent bonding.

Table 2.1 Comparative study of covalent bonding techniques including different performance parameters of biosensor with respect to detection limits, selectivity .and merits.

Author/Year	Methodology	Performance parameters	Merits
Chen et al.2008 [50]	Covalent Bonding	Output range =5.10 x 10^{-7} to 1.67 x 10^{-3} M Limit of detection= 2.11 x 10^{-7} M	Better storage stability. higher enzymatic activity Good affinity for H_2O_2,
Lio et al.2020[51]	Covalent Bonding	Output range = 0.005 to 1 U mL-1 Limit of detection =6.97 x 10-4 U mL-1.	High sensitivity High stability Low detection Limit
Gomes et al.2020[52]	Covalent Bonding	Output range =4.98–50.0 x 10-8 mol L-1, Limit of detection = 2.76 x10-8 mol L-1 SD = 3.47%	Fast response times Chemical selectivity Cost-effective and non-polluting.
Murphy et al.2019[53]	Covalent Bonding	Output range =20 to 892 µM, Selectivity=0.142 µA µM−1 cm−2 Limit of detection = 6.21 µM,	High stability with good reproducibility. Excellent selectivity and sensitivity.
Bocanegra et al.2020 [54]	Covalent Bonding	Output range = 0.02 µM Linear range = 0.06−10 µM RSD = < 6 Response time=10s	High linear range Lower detection limit, Fast response time
Murphy et al.2020 [55]	Covalent Bonding	Output range =0.02 to 4.30 mM Sensitivity= 160.6 µA mM-1 cm-2 Limit of detection = 6 µM,	Excellent stability Better reproducibility
Thenmozhi et al. 2017[56]	Covalent Bonding	Output range = 0.429 µM to 0.455 mM Limit of detection = 0.171 µM,	Better sensitivity Larger linear range

2.2.2.3 Physical Entrapment

The enzyme is actually physically confined inside the porous matrix using this method. The support matrix used in this process is often a water-soluble polymer, and the chemical bonds created might be covalent or non-covalent. The main advantage of this application

is that it adjusts the pore size of the support matrix utilizing the help of the polymer concentration to prevent enzyme loss. Agar-agar and carrageenan are two polymers that have greater pore diameters. This method's main drawback is the potential for low molecular weight enzymes to leak from the matrix. Figure 2.5 shows the fundamental layout of the physical trapping approach.

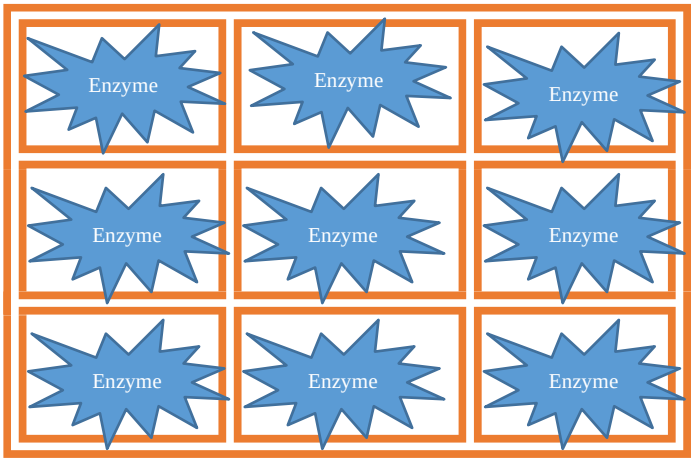


Figure 2.5 Basic structure of physical entrapment method which involves physical confinement of enzyme inside the porous matrix.

The various design and development efforts for physical entrapment approaches for biosensor design are outlined in this section. The 3D matrices physically entrap biorecognition components using covalent or non-covalent linkages. The enzyme is dissolved in a monomer solution, and the monomer solution is subsequently polymerized either chemically or experimentally. The biorecognition components are used to join the organic materials' 3D network. Materials such as “poly dimethyl siloxane, photopolymer, gelatin, alginate, cellulose, acetate phthalate, modified polypropylene, and polyacrylamide's” inorganic activated carbon, as well as permeable ceramics, are employed. Electro polymerization, the sol-gel method, and microencapsulation are often employed techniques in this process. A basic procedure called electro polymerization involves applying a current or voltage to an electrolyte or aqueous resolution that contains both molecules and monomer molecules. On the electrode's surface in the electrolyte, the monomer undergoes either reduction or oxidation. This results in reactive fundamental species, which combine to create a polymer that traps the biorecognition components

(enzymes) close to the electrode in the aqueous resolution. “Aniline, pyrrole, and thiophene” are the electropolymerized materials utilized to create enzyme immobilization films. The likelihood of enzyme leakage, which makes this approach less suitable for commercial use, is its main drawback [56].

2.2.2.4 Sol-Gel techniques

The most popular method for trapping enzymes at low temperatures is the sol-gel procedure. Metal-alkoxides are hydrolyzed and reduced to yield a nano permeable material. The bio-elements are enclosed in a 3D matrix as the network expands with time and temperature. These are the benefits of this method: chemical and thermal resistance. The capacity to enclose dense biomolecular mixtures under barely immobilizing circumstances a more approachable method of synthesis using the sol-gel method, Juan Li *et al.* [57] created an amperometric electrode with hydrogen peroxide using simple and efficient enzyme immobilization. “Horseradish peroxidase (HRP)” is immobilised in a sol-gel silica matrix on a carbon adhesive electrode for the “enzyme electrode (CPE)” which acts as a bridge for the transfer of electrons between the electrode surface and hydrogen peroxide, was present when hydrogen peroxide was identified. Using an amperometric approach, the impacts of working factors such as the working conductor's functioning potential, arbitrator absorption, pH, and thermal stability were examined for optimum analytical performance. Under the ideal circumstances, direct standardization for hydrogen peroxide was achieved $2.1x\ l0^{-5}$ to $2.61\ x\ 10^{-3}$ M in the range. The enzyme electrodes apparent “Michaelis-Menten constant was 4.81 mM”. It was also looked at how well this enzyme electrode performed using flow-injection analysis. The enzyme electrode engaged around 60% of its activity after 35 days of storing in a phosphate buffer at 4-degree centigrade. HRP has been effectively mounted on a CPE by the sol-gel procedure, and it now serves as a sensitive and specific H_2O_2 biosensor. The mediator “hexacyanoferrate (I1)”, which shuttles electrons between the CPE and the HRP in the silica sol-gel matrix, is the foundation of the H_2O_2 biosensor. A benefit for reducing interferences is the enzyme electrode's low operating voltage. Since the “sol-gel immobilised HRP CPE” improves the CPE's stability as well as its sensitivity for H_2O_2 detection, the enzyme electrode displays an outstanding electrochemical reply in terms of permanence, sensitivity, and repeatability. As a consequence, it can be used as a real-time PIA electrochemical detector. The outcomes

show that immobilising enzymes for use in electrochemical biosensors using the sol-gel procedure is a needed strategy. Important neuroactive chemicals known as "organophosphorus" compounds provide difficult analytical problems. "Acetylcholinesterase (AChE)-based amperometric biosensor" was proposed by Raghu *et al*. [58]. It was created by immobilising the enzyme on a carbon paste electrode using a silica sol-gel layer. In 0.11 M phosphate buffer, the mono enzyme biosensor was utilised to measure two "organophosphorus chemicals, primarily methyl parathion (MP) and acephate (pH 7.0)". "Acetylthiocholine chloride (ASChCl)", the substrate that was employed, proved that thiocholine had really formed. It was electrochemically oxidised, which greatly raised the anodic peak current relative to the calomel electrode about 0.60 V. On the biosensor response, the properties of pH, enzyme stacking, and substrate absorption were identified. With incubation times of 20 minutes and 4 minutes, respectively, the monoenzyme biosensor established linearity for methyl parathion and acetate in the concentration ranges of 0.11-0.55 ppb and 52-755 ppb. For methyl parathion and acetate, detection limits under ideal occupied circumstances were create to be 0.081 ppb and 88 ppb, individually. For 60 frequent dimensions, the sensor has good working stability, preserving 89% of its original activity. This article's research proposal produced a "voltammetric approach" for identifying "organophosphorus insecticides". It takes little time to build a biosensor utilising the straightforward sol-gel method to immobilise the "acetylcholinesterase". The ability of the pesticide to inhibit AChE was directly correlated with the limit of detection of the "AChE sensor". The novel sensors can be made inexpensively and are single-use devices. The AChE biosensors have definite benefits, such as the ability to detect hydrophobic substrates and very humble enzyme immobilisation. ChEt have been covalently immobilised against tetraethyl orthosilicate (TEOS) sol-gel films in a biosensor created by Singh *et al*. [59]. Tetraethyl orthosilicate sol-gel/ChEt/ChOx enzyme films remained shaped in this way, and they were characterised by amperometric, scanning electron microscopy, UV-vis, and FTIR spectroscopic methods, respectively. "Tetraethyl orthosilicate sol-gel/ChEt/ChOx" has heat constancy up to 56 °C, rendering to photometric trainings. The limit of detection was 121 mg dL1, the response time was 181 s, the linearity was up to 788 mg dL1 (11 mM), the shelf life was 30 days, and sensitivity was 5.41×10^{-5} Abs. mg^{-1} dL^{-1}"Tetraethyl orthosilicate" was used to produce sol-gel films

that were optically rich among 300 and 900 nm. These films were originated to be absorbent, which made them appropriate for immobilizing cholesterol oxidase and cholesterol esterase. Sol-gel films inclined to crash when their breadth increased or they were frenzied. The films may crack at critical thickness due to the effects of biaxial stress. It has been demonstrated that "tetraethyl orthosilicate sol-gel films" may effectively immobilise both cholesterol oxidase (ChOx) and cholesterol esterase (ChEt). These "tetraethyl orthosilicates sol-gel/ChEt/ChOx" enzyme films have been investigated by "UV-vis, FTIR, SEM", and electrochemical methods. It has been established that these "tetraethyl orthosilicate sol-gel/ChEt/ChOx" enzyme films are reliable for measuring cholesterol oleate up to 780 mg dL^{-1} (mM). These TEOS sol-gel enzyme films allow for precise directing of "glucose, lactate, uric acid and ascorbic acid". The total cholesterol levels in serum and blood samples should be resolute using these "tetraethyl orthosilicate sol-gel/ChEt/ChOx films". It might be interesting to alteration the composition of the TEOS sol-gel film to produce cholesterol biosensors. Sol-gel-based fiber-optic biosensors with "fluorophore-acetylcholinesterase" conjugates that remained pH-sensitive and intended to monitor acetylcholine were rapidly prepared by Doong *et al.* [60] by this method. A florescent indicator sensitive to change in pH secure with an enzyme in a network of sol-gel, a cap of glass that can be mounted to an optical fiber, and a flow-through reactor built in for incessant monitoring make up the sensor. In order to maintain a steady pH value and enzyme activity, the sol-gel manufacturing technique has been altered. The most promising option for biosensors was found to be FITC-dextran out of the nine fluorescent indicators that were looked at. The ideal "enzyme-dye and TMOS: HCl: H2O": sol solution ratios were 1:3.6 and 3:5, respectively, for the speedy production of sol-gel film. A buffer with a 1 mM pH 8.5 capability and a substrate concentration of less than 50 mM are both necessary for the production of acetylcholine. The designed biosensor demonstrated a highly repeatable response to acetylcholine (R.S.D. was found to be 3.5%, n was recorded as 8). In the concentration range of 0.5 to 20 mM, acetylcholine likewise displayed excellent linearity (R2 = 0.98). Furthermore, inhibition of up to 30% can be reached in roughly half an hour by adding paraoxon of 0.54 molarity (152 ppb) to the system [61]. This shows that the biosensor is capable of evaluating "acetylcholine and organophosphorus neurotoxins". The current study demonstrates how to swiftly and efficiently use sol-gel mechanism to

encapsulate “AChE and FITC-dextran” to produce a fiber-optic biosensor based on AChE with excellent results and sensitivity. The variables, such as the “TMOS: HCl: H2O ratio” and the buffer solution, were carefully adjusted to improve the study's results for 246 R.-A The electrode showed great sensitivity to acetylcholine with a response linearly in the range of 0.5-20 mM. The biosensor responded to ACh in a completely revocable manner that was also remarkably repeatable (R.S.D. was found to be 3.5%). An inhibition of 30% can be attained with 152 ppb of paraoxon. Increased sensitivity can be achieved through longer pesticide incubation periods, slower flow rates, and stopped-flow operation. The biosensor has many advantages like its quick response time, reasonably priced equipment, need for little sample preparation, and high sample throughput. These characteristics can be used to track O.P. levels in a variety of samples, including water samples from the environment and drinking sources, agricultural resources and many others. They are wanted due of the possibility of miniaturization. In order to preserve the enzyme's function, the biosilica for enzyme immobilisation combines a silica matrix's exceptional support characteristics with a benign immobilisation technique using a cholinesterase-specific buffer and indophenyl acetate (molarity of 2×10^{-4}) as the substrate, butyrylcholinesterase activity was measured spectrophotometrically at 630 nm. At pH 8.0, cholinesterases perform hydrolysis the yellow indophenyl acetate to yield the” blue 4-(4-hydroxy-phenylimino)-cyclohexa2, 5- dienone”. The product's absorptivity was calculated to be 8,100/M/cm on the basis that butyrylcholinesterase will totally convert it. Prior to determining absorbance, SiO_2 units were eliminated using centrifugation for ten seconds. All tests were conducted at STP. The quantity of protein using a bicinchonic acid protein test kit. Betancor *et al.* [62] investigated the trapping of previously immobilised $C_6H_{12}O_6$ oxidase using a sol-gel technique. The immobilization was performed using a (porous support)ie sepharose beads derived with functional group called glutaraldehyde. The enzyme activity could be preserved once the sol-gel was created since it had already been immobilized. Initially only 10% of the enzyme activity was lost compared to the enzyme which reduced sixty percent of its initial activity. The enzyme leak’s reduction from the sol-gel matrix, which was problematic when encasing the enzyme (the enzyme activity was lost up to 39% after 16 hours in a buffer solution), was another advantage. Additionally, there ought not to be any contact between the sol-gel inside the porous support and the immobilized enzyme. This allowed for the

maintenance of the stability attained through the covalent connection on the support of Sepabeads. Using this porous support, it inhibited the soluble enzyme from negatively reacting with the silica matrix as opposed to encasing it, which triggered high recovery of activity of enzyme. Additionally, regardless of the matrix parameters, the absence of interactions among proteins and matrix when using porous supports may prevent any changes to the enzyme properties once the enzyme has been immobilised in the sol-gel. Last but not least, this method totally removed the activity loss brought on by enzyme leakage, which was significant when using the soluble enzyme. Furthermore, regardless of the trapping matrices used, a good immobilization technique can significantly stabilize the enzyme and maintain this stability even after the enzyme has been trapped. The enzyme may therefore be pre-immobilized on a porous support as an initial step in the sol-gel preparation. Sol-gel matrices are an excellent alternative for developing biosensors, while multipoint covalent attachment to pre-existing supports helps stabilize enzymes, according to a comparison of the two techniques. The comparative analysis of all modern Sol-Gel processes is shown in Table 2.2.

Table 2.2 Comparative study of all state-of-art Sol-Gel techniques comprising of performance biosensor including its merits and demerits

Author/Year	**Methodology**	**Performance parameter**	**Merits**	**Demerits (if any)**
Juan *et al.* 1996 [57]	Sol-Gel technique	An amperometric electrode for H_20_2 using the sol-gel mechanism	Linear calibration range = 2x $l0^{-5}$ to 2.6x 10^{-3} M Michaelis-Menten constant = 4.8 mM. "60% of electrode activity was retained after 35 days of storage in a phosphate buffer at 4°C". Good sensitivity and reproducibility. Applied as an electrochemical detector in PIA	---
Raghu *et al.*2012 [58]	Sol-Gel technique	Amperometric biosensor based on film-based silica sol–gel enzyme over the electrode based on carbon material	Limit of the detection = 0.08 ppb for methyl parathion Limit of detection = 87 ppb for acephate. Operational steadiness 89% of its unique activity for 60 succeeding	---

			dimensions. Compensations comprises observations of hydrophobic substrates and comparative comfort of the enzyme immobilization	
Singh *et al.* 2006[59]	Sol-Gel technique	ChOx and ChEt have been covalently immobilized on TEOS sol–gel films.	Present steadiness = 55 ◦C, Response time= 18 s, Linear range = "780 mg dL−1 (12 mM)" Life span of 30 days, detection boundary of 12 mg dL−1 Compassion= 5.4 × 10−5 Abs. mg−1 dL−1	---
Doong *et a.*2001[60]	Sol-Gel technique	"Fiber-optic biosensor with condensed pH-sensitive fluorophore-acetylcholinestera se conjugates is established for the resolve of acetylchlorine"	"Reaction of the advanced biosensor to acetylcholine was extremely reproducible (R.S.D. = 3.5%, n = 8). Range of the linearity range of acetylcholine = 0.6 to 20 mM. 35% inhibition can be attained inside 30 minutes when 0.54µM (152 ppb) paraoxon was extra into the system" Wild reaction times, low-cost composition, least sample pretreatment and high sample output.	---
Luckarift *et al.* 2004 [61]	Sol-Gel technique	Determination of the appropriateness of biosilica as an immobilization Medium with the "enzyme butyrylcholinester ase".	At 8.0 pH hydrolyze the yellow indophenyl acetate to an azure response product. butyric absorptivity of cholinesterase= 8,200/M/cm	Exacting situations compulsory for biochemical synthesis limit.
Betancor *et al.* 2005[62]	Sol-Gel technique	Contrast of the entrapment of permitted or beforehand immobilized glucose oxidase by a sol-gel method	Extraordinary retrieval of enzyme activity. Decent enzyme steadiness by multipoint covalent attachment on pre-existing backings	---

2.2.2.5 Microencapsulation techniques

A simple and inexpensive method is used to enclose biorecognition components (enzymes) in a membrane. Of the spherical semi-permeable. The membrane may be lipoidal, non-ionic, polymeric, based on lipoproteins, or it may be anionic. The detecting surface of a pH glass anode was configured by Sahney *et al*. [63] to immobilise the urease enzyme using a number of approaches, comprising physical adsorption, entrapment, sandwiching, and microencapsulation. The sandwich films containing the microencapsulated and materially deceived enzyme do not meaningfully escape enzyme. Potentiometric techniques were used each time to estimate the urea concentration. The outcomes specify that the microencapsulation technique is a more successful means to immobilise enzymes in sol-gel films manufactured from TMOS across a wide range of biosensor performance criteria. Microencapsulated biosensors are superior to alternatives because of their advanced sensitivity (dpH/dp(C)=2.4), lower detection limit of 52 g mL-1, broader linear range of urea extent (0.02-30 mM), and reasonable enduring stability of around twenty-five days with eighty percent reaction indicator. With blood serum samples,the microencapsulated enzyme electrode device produces more favorable results. In order to detect glucose, Cosnier *et al*. [64] created a unique enzyme electrode grounded on artificial “hydrophilic latex matrices”. $C_6H_{12}O_6$ oxidase was micro-encapsulated and immobilised using the simple adsorption of enzyme-latex solutions on the surface of a Pt electrode. The 2 latex films have been either functionalized with gluconamide or hydroxylated. Potentiostats were employed to calibrate the response of these biosensors to glucose additions to the modified electrodes at 0.6 V/SCE. Thus, the Hydrogen peroxide is oxidised via the enzymatic oxidation of glucose in the existence of dioxygen. Such electrodes' reaction was assessed in relation to the temperature and film thickness. Latex-based, a two-layer biosensor with a response time of 3-5.1 s and a compassion of 38.78 mA M^{-1} cm^{-2}. Moreover, a substantial development in the biosensor's updraft stability was seen. The enzyme is only reduced to deskbound and denatured at infections above 65 °C. Also, the succeeding biosensors have a good sensitivity due to the unique physiognomies from these latex sheets. “Tyrosinase” was immobilized in a “titania sol/gel matrix” by Yu *et al.* [65] to generate a new and novel amperometric phenol sensor. This medium thus helped as both a small mass transportation block to the enzyme substrates and a suitable environment for preserving the natural assembly and purpose of the entrapped enzyme. A vapour deposition technique wasused to

create the “tyrosinase-entrapped sol/gel film, simplifying the traditional sol/gel” procedure and preventing the reduction and breaking of glasses made from traditional sol/gel. “Tyrosinase” immobilised in the presence of oxygen could oxidise phenol to produce a quantifiable product that was measured at 150 mV without the use of an intermediary. With an exposure boundary of 1.0 $x/10^{-7}$ M, the lined variety for phenol determination was meanwhile $1.2x10^{-7}$ to $2.6x10^{-4}$ M. Tyrosinase that has been encapsulated has a deceptive Michaelis-Menten constant of (0.2990.02) mM. As a result, the biosensor's stability was also measured. The porous enterprise and extraordinary enzyme filling of the sol/gel matrix controlled to the phenol sensor's rapid response (fewerthan 5 s) and sensitivity of up to 104 mA/mM. A small amount of conveyance barrier to “tyrosinase's substrates”, an appropriate loading and high catalytic activity of the enzyme,and a quick reaction time from the sensor are all produced by the absorbent structure of the“titania sol/gel matrix”. For preventing tyrosinase leaking out of the film, this film is quiteeffective. Because of this, the sensor has excellent output and long-life span stability. They also show that the “titania sol/gel matrix” may be used to produce biosensors and immobilise enzymes. Using assistance of sol-gel methods, Wang *et al.* [66] shaped an amperometric tyrosinase enzyme electrode for the rapid and precise immobilisation of phenols. This process involved adding a grafting copolymer to the solution of sol-gel to optimise the configuration of the resulting carbon-base and inorganic composite material. Tyrosinase was still active in the sol-gel thin film, and its reaction to numerous phenol complexes was assessed at 0.2 mV vs. sat. KCl. It was investigated how pH, oxygen content, and temperature affected current responsiveness. Additionally assessed was the biosensor's stability. For catechol, phenol, and p-cresol, the biosensor's sensitivity was 59.6,23.1, and 39.4 $\times 10^{-3}$ A/mM, individually. When kept in a dry state and reserved at 4°C, theenzyme electrode reserved 73% of its original activity after being used occasionally for 3 weeks. A good matrix for immobilizing tyrosinase is sol-gel hybrid materials provided. Thebiosensor made using a hydrophilic sol-gel ancestor returns more powerfully, and the difference in oxygen level has slight effect on the measurement of phenolic substances at the mM nearby. Premkumar *et al.* [67] created a sensing component by immobilizing bioluminescent E. coli reporter cultures in dense silicate films and encapsulating them in sol-gel produced silicates. The findings demonstrated that the captured bacteria retain the advantageous organic traits of the free culture as well as their metrological characters. Moreover, they improved compassion, repeatability, life span

stability, and overall varietyof response to a broad period of hazardous chemicals. The microorganisms that are enclosed in silicate can be used in multiple-use sensing components or in continuous operation. The bacteria-silicate hybrids are utilised in test kits for multiple-use sensing or as disposable sensors. They can also be incorporated with early warning systems that function under continuous flow conditions. Acetylchlorinesterase (AChE)", an enzyme, wasimmobilised on gold nanoparticles to create an amperometric biosensor, according to Buiculescu *et al* [68] designs of a novel sensor. "(Au NPs)". The active enzyme is covalently attached to the gold nanoparticles' surface through a thiol bond. This immobilisation gives the colloidal Au NPs, the catalyst, and the transducer surface increased stability and exceptional electron transport. The "Au NP enzyme nanocomposite" was surrounded by a layer of biosilica to further increase the stability of the biosensor by protecting the enzyme from denaturation and protease attack. Each procedure is observed and verified using "ATR-FT-IR" spectroscopy, including the coupling of the enzyme to the gold nanoparticles and the encapsulation in biosilica. Even though the biosensor's performance was tracked for 4 months, the stabilising effect of the entrapment was determined amperometrically. Initially sensitive to 27.58 nA mM1, the biosensor reacted exponentially to substrate concentrations within 0.04 and 0.4 mM. In contrast to the existing AChE biosensor, the nanocomposites of biosilica combined with Au NPs-AChE conjugates produce a system that offers both a strong enzyme stabilisation signal and mediation. The biosensor's activity was fully sustained after 4 months, in contrast to the typical AChE biosensor, wherein performance only reached 50percent after six weeks of operation. The AChE amperometric biosensor is no longer a viable option for acetylthiocholine detection because the AChE has been immobilised on the Au - Nanoparticles and subsequently encapsulated with the conjugates into biomimetic silica. These findings imply that AChE inhibitors could also be identified with great sensitivity utilising a system madeup of Au NPs-AChE conjugates trapped in biosilica; studies for this purpose are now being developed. Acetylcholinesterase is hence of substantial bio-analytical interest, and Sotiropoulou *et al.* [69] thus postulated the outcome of the microenvironment of sol-gel on activity results of this enzyme, which is further associated to the total analytical performance of matching biosensors. At the beginning, the "TEOS: H_2O" ratio (r) and the catalyst are utilised to enhance the sol-gel membranes' porous, mechanical properties, and surface properties in enzymatic activity. FT-IR and electrochemical impedance spectroscopy (EIS) are utilised

to investigate the rotational and conformational motion of the enzyme in the sol-gel matrices. All things considered; analysis has shown that the rotating movement of proteins can influence the biosensor's sensitivity. The optimum performance for a biosensor is achieved by base-catalyzed sol-gels with r values near to 2. The biosensor exhibits 3% sensor-to-sensor reproducibility, a linear range of 1 to 3 mM, and a reaction time of about 30seconds (RSD). Its sensitivityis 2.5 mA/mm. Comparing these analytical qualities to previously published sol-gel biosensors, they are significantly better. When the physicochemical properties of sol-gel membranous structures were carefully optimized, it became clear that sol-gels that were base-catalyzed provide the best conditions for "acetylcholinesterase immobilisation". The biophysical analysis came to the conclusion that the main element influencing enzyme functionality and, consequently, biosensor response is the movement of the enzyme inside the mesopores of the films. A pesticide biosensor was created using the suggested system. Two separate "microorganisms' acetylcholinesterase" (from "Electrophorus electricus and Drosophila melanogaster respectively") were captured, and in both cases, the enzyme maintained high operational abilities while still being able to detect low levels of the model"pesticide Dichlorvos". The comparative analysis of microencapsulation methods is shownin Table 2.3

Table 2.3 Comparative study of the Microencapsulation techniques with respect to different performance parameters, merits and demerits

Author/Year	**Methodology**	**Performance parameter**	**Merits**	**Demerits (if any)**
Sahney *et al*.2006[63]	Microencapsulation	"Higher sensitivity (dpH/dp(C) = 2.4)", lower detection limit = 52 g per mL, Larger linear range i.e. 0.01–30 mM Stability=80% for about 25 days.	Higher sensitivity Lower detection limits. Long term stability	
Cosnier *et al.*	Microencapsulation	Sensitivity =38.78 mA M-	High sensitivity	For temperature

2000[64]		1 cm-2 Time of response = 3-5 s	complemented with significantly improved thermal stability	higher than 65°C the enzyme denatured and became inactive.
Yu *et al.* 2003[65]	Microencapsulation	Sensitivity =103 A/mM Linear range = " $1.2x10^{-7}$to $2.6x10^{-4}$ M Detection limit" =$1.0x/10^{-7}$ M. Michaelis-Menten constant = (0.299±0.02) mM. Response = <5s	High catalytic activity of the enzyme along with good loading.Quick response rate of the sensor.	
Wang *et al.* 2000[66]	Microencapsulation	Sensitivity for catechol, phenol and p-cresol = 59.6, 23.1 and 39.4 mA/mM, respectively. Activity=71% after 21d of storage	High sensitivity Good stability Sol-Gel networks increase the thermal stability of immobilized enzyme	
Premkumar *et al.*2000[67]	Microencapsulation	Applicable under constant operation or in a multiple use sensing elements. Used in multiple use sensing test-kits and as sensors that are disposable.	Shelf life, repeatability, sensitivity, and larger range response to a wide class of toxic substances.	
Buiculescu *et l.*2012[68]	Microencapsulation	Sensitivity=27.58nA M−1 Linear response range =0.04 to 0.4 mM. Activity=50% after 42d of operation.	Good sensitivity Significant enzyme stabilization	
Sotiropoulou *et al.*2005 [69]	Microencapsulation	Sensitivity = 2.5 mA/mM (RSD) = 3%. Response time= 30s	"Enzyme retained high functionality. Could not detect the model pesticide Dichlorvos to very high amounts"	

2.2.2.6 Cross-linking (copolymerization)

This approach does not require a matrix or support for immobilization because the covalent bonds between different groups are used to connect the enzymes directly. This approach is simple to apply and appropriate for pure enzymes used in industrial and commercial applications. The method's biggest drawback is that the polyfunctional regulator used to cross-link the enzyme may cause it to lose its catalytic characteristics via denature or structurally altering the enzyme. Figure 2.6 depicts the cross-linking method's fundamental composition. This approach is simple to apply and appropriate for pure enzymes used in various commercial and industrial applications. The primary drawback of this technique is that the polyfunctional regulator used to cross-link the enzyme may cause it to denature or structurally change, resulting in the loss of catalytic activity.

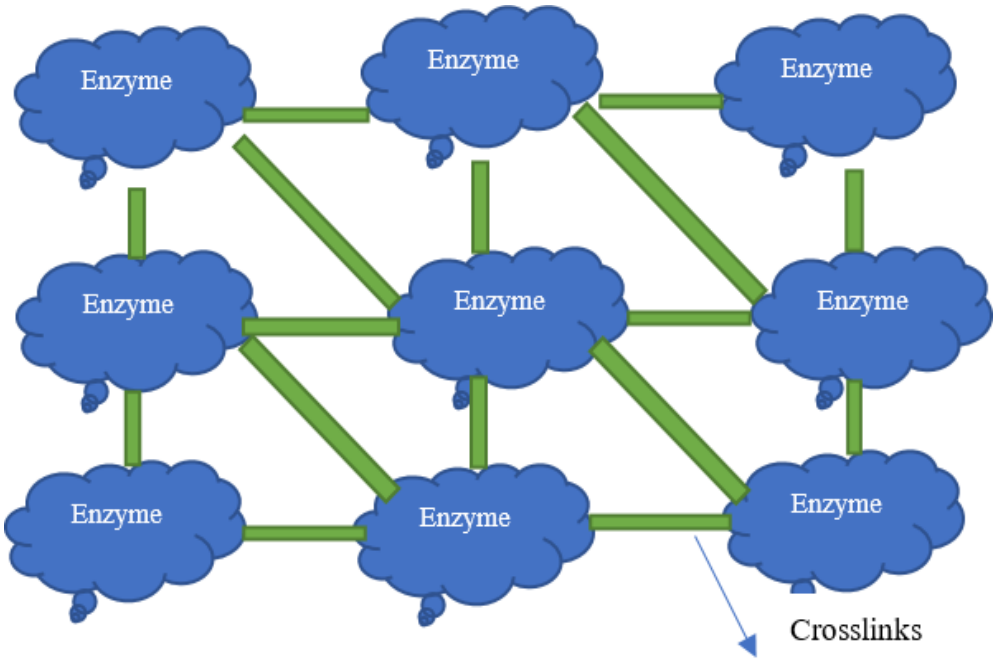


Figure 2.6 Cross Linking immobilizations techniques is based on the principal of formation of covalent bonds between different groups which are used to connect the enzymes directly.

Crosslinking, entrapment, and layer-by-layer assembly were used to create a glucose biosensor by Nenkova *et al.* [70] with an aim to study the effects of different immobilization techniques on performance. To investigate their influence on the functionality of glucose biosensors, nano zeolites, magnetic nanoparticles, and multi-walled nanotubes were

integrated in the enzyme. The biosensors underwent more cyclic voltammetric analysis. The most electroactive electrodes were made by crosslinking materials like acrylonitrile copolymer/nano zeolite/carbon nanotube and acrylonitrile copolymer/nano zeolite/magnetic nanoparticle. These findings displayed that the combination of multi-walled carbon nanotubes, magnetic nanoparticles, and nano zeolites considerably increased the electron transferability of the sensors. The glucose oxidase electrode with the acrylonitrile copolymer/nano zeolite/carbon nanotube composition had the maximum sensitivity in amperometric experiments (10.959 microamperes per millimolar). By means of a linear dynamic range of up to three millimolar, the lowest detection limit for glucose was 0.02 millimolar. The response was 81% of the initial current after thirty days. In comparison tothe other approaches, the crosslinking method increased biosensor sensitivity by a factor of 6. The electrode's sensitivity was further improved by adding multi-walled carbon nanotubes, magnetic nanoparticles, and nano zeolites to its matrix of acrylonitrile copolymer. The sensor's ability to transmit electrons was considerably enhanced by the synergistic interactions of magnetic nanoparticles and multi-walled carbon nanotubes with nano zeolite. The nanoparticles made the polymer film more electroconductive and porous by ensuring a advanced specific surface area and a higher concentration of the immobilised enzyme. Diffusion of the substrate was thereby made simpler and guaranteed. A brand-new enzyme-based amperometric biosensor for "hydrogen peroxide (H_2O_2)" was proposed by Miao *et al.* [71] and constructed using a quick and efficient method of enzyme immobilisation consuming chitosan film cross-linked with glutaraldehyde. The horseradish peroxidase enzyme was immobilised on the carbon paste electrode's surface. The solution contained a mediator by the name of "hexacyanoferrate (II)". The biosensor was extremely stable, retaining around 85% of its activity even after a month of storage at 4°C in phosphate buffer. Numerous operating parameters and the effect of the chitosan film thickness were tuned. The data produced via this biosensor sufficiently supported the "traditional iodometric titration approach", demonstrating the biosensor's usefulness through the analysis of real samples. Chitosan film that has been crosslinked with "glutaraldehyde" can be easily immobilised to create a reliable and "affordable HRP electrode". When there is a mediator in the solution, the biosensor can function at low applied voltage. The best situations for the creation and use of the biosensor have been researched. The biosensor

that was produced has great reproducibility and stability. The study by Fernandes *et al*. [72] describes the development of a biosensor for the determination of rutin in pharmaceutical formulations using square wave voltammetry. The biosensor is constructed by immobilizing laccase on chitosan microspheres cross-linked with tripolyphosphate. Laccase catalyzes the reduction of rutin back to rutin at +0.35 V vs Ag/AgCl, and also catalyzes the oxidation of the corresponding quinone. The reduced current generated is used to determine the concentration of rutin. With a solution containing 2.0 x 106 mol L1 rutin, the biosensor demonstrated excellent recovery (90.7 to 105.0%) and precision (relative standard deviation of 3.1% for n = 9). Also, it was discovered that the biosensor had a 320-day lifespan (at least 930 determinations). With a 95% confidence level, it was discovered that the results for rutin in pharmaceutical formulations acquired using the biosensor matched those obtained using the traditional technique. Overall, with good sensitivity, selectivity, and long-term stability, the biosensor created by Fernandes *et al*. [7] seems to be a viable instrument for the detection of rutin in pharmaceutical formulations. It may have a great repeatability, low detection threshold, high sensitivity, outstanding long-term stability, and a rapid response time. The biosensor's inexpensive price, ease of use, and quick development make it superior to current techniques for identifying flavonoids. The rutin determination was successful in three pharmaceutical formulations without the need for any preliminary separation steps before the electrochemical test. In light of the established approach and labelled values, the results were satisfactory. Gilo crude extract was immobilised in chitosan matrix by De Oliveira *et al*. [73].

This led to the development of a reagentless biosensor for the detection of rutin in pharmaceutical formulations. The peroxidase enzyme employed in this biosensor was extracted from gilo tissue and immobilised in chemically cross-linked chitosan with glutaraldehyde and epichlorohydrin integrated in a carbon paste electrode. After the peroxidase was thus immobilised in the chitosan matrix, the biosensor performed at its peak level. The biosensor responded linearly to rutin concentrations from 3.4 x 10^7 to 7.2 x 10^6 M. The limits of quantification and detection were 2.0 x 10^8 and 6.3 x 10^8 M, respectively. The biosensor was found to have good recovery of rutin from the samples, ranging from 96.2 to 102.4%. Overall, the biosensor developed by de Oliveira *et al*. [73] appears to be a promising tool for the detection of rutin in pharmaceutical formulations, with good sensitivity and specificity. Further research could be done to optimize the

performance of the biosensor and to investigate its application in real-world samples. For solutions containing 3.4 x 10^{-7} to 7.2 x 10^{-6} M "rutin in 0.1 M phosphate buffer solution at pH 7.0 (n = 10), the relative standard deviation was less than 1.0%". This biosensor has an eight-month lifespan (at least 500 determinations) and the performance of the immobilised peroxidase on the chitosan biopolymer, which was made from gilo tissue, was evaluated. The biosensor can immobilize the efficient interactions between the enzyme and cross-linked biopolymer. The bioelectrode for biosensors provided beneficial analytical characteristics, such as high sensitivity, quick response, long-term stability, and low detection limit. With this method, a novel class of electrochemical biosensors for rutin can be developed in a simple and low-cost manner. To create an Amperometric biosensor to measure caffeine levels in liquids, Babu *et al*. [74]engineered a microbe that can break down caffeine. "Pseudomonas alcaligenes MTCC 5264 whole cells" that are capable of degrading coffee were immobilised on a cellophane membrane. Gelatin was used as a protein-based stabilising agent and glutaraldehyde servedas a bifunctional crosslinking agent to achieve the molecular weight cut-off" "(MWCO) of3000-6000. (PBSA)". Caffeine was detected in solution using the biosensor device at concentrations between 0.1 and 1 mg $mL^{-1.}$ With read-times as fast as three minutes, this caffeine biosensor serves as a quick analysis tool for solutions containing caffeine. Interestingly, it was simpler to isolate and immobilize potent caffeine-degrading bacteria for the analysis of caffeine described here thanks to an inventive selection strategy that included the isolation of bacteria from soils that could break down caffeine and use it as the only source of carbon and nitrogen. This strategy also involved inducing caffeine-degrading capacity in bacteria for the development of the biosensor. Thus, it was found that this biosensor is particularly sensitive to sugars, theophylline, theobromine, paraxanthine, and other interfering compounds. Although afew caffeine biosensing techniques have been identified, their use for commercial samples are restricted. New caffeine detection techniques are still being developed and put to use, particularly in food and clinical chemistry. Different substrates were discovered to interfere with the analysis of caffeine, though not significantly. The results of the biosensor's examination of the caffeine level of marketable tasters of direct tea and coffee and HPLC analysis were highly comparable. This tool aids in avoiding the laborious and time-consuming sample preparation and analytical procedures frequently found in traditional analysis methods. The biosensor proposed by Kurbanoglu *et al.* [75] is an amperometric

biosensor that utilizes alcohol oxidase immobilized in a conducting polymer matrix for the detection of ethanol in actual samples. The conducting polymer was polymerized onto a platinum electrode using an electrochemical method. Glutaraldehyde was used to bond the alcohol oxidase enzyme to the conducting polymer matrix. The biosensor was optimized by studying the effects of variables such as conducting polymer thickness, enzyme loading, pH, and the introduction of nanoparticles such as carbon nanotubes and gold nanoparticles. The biosensor demonstrated good repeatability, linear range, and great functional stability. The linear analytical range for ethanol was between 0.1 and 5 mM, and only 7% of the biosensor's activity had been lost after 25 tests over 6 hours. The biosensor was successfully used to detect ethanol in actual vodka and whiskey samples, indicating its potential for practical applications. The biosensor's quick response time makes it a hopeful tool for the rapid estimation of caffeine in samples.

A functional "NH_2 group-containing CP surface" was used as the ideal microenvironment in the proposed method to immobilize the biomolecule. The "electropolymerized SNSNH2 was given attractive matrix" properties with special and adaptable qualities for "biomolecule immobilization by the inclusion of CNT dispersion and AuNP". It can be said that "CP and AuNP" work together to enhance the bioactivity of the immobilized enzyme, resulting in fast, reliable, and sensitive reactions to the substrate. Colak *et al*. [76] created a new amperometric glucose biosensor by electrochemically polymerizing polypyrrole-poly (vinyl sulphonate) (PPy - PVS) sheets onto a platinum (Pt) electrode. Pyrrole and poly (vinyl sulphonate) were electropolymerized on the Pt surface using a "electrochemical cell containing pyrrole and poly (vinyl sulphonate) and cyclic voltammetry" at a scan rate of 50 mV/s on the Pt electrode "between -1.0 and +2.0 V (vs Ag/AgCl)". The electrochemical measurementof H_2O_2 produced in the enzymatic reaction of glucose served as the basis for the amperometric determination. The "oxidation of enzymatically generated H_2O_2 at 0.4V vs.Ag/AgCl" was used to measure glucose. The optimal pH and temperature were found to be 7.5 and 65 °C, respectively, after studies into their effects. A value of 0.4 V was discovered to be the appropriate potential after the impact of working potential was investigated. The operating stability of the glucose electrode was also examined. The "PPy/PVS-GOX glucose biosensor" response showed a decent degree of repeatability with a relative standard deviation (RSD) of 2.48%. The glucose biosensor maintained 63% of its initial activity after being stored in a 0.1 M phosphate buffer solution with a pH of 7.5

for 93 days at 4 °C. With its low working potential, the biosensor showed slight interference from potential interferents. Theglucose biosensor was found to have excellent operating and long-term storage stability, aswell as being exceedingly sensitive and selective. For creating a strong chemical interactionwith enzymes, which is essential for minimizing enzyme leakage and ensuring good operational stability, this composite "(PPyPVS)" offers more advantageous advantages. Additionally, "PPy-PVS" may provide an electrochemical and biocompatible milieu to immobilize the enzyme, which makes this substance very effective for creating extremely sensitive and selective glucose biosensors.

An "enhanced l-lysine amperometric biosensor" based on lysine oxidase immobilized on a platinum electrode by "glutaraldehyde co-crosslinking" with "bovine serum albumin" was proposed by Guerrieri *et al*. [77]. The sensor has been tested to determine the amount of lysine in a pharmaceutical sample, and the results show good agreement with the estimates. A fast response biosensor with excellent sensitivity and increased stability was created thanks to careful tuning of the enzyme immobilization process. In batch addition trials, the response time, t 0.95, was tested and found to be less than 6 s. The detection limit at S/N = 3 was one μM and linear lysine responses up to 0.6 M were detected with a sensitivity of 4.4 A mM. A biosensing device was able to exhibit previously unreported fast reaction time, high sensitivity, and stability by optimizing the enzyme immobilization process. Since this possibility is unlikely to be found when enzyme immobilization is carried out using alternative techniques like the electrochemical method, it was suggested by a consideration of its hydrodynamic behaviour as well as pH that it is possible to switch from an enzymatic to a diffusive kinetic control by adjusting the pH of the "supporting electrolyte" at the right time and by controlling the mass transport. However, the selectivity of both the "enzymatical and electrochemical transduction" steps remain to be discussed.

Cross-linking is most powerful immobilization technique used to form covalent bonds between the biomolecules and the support. This technique consists of several merits like stability, high retention of biological activity, versatility and resistance to harsh conditions. Despite of these merits this method consists of some limitations like complex immobilization process which requires use of chemical agents. This technique is also suffered due to non-specific binding of additional reactive group on solid support and sometimes loss of proximity of cross-linking agent on active side.

Table 2.4 Comparative study of the Cross-Linking techniques highlighting the performance parameters ofthe sensor including merits and demerits

Author/Year	Methodology	Performance parameter	Merits	Demerits (if any)
Nenkova *et al.* 2015[70]	Cross Linking	Sensitivity=10.959 microamperes per millimolar Detection Limit=0.02 millimolar	Six times higher biosensor sensitivity than the other methods. rapid biosensor response.	---
Miao *et al.* 2000[71]	Cross Linking	Fast response = < 10s Activity=85% after 30d of storage	Good stability of biosensor Robust and low cost HRP electrode Good reproducibility and stability.	---
Fernandes *et al*.2008 [72]	Cross Linking	Detection limits = 6.23×10^{-8} and 7.12×10^{-7} mol L−1 SD=3.1% Lifetime=320 days	"High sensitivity, Good reproducibility, Low detection, Rapid response and excellent long-term stability"	---
De Oliveira *et al.* 2006[73]	Cross Linking	Detection Limit= 2.0×10^{-8} Quantification limits = 6.3×10^{-8} M SD =1% Lifetime= 8 months	"High sensitivity Good reproducibility Long-term stability, Low detection limit Rapid response."	---
Babu *et al.* 2007 [74]	Cross Linking	Detection Limit=0.1 to 1 mg mL−1 Optimum pH = 6.8 Temperature measurement=30± 2 ◦C	Fast response time Low detection limit	---
Kurbanoglu *et al*.2015 [75]	Cross Linking	Linear analyticalrange =0.1-5 mM ethanol	Good linear range Repeatability Fast, high operational stability and sensitivity.	---
Colak *et al.* 2012 [76]	Cross Linking	RSD= 2.48% Retention Activity =63% after 90days of storage.	Highly sensitive, selective. Good operational stability. Good long-term storage stability.	---
Guerrieri *et al.* 2007[77]	Cross Linking	Sensitivity= 4.4μA mM−1, Detection limit at S/N = 3 was 1 μM.	Quick response biosensor with high sensitivity and stability.	Selectivity of both the enzymatical and electrochemical transduction steps needs to be improvised

2.2.2.7 Adsorption

This method of immobilising enzymes is the simplest and oldest since the enzyme is absorbed by the external layer of the matrix or support. Various types of carriers or supports are utilized in this method, including ion exchange resins occasionally, organic supports like starch, and mineral supports made of clay or aluminium oxide. Between carriers and support, there is no long-term connection in this process. Using hydrogen bonds or ionic contact, a weak connection stabilizes the enzyme to the mount. Figure 2.7 shows the fundamental composition of adsorption.

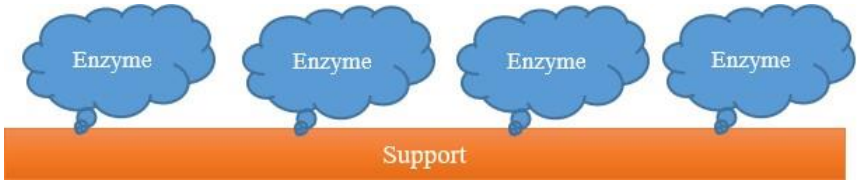


Figure 2.7 Adsorption (enzyme immobilization) technique in which the enzyme is adsorbed by the exterior surface of the matrix or support.

Tsai *et al.* [78] proposed a new amperometric glucose biosensor by further immobilisation of "glucose oxidase (GOx) in platinum-multiwalled carbon nanotube-alumina-coated silica (Pt-MWCNT-ACS) nanocomposites" improved glossy carbon electrode. Glassy carbon electrode that was created using "GOx-Pt-MWCNT-ACS nano biocomposites" was examined using "field emission scanning electron microscopy, energy-dispersive X-ray spectroscopy, cyclic voltammetry, and amperometric to determine its morphology, nature", and performance. To identify the best analytical execution, the effects of various experimental situations were examined. The optimised glucose biosensor had different characteristics including, broad linear range of up to 10.5 mM, high sensitivity of 113.13 mA M1 cm2, and a reaction time of less than 5 s. Compared to "conventional GOx-Pt-CNT nano biocomposites modified electrodes", the sensitivity for glucose measurement at the "GOx-Pt-MWCNT-ACS nano biocomposites modified glassy carbon electrode" is more useful. After being coated with Nafion, the improved biosensor has natural anti-interferents properties and long-term storage stability, thus it is functional to measure the amount of glucose in synthetic serum. The "electrodeposition of Pt nanoparticles" in a glassy carbon electrode modified with MWCNT-ACS nanocomposites, along with the adsorption of

“GOx at the Pt-MWCNT-ACS nanocomposite, enabled” a sensitive and focused response to glucose. “MWC NTs serve as effective supports for Pt nanoparticles and dynamic electron conduits”. Due to the integration of “Pt nanoparticles in MWCNT-ACS nanocomposite, hydrogen peroxide” can be electrochemically oxidised with greater efficiency. By adsorbing to a “Pt-MWCNT-ACS nanocomposite modified glassy carbon electrode, GOx” is immobilised while retaining its bioactivity and a favourable microenvironment. An efficient method for creating amperometric glucose biosensors is provided by the electrocatalytic performance of the “Pt-MWCNT-ACS nanocomposite, the biorecognition property of GOx”, and the advantages of the electrochemical methodology. The final "Nafion/GOD/Pt/CNT electrode" exhibits positive qualities like a wide determination range, a quick response time, and a high current density. The final “Nafion/GOD/Pt/CNT electrode” exhibits desirable properties such a wide determination range, quick response time, and a high current density. By utilising glucose oxidase (GOD) adsorption onto a carbon nanotube (CNT) electrode that has been improved with platinum nanoparticles, Tang *et al.* [79] developed a novel "amperometric biosensor" . For CNTs' growing medium, graphite substrate was utilised. After that, a thin layer of nafion was applied to the finished GOD/Pt/CNT electrode to increase its anti-interferent qualities and avoid GOD loss during measurement. “The morphologies and electrochemical performance” of the "CNT, Pt/CNT, and Nafion/GOD/Pt/CNT electrodes" have all been examined using "scanning electron microscopy, cyclic voltammetry, and amperometric techniques." Excellent qualities including a wide determination range are produced by the enzyme electrode's “exceptional electrocatalytic activity and distinctive three-dimensional structure (0.1–13.5 mM),” and high sensitivity and stability. In addition, the enzyme electrode has good reproducibility and applicability to whole blood analysis.8% mole yttrium doped “SrTiO3 (YST08)” was utilised to build a “Horseradish peroxidase (HRP)” biosensor that was intended to detect hydrogen peroxide (H2O2), according to a technique developed by Silva *et al*. [80]. “Energy-dispersive X-ray spectroscopy (EDS), scanning electron microscopy (SEM), Brunauer-Emmett-Teller N2 physisorption (XRD), zeta potential, atomic force microscopy (AFM), cyclic voltammetry, Fourier-transform infrared spectroscopy (FTIR), and chronoamperometry” was utilized to characterise the perovskite. Due to particle buildup and the high temperature of calcination, the structure showed a low

specific surface area. This quality was confirmed by SEM examination, which also showed that the grains were even and uniform. Zeta potential data indicate effective “HRP” immobilisation on the “YST08” surface due to the immobilised sample's zeta potential. Due to surface topographical changes and the identification of functional groups,“FTIR spectra and AFM micrographs demonstrate a uniform HRP adsorption”. Under electrochemical investigation, the “YST08 + HRP electrode demonstrated effectiveness insensing H_2O_2” at various scan speeds and background electrolytes. The reaction is surface-controlled, as evidenced by the low reduction potential for H_2O_2 at pH = 7, which is roughly 0.31 V. The “YST08 matrix” had a significant impact on the dispersion mechanisms, displaying a detection limit of 14.97 M, enhancing the enzyme's long-term activity and maintaining 14.97% of its specific capacity after 6 months. Due of the higher calcination temperature during the production process, this perovskite showed a low specific area of 2.177 m^2/g.

However, it was sufficient to create a microenvironment for HRP stabilisation. Covalent functionalization of the YST08 surface, which increased the concentration of functional groups to form chemical interactions between the HRP and YST08 surface, made the immobilisation more effective. “Zeta potential, FTIR, and AFM findings were used to confirm HRP adsorption on the YST08 surface”. The results of electrochemical studies support an irreversible surface-controlled mechanism with a low reduction potential for H_2O_2 of about 0.31 mV “(V against Ag/AgCl) at pH = 7.0”. The likelihood of effective urease adsorption on silicate for the development of biosensors was reviewed by Kucherenko *et al.* [81]. The urease adsorption technique on silicate is remarkable since it has benefits like quick, simple operation and the lack of harmful or auxiliary substances. The best circumstances for silicate modification of transducer surfaces and urease adsorption on these surfaces were determined. The operational parameters for the developed biosensor were optimised. "High intra-reproducibility (RSD - 4.5%)," "enhanced inter-reproducibility (RSD of urea determination is 9%"), and "operational stability (less than 10% loss of activity after ten days")" were all features of the improved biosensor with adsorbed urease.

The modern technique for “enzyme adsorption on silicate” in biosensors was contrasted with the conventional techniques for urease immobilization. The developed biosensor's operational characteristics (pH and ionic strength) were found to be quite similar for those of the biosensor based on urease immobilised in GA vapour. Thus, after trials it was decided

that the enzyme adsorption technique on silicate is well-matched for calibration of biosensor intended for future manufacture for these reasons. This urease adsorption on silicate method is quick and simple. It is reproducible and does not use any harmful chemicals. According to a process created by Fereira *et al*. [82], “glucose oxidase (GOD) is immobilized in Layer-by-Layer (LbL) films” and consecutively adsorbed with layers of “poly (allylamine) hydrochloride (PAH)” on top of an ITO substrate that has been treated with a Prussian Blue (PB) layer. The amperometric glucose biosensors using the "ITO/PB/GOD-PAH heterostructures" showed a high sensitivity of 16 A mmol l cm^{-2} and a detection limit of 0.20 mmol l^{-1}. Consequently, the ultrathin nature of the film and the low operating potentials that may be used as a result of the "efficient catalysis of H_2O_2" created in the enzymatic reaction in the presence of "Prussian Blue" are related to the increased sensitivity. The lack of interference from potential interferents like shows that biosensors are very selective to glucose. The biosensors' stability was examined by keeping track of their sensitivity for roughly 20 days, which showed stable “GOD adsorption”. Constructed on the immobilization of“horseradish peroxidase (HRP)” onto a “gold-nanoparticle (GNP)-adsorbed conducting poly (thionine) (PTH) film”, An amperometric hydrogen peroxide (H_2O_2) biosensor was developed by Ahammad *et al*. [83]. The modification process was examined using “electrochemical impedance spectroscopy (EIS), cyclic voltammetry, and scanning electron microscopy (SEM)” (CV). With regard to potential interferences and the use of the biosensor in real-sample analysis, the impacts of laboratory parameters, such as the concentration of the biosensor performance, were assessed. For optimum analytical performance, the mediator (hydroquinone, HQ), the solution's pH, and the working potential were investigated. The "immobilised HRP displayed excellent electrocatalytic" activity for the reduction of H2O2 in the presence of the mediator. A regression coefficient of 0.999 allowed for the achievement of a linear dynamic range of 5-150 M. The detection limit with a signal-to-noise ratio of 3 was found to be 1.5 M. Researchers successfully looked into the reproducibility, repeatability, and stability of the biosensor. Nanduri *et al.* [84] presented a biosensor depend on landscape phages entrapped by physical adsorption on the exterior of a quartz crystal microbalance used to detect "galactosidase from Escherichia coli”. The sensor's detection range was between 0.002 and 210 nM , its reaction time was roughly 100 s, and its detection limit was only few nanomoles. At a concentration

of roughly 200 nM of – "galactosidase, the signal" was saturated and the required dose-response curve showed a typical sigmoid shape. Even when the concentration of BSA were 2000 times higher than the concentration of – "galactosidase", a clear preference for – "galactosidase" was seen in mixed solutions. Three binding sites were required for a single molecule of – "galactosidase" to bind, indicating that the binding was specific. The phage and monoclonal "anti-galactosidase antibodies" respective Kd values from the enzyme-"linked immunosorbent assay" (ELISA) were 21 ± 2 and 26 ± 2 nM. The performance of the sensors is comparable, despite the physical adsorption approach being more effective and affordable than the "Langmuir-Blodgett and molecular assembly methods". Phage can be employed as a recognition component in sensors because of the physical adsorption process used to immobilize it on the sensor surface. At 63°C and 76°C, the phagemaintained the detectable binding ability for longer than six weeks and three days,respectively. Phage degradation was found to have an activation energy of 1.34 $x10^5$ J/mol.These findings demonstrate that phage probes are highly thermostable and continue to workeven after being exposed to extremely high temperatures. The capacity to use phage in biosensor technology for selective and specific recognition of relatively big protein molecules is demonstrated by the phage used to "bind –galactosidase". The different methods of absorption techniques are depicted in Table 2.5.

Table 2.5 Comparative study of the Adsorption techniques with respect to performance parameters, merits and demerits.

Author/Year	Methodology	Performance parameter	Merits
Tsai *et al* .2009[78]	Adsorption	Linear range =10.5 mM Sensitivity = 113.13 mA M−1 cm−2, Response time= <5s	Long-term storage stability. Sensitive and selective
Tang *et al.* 20044[79]	Adsorption	termination range = 0.1–13.5 mM ponse time = 5 s h sensitivity =91 mA M¡1 cm¡2 bility = 73.5% after 22 days	Large determination range. Time for response is small Large current density along with high sensitivity and

		of storage.	stability
Silva *et al*.2020[80]	Adsorption	Detection limit = 14.97 µA Specific capacity= 14.97% after 180 days.	Low specific surface area. Efficient in sensing H2O2 in different can rates.
Kucherenko *et al.* 2012 [81]	Adsorption	RSD = 9% Stability = <10% after 10days of storage	Simple, quick and repeatable It does not involve any toxic reagents
Ferreira *et al*. 2004 [82]	Adsorption	High sensitivity = 16µA mmol−1 l cm^{-2} Detection Limit = 0.20 mmol l^{-1} Constant Sensitivity = Approx. 20days	High sensitivity Highly selective to glucose
Ahammad*et al*.2011[83]	Adsorption	Linear dynamic range = 5–150 µM Regression coefficient = 0.999 Detection Limit= 1.5 µM	Good stability, reproducibility, repeatability, and selectivity.
Nanduri et al.[84]	Adsorption	Response time = ∼100s Range of 0.003–210 nM. Activation energy of phage degradation = 1.34 × 105 J/mol Stability = 6 weeks at 63 ◦C, and 3 days at 76 ◦C.	Selective and specific recognition of relatively large protein molecules. Highly thermostable Method is simpler and more economical

2.3 Cadmium detection by conventional methods and Biosensors

The most widely used, reliable, and sensitive technique for cadmium detection in milk is this one. The four approaches for cadmium detection that are most frequently employed were discovered throughout the literature review: “ICP-MS, GFAAS, and FAAS” are acronyms for inductively coupled plasma mass spectrophotometers, flame atomic absorption spectrophotometers, and graphite furnace atomic absorption spectrometry. These techniques' key competency is the simultaneous, highly sensitive, and accurate finding of numerous components. In this part, comprehensive evaluations of each step are provided.

2.3.1 Flame atomic absorption Spectrophotometer (FAAS)

FAAS is the most popular method for detecting cadmium in milk since it is reasonably affordable. It is a method of quantitatively analyzing light absorption by atoms in both their free and ground states. This method involves the excitation of ground state atoms by

electromagnetic radiation, which absorbs photons with an equivalent excitation energy and wavelength. The fundamental advantage of this method is that more than sixty characteristics can be determined at once. As a result, Norouzirad *et al*. [85] in Iran found that milk from cows and buffaloes included higher concentrations of the heavy metal cadmium. Using FAAS to find cadmium in dairy cow's milk, Bangladeshi researchers Muhib *et al*. [1] found that the limit of cadmium is higher in all samples. El-Ansary *et al*. [86] used the same method for cadmium detection and discovered that cow's and buffalo's milk contained higher levels of cadmium metal than allowed. A. Ismail *et al*. [87] conducted a similar investigation on milk samples obtained by FAAS in Pakistan and discovered that the cadmium metal concentration was within acceptable limits. Additionally supporting FAAS to detect cadmium limits, Betancourt *et al*. [88**]** and Pilarczyk *et al.* [89] discovered it was well within limits. The majority of writers employ FAAS to detect cadmium, although this technology has drawbacks in terms of expense, limited precision, and the need for expert operators. Table 2.6 provides the results of the quantitative study of Cadmium by FAAS.

Table 2.6 Quantitative analysis of Cadmium detection by FAAS representing range of detection of different milk samples

Reference	Year	Country	Milk type	Range of detection. (mg/kg)
Norouzirad *et al.* [85]	2015	Iran	Cow milk	.001±0.001
			Buffalo milk	0.00±0.001
Muhibe *et al.* [1]	2016	Bangladesh	Cow Milk	0.053±0.022
Ansary *et al.* [86]	2017	Egypt	Cow milk.	0.3067 ± 0.0086
			Buffalo milk	0.3084 ± 0.0087
A. Ismail *et al.* [87]	2017	Pakistan	Cow Milk.	0.001
Betancourt *et al*. [84]	2018	Mexico	Cow milk	0.0181

2.3.2 Inductively coupled plasma mass spectrophotometer (ICP-MS)

The utmost popular method for finding multi-element and isotopic analysis traces is "inductively coupled plasma-mass spectrometry (ICP-MS)". It has stood frequently utilized to analyze samples that are metallic, inorganic, organic, and biological. It is the most recommended method among the mass spectrometry techniques because of its great

sensitivity and capacity to ascertain the isotope makeup of a sample. This technique, which is based on mass spectrometry, ionizes the given sample using the idea of inductively coupled plasma, which further atomizes the material and produces atomic and tiny polyatomic ions, which are also identified. Pilarczyk *et al*. [89] used ICP-MS to analyse 20 samples of Simmental and Holstein-Friesian cow's milk to determine the concentration of harmful heavy metals like cadmium. They discovered that the level of cadmium was below the allowable limits. Similar efforts were made by Patel *et al*. [90] in India to determine the level of cadmium in dairy milk using the same methodology, and they discovered that the level was on the higher side. In a different region of Poland, Monika Sujka *et al*. [91], another Polish author, used ICP-MS to analyse cow's milk and discovered that 50% of the samples had higher limits of cadmium concentration. According to Miedico *et al*. [92], who measured the cadmium levels in goat milk in Italy and discovered that the concentration level is below the allowed threshold, drinking goat milk is always safer. This method offers many advantages over other methods, including increased sensitivity, a larger dynamic range, a smaller detection range, and good precision. However, it is more expensive and limits interferences brought on by sample matrix components. Quantitative analysis of this method for detection of cadmium are given in Table 2.7.

Table 2.7 Quantitative analysis of ICP-MS method for Cadmium detection representing range of detection of milk samples

Reference	Year	Country	Milk type	Range of detection.
Pilarczyk *et al*. [89]	2013	Poland	Cow milk	0.1 µg/L
Patel *et al.* [90]	2018	India	Cow milk	0.00063 mg/l
Monika Sujka *et al*. [91]	2019	Poland	Cow milk	0.0067 to 0.0058 mg/kg.

2.3.3 GF- AAS: Graphite Furnace Atomic Absorption Spectrometry

The sample is vaporized in a graphite-coated furnace during a process known as graphite furnace atomic absorption spectroscopy (GFAAS), often referred to as electrothermal atomic absorption spectroscopy (ETAAS). By absorbing ultraviolet or visible light, the atoms in this process move to higher electronic energy levels. Typically, the light is transmitted longitudinally through the tube from a line source that is a feature of the determining element. Measured is the absorbance brought on by unbound analyte atoms in the gas phase. In comparison to FAAS, this approach has a superior detection level and greater sensitivity. When Najarnezhad *et al*. [93] employed the GF-AAS technique to find

cadmium in milk, they found that the limit was higher (4.7 ± 1.0 mg/kg) than what was allowed. Additionally, this strategy is costly, imprecise, and requires high operator skills.

2.3.4 Fluorescence Spectroscopy

Fluorometer can measure fluorescence. A fluorometer is a device used to gauge the intensity and distribution of the emission following excitation, among other fluorescence-related factors. For a variety of uses, including biochemical detection, water quality monitoring, biological sensing, and others, portable fluorescence sensors have been created. According to Shin *et al*. [94], typical fluorescence-based instrumentations are now incorporated into portable sensing devices for remote and resource-constrained contexts. The advancement of a urease optical biosensor for the detection of heavy metals based on the sol-gel immobilization process was done using a novel strategy by Tsai *et al*. [95]. FITC- dextran, a fluorescent dye, was encapsulated, and variables such the probe's optical characteristics, the relative enzyme activity, the initial pH value, and the buffer were taken into consideration.

2.3.5 Principle findings from the analytical chemistry methods

1. Four methods are frequently employed in the laboratory for the identification of heavy metals in milk: FAAS, ICP-MS, GF-AAS, and electrochemical detection.
2. The capacity to simultaneously detect numerous metal ions from the milk samples is the key benefit of all these approaches.
3. All of the methods offer appropriate detection limits and dynamic linear ranges, but because these values rely on the concentrations of the milk samples, they cannot be compared on an equal footing.
4. Overall, ICP-MS discovered a highly dependable method that may be employed for validation purposes in terms of sensitivity and detection limitations.

2.4 Biosensor detection

Due to its benefits, such as being simple to construct, inexpensive, and small in size, this method is the most promising for finding cadmium in milk. Similar to the previous example, this technique works well for online and in-person detection of heavy metals in milk without a trained operator. Biosensors are often divided into six categories: Whole-cell biosensors, immune-biosensors, electrochemical biosensors, thermometric biosensors, optical biosensors, and piezoelectric biosensors.

2.4.1 Electrochemical biosensor

Antigen-antibody interactions and enzyme-substrate reactions are two examples of biological events that are converted into electrical signals using a scientific instrument known as an electrochemical biosensor (e.g., current, voltage, impedance, etc.). The basic concept of electrochemical method is depicted in Figure 2.8

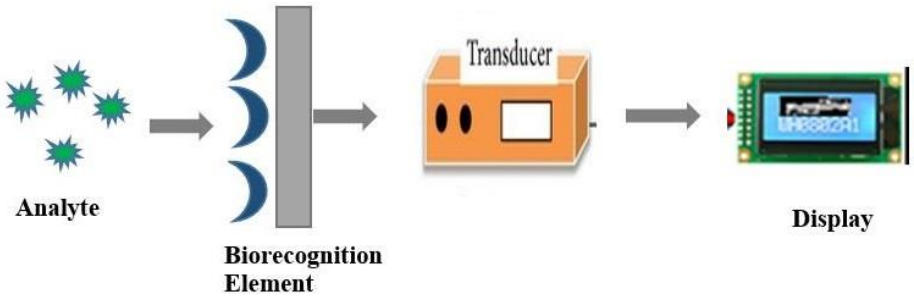


Figure 2.8 Basic concept of electrochemical biosensor comprising of various component to measure biorecognition event into an electrical signal.

An electrode is a crucial element used as a solid substrate for immobilizing biomolecules (enzyme, antibody, and nucleic acid) and electron movement in an electrochemical biosensor. It is made up of three crucial components: a transducer to turn the reaction into an electrical signal that can be measured and a receptor that binds the sample. Cho *et al.* [96], the electrode serves as the transducer in electrochemical sensors. Four categories of electrochemical biosensors are recognized. Biosensors with amperometric. Biosensors with potentiometric. Biosensors with an impedance. Voltammetric biosensors and nanoparticle biosensors. A single-walled carbon nanohorns modified screen-printed electrode with bismuth layer that exhibits outstanding electrochemical activity was proposed by Yao et *al.* [97]. Cadmium (II) ions could be determined with greater practicality thanks to the detection limit of 0.2 μg L^{-1}.

To find the cadmium in powdered milk, Munoz *et al*. [98] created stripping potentiometric using a handmade flow cell sensor. This procedure employs a supporting electrolyte of an acetic acid-acetate buffer solution (pH 3.4), an electrolysis potential of - 1.1 V, and a flow rate of 3 ml min-1. Thus, the sensitivity and detection limits offered by this approach were good. Electrochemical sensing based on graphene holds great promise for common sensing applications. Ping *et al*. [99] created an electrochemical sensing platform based on a screen-printed electrode modified with an electrochemically reduced graphene oxide sheet that

demonstrated high conductivity and quick electron transfer kinetics. Cadmium had a detection deposition potential of 1.2 V and a linear range detection limit of "1.0 μg L^{-1} to 60.0 μg "L^{-1}

The threshold was reached at 0.5 μg L^{-1}. As a result, this method was able to detect low concentrations of cadmium ion in milk (μgL^{-1}) because disposable bismuth film electrodes made of graphene are sensitive, stable, and dependable platforms for measuring heavy metals. This specially created electrode had a high conductivity and quick electron transfer kinetics. To obtain the highest stripping performance, a number of factors, such as the electrolyte environment and the electrode position circumstances, were carefully tuned. This approach produced good linear range and detection limits. The amperometric biosensor is a different kind of biosensor. They are independent and integrated devices that gather precise quantitative analytical data by measuring the current that results from the oxidation or reduction of an electroactive biological ingredient. The basic working aim of the Amperometric biosensors are highlighted in Figure 2.9.

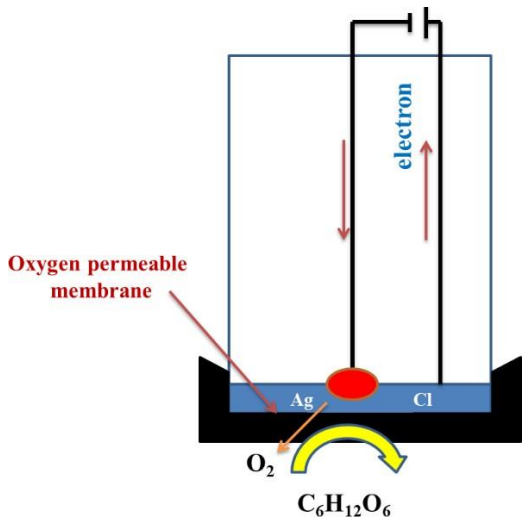


Figure 2.9 Basic working principle of the Amperometric biosensor which measures the current developed due to a chemical reaction of the electroactive materials on the transducer surface

While a continuous potential or current is supplied, it measures the current or potential that results from a chemical reaction of electroactive materials on the transducer surface. The concentration of the target species has an impact on how the current changes. S.S. Babkina *et al.* [100] used this biosensor to determine the occurrence of heavy metals in H_20, milk, and blood serum with a selectivity factor of 1:10. The stationary mercury-film electrode

(SMFE) with silver support and the cellulose nitrate (CN) membrane with immobilised single-stranded DNA were the foundation of the amperometric biosensor "(BS) (ssIDNA)". Preconcentration of metal ions on the "BS, DNA-metal complex destruction with ethylenediamine tetra acetate (EDTA)", and "voltammogram" recording were the steps used to identify heavy metals.

The minor limits of detectable levels for "Pb (II), Cd (II), and Fe (III)" are 1.0×10−10, 1.0×10^{-9} and 1.0×10^{-7}, respectively. A comparable and innovative strategy was put forth by Silwana *et al*. [101], who first developed a method for the measurement of metal concentration in the existence of specific metals of "Cd^{2+}, Pb^{2+}, and Hg^{2+}" before demonstrating the effectiveness "of the amperometric enzyme sensor system via measuring the oxidation current" of "hydrogen peroxide". The use of an "amperometric urea biosensor" by Kuralay *et al*. [102] for the "Inhibitive measurement of Hg^{2+} ion" employingimmobilised enzyme in "poly(vinylferrocenium)" film was established. "A PVF+ClO4− "thefilm was coated on Pt electrode at +0.7 V versus an "Ag/AgCl by electrooxidation of poly(vinyl ferrocene) in methylene chloride" containing 0.1 M "tetrabutylammonium perchlorate (TBAP)".

Another type of biosensor in use is a potentiometric biosensor. Potentiometric biosensors are designed to detect specific analytes (e.g., ions, molecules, biomolecules) by converting the chemical information from the analyte-receptor interaction into an electrical signal. It is a device with an integrated biological sensor and a potential electrochemical transducer. Potentiometric biosensors often depend on a biochemical process that results in a simpler chemical specieand its subsequent electrochemical detection (NH_4OH, CO_2, pH, H_2O_2). Electrical potential is the analytical signal produced by a potentiometric biosensor. Potentiometric sensors primarily measure the variation of potential difference between working and reference electrodes at various analyte concentrations to determine the analyte concentration. Y. Yang *et al*. [103] proposed this kind of regenerative potentiometric urease inhibition biosensor based on self-assembled gold nanoparticles to measure mercury ions. On the "PVC-NH2 matrix membrane pH electrode surface", which also contained "N, N-didecylaminomethylbenzene (DAMAB)" as a neutral carrier, "gold nanoparticles" were chemically adsorbed. Urease was then immobilised on the gold nanoparticles. In-depth research has been done on the response properties of the "DAMAB/PVC-NH2 pH-sensitive

membrane" and the impacts of nanoparticle size. With a detection limit, the linear range of Hg^{2+} determination was 0.09–1.99 μmol L^{-1} with a detection limit of 0.05 μmol L^{-1}. Low detection threshold, quick reaction, and simplicity of regeneration are benefits of self-assembled immobilisation. By using urease immobilised on nylon membrane by "hydrosol gel technique" as the bio component and phenol red as the pH indicator, Kaur *et al*. [104] created a unique method for demonstrating the impact of metals on enzyme activity. The bottom limit detection for the milk was 38.6 m, and the enzyme membranes remained stable at 4°c for more than two months. To reach a detection limit of 9.66 m in the potentiometric technique, the response of an "ion-selective electrode (ISE)" to changing ammonium ion concentration as a result of urease inhibition by "Pb (II) ions" was investigated. "Impedimetric biosensors", a different kind of biosensor that measures the changes in charge conductance, and capacitance at the sensor surface as the selective binding of the target occurs. Khedimallah *et al*. [105] used an electrochemical impedance spectroscopy technique to determine the presence of heavy metals, specifically "Cd^{2+}, Zn^{2+}, and Ni^{2+}", utilizing a gold "electrode functionalized with a PVC-tannin bio membrane". e. Compared to existing analytical techniques used to detect heavy metals, the suggested analytical methodology was quick, easy, and affordable. According to this investigation, the three heavy metals had good sensitivity and a low detection limit of 10^{-9} M. The Zn-binding and response sensitivity of the tannin bio membrane were both quite high (II). Impedimetric responses for "Cd^{2+}, Zn^{2+}, and Ni^{2+}" between concentrations of 10^{-9} to 10^{-3} M were demonstrated to be linear. Due to their small size, high sensitivity, and ability to concurrently and individually detect heavy metals from solutions, nanoparticle-based electrochemical sensors are gaining popularity. Palladium nanoparticles (Pd NPs) on porous activated carbons (PACs), which are obtained from waste biomass feedstock (fruit peels), were used to create a simple sensor by Veerakumar *et al*. [106]. The PACs have desirable textural properties and porosities that are conducive to Pd NP dispersion on the graphite PAC substrate. This was employed to find the hazardous metal ions of cadmium. This method has produced good detection limits, selectivity, and sensitivity. A different method of cathodic-anodic stripping Shahbazi *et al* [107] voltammetric measurements were performed using a voltammeter. A hanging mercury drop electrode (HMDE) in a three-electrode arrangement used to help with this measurement. A platinum wire with a

significantly bigger surface area than HMDE served as the auxiliary electrode. As a reference electrode, silver/silver chloride (KCl 3M) was utilised. Using a big Teflon rod spinning at 2000 rpm, the mixture was stirred. Before each experiment was run in a nitrogen environment, solutions were purged with high purity nitrogen for 3.0 minutes. In order to investigate the levels of cadmium in dairy products obtained from five industrial zones in Iran (n = 250 samples) throughout the summer and winter of 2013, this study was carried out. Cadmium results were within acceptable limits. Using the drop-coating technique, Palisoc *et al*. [108] created pencil graphite electrodes that combined bismuth nanoparticles and Nafion. The ultrahigh temperature processed milk was tested using this technique to find any amounts of cadmium. Anodic stripping voltammetry was employed for detection, with the modified PGE serving as the working electrode. Cadmium was less than the allowed level, according to scanning results. The modern world requires automation and online measurements. Different flow systems are created for electrochemical stripping analysis that are ideal for sensitive, rapid, and selective determination. This approach also has the benefit of functioning well in batch systems. Suturovic. Z *et al*. [109] suggested a straightforward, home-made flow system comprised of glassy carbon tubes that acted as both a working and auxiliary system for detecting cadmium in skimmed milk powder. To understand comparative analysis of different electrochemical sensors in terms of detection limits, linear range and sensitivity, all the surveyed methods for cadmium are highlighted in Table 2.8

Table 2.8 Quantitative analysis of electrochemical methods for cadmium detection with respect to the sensitivity, detection limits of different milk samples.

Reference	**Year**	**Country**	**Milk type**	**Range of detection. (µg/l)**		
Munoz *et al*. [98]	2004	Spain	Raw milk.	6.51		
Ping *et al*. [99]	2013	China	Raw Milk	1-60		
Bakina *et al*. [100]	2015	Taiwan	Raw milk.	Linear range in µm	Sensitivity µm/µA. cm^2	Detection limit nM
				0.5-5.5	66.7	41
Shahbazi *et.al*. [107]	2016	Iran	Raw milk.	Linear range in ppb	LOD in ppb	LOQ in ppb
				0.3-220	0.12	0.3

Palisoc *et al*. [108]	2019	Philippines	UHT milk	7.31 µg/L.
Suturović, Z *et al.* [109]	2019	Serbia	Milk	0.11mg/L

2.4.2 Thermometric Biosensor

Thermometric biosensors take advantage of the variation in heat evolution or absorption that happens during biochemical activities. The temperature of the reaction medium changes as a result of this heat fluctuation [110]. The temperature change is monitored using sensitive thermistors. Exothermic reactions are the norm in biology. The enthalpy changes that accompany enzyme reactions range from 20 to 100 kJ/mol. Sometimes insufficient particular enthalpy increases can be amplified by subsequent reactions with larger enthalpy changes. Coimmobilizing enzymes and high-protonation enthalpy buffers, such as TRIS, can enhance assays for signal amplification. [111] Thermocouples, resistance thermometers, thermistors, and diodes are only a few examples of thermometric sensors. However, there is some encouraging continuing research in this area. In contrast, magnetic approaches (such as the Hall Effect or magneto resistance) are infrequently used in the design of disposable sensing devices [112-113]. In large magneto resistance sensors, for instance, the binding of magnetic nanoparticles (as reporters of a biological event) onto the surface of a sensor results in a change in its electrical resistance, enabling quick and real-time quantification of biomolecules. [114].

2.4.3 Optical Biosensors

The optical biosensor has gained more popularity from last few decades due to its smaller size and fast response. For the purpose of detecting cadmium in milk, Verma *et al*. [115] created a brand-new absorption-transmission-based fibre optic biosensor. This biosensor is made up of complete Bacillus badius cells that have been co-immobilized onto spherical plastic discs using a sol-gel technique with a fibre optic transducer system as an indication. The main benefit of this approach was its 90-day storage stability, which allowed for an excellent detection limit of 8.1 µg/l.

Based on "Escherichia coli DH5 technology", Kumar *et al.* [116] suggested another optical biosensor for detecting cadmium (pNV12). The "Staphylococcus aureus plasmid pI258 gene"S and the cad promoter were responsible for this sensor's fundamental operation, which involved gene expression. In Punjab State, this biosensor was tested on several

samples using two samples from the Industrial area and found cadmium levels more than the permissible limits. A comparative analysis of all reviewed optical biosensors is given in Table 2.9.

Table 2.9 Quantitative analysis of optical detection methods for cadmium detection representing linear range of detection and Biocomponent used in biosensor

Reference	Year	Country	Milk type	Range of detection. (µg/l.)	Biocomponent
Verma *et al.* [115]	2010	India	Raw milk	8.1 Linear Range -0.1 to 10	*Bacillus badius isolate*
Kumar *et al.* [116]	2017	India	Raw milk	10 Linear Range -10-50 Reduced detection limit-5	Escherichia coli (pAD123)and Escherichia coli DH5α.

2.4.4 Piezoelectric biosensor

Piezoelectric biosensors are a group of analytical devices that monitor interactions between affinities. A piezoelectric platform, also known as a piezoelectric crystal, is a type of sensor that works on the principle that oscillations can change depending on the mass that is attached to its surface. Since this form of sensor is mass produced for the electronic industry, making it economical and readily available from any manufacturer, quartz crystal microbalances (QCM) are often used tools for the development of biosensors. Their common fundamental mode frequency varies from 1 to 20 MHz, and they are currently used as attenuators in electrical equipment. For piezoelectric biosensors, direct, label-free interaction with the analyte is the most effective method of operation.

In this way, antigens and antibodies are presented as potential biomolecules that can work with a piezoelectric sensor [117]. In this investigation, “white BALB/c mice” were used to produce a polyclonal antibody against “Francisella tularensis”, which was then immobilised on the surface of a “10 MHz QCM”. Antigen-antibody interaction with “F. tularensis” caused the bacteria to bind to the sample's surface, which was followed by a change in frequency of up to 40 Hz. Extreme pH can split the intricate bacterial-antibody complex, allowing the biosensor to be reused. “Bacillus subtilis and Escherichia coli” did not interact with the biosensor surface in any way. Even if the biosensor functioned properly, the frequency change was not much. Through hybridization, which causes a mass

increase on the biosensor surface, genetically modified organisms were detected using a "QCM biosensor containing immobilised DNA" [120]. His tagged proteins can interface directly with the sensor surface, allowing for the measurement of these macromolecules through the interaction. Li and colleagues tested the proteins on a QCM biosensor using the His-tag interaction with metals [118]. The acid analysis in food samples was carried out using an immunosensor with a 10 MHz AT-cut based QCM that was coated with antibodies against okadaic acid [119]. The lowering of oscillations up to 900 Hz was seen after the exchange and was confirmed to be caused by the direct interaction. In addition to commercial sensors, in-lab generated materials can be used to create piezoelectric biosensors very thin and flexible membranes with piezoelectric properties synthesized by Spanu and coworkers from "polyvinylene fluoride" [120-121].

Zhao and colleagues [122] effectively conducted the determination of immunoglobulins G using nanowires made of "SiO2 with a ZnO-coated surface" [123]. A "(K, Na) NbO3 microstructured fiber-based prototype" sensor was created as a viable platform for analytical and diagnostic uses [124]. Another way to use the piezoelectric sensors is to coat their surface with molecularly imprinted polymers, which can detect interactions that are similar to those between the antibody and the antigen but without the antibody present. This enhancement can lower the cost of the biosensor while also making the production process more repeatable.

2.4.5 Whole-cell biosensor

Receptors, ion channels, and enzymes that have naturally evolved in cells can be employed as targets for biological or biologically active analyses. Therefore, functional information and the impacts of the analyte on the physiological function of living cells can be measured using whole cell-based biosensors. Bacillus badius cells were immobilized onto nylon membranes using the sol-gel method with alcohol and TEOS, according to a method Verma *et al*. [125] suggested. Sol-gel was combined with the cells before being placed onto a nylon membrane and dried. Immobilized cells in hydrosol-gel were used in the investigation as a bio component. With a bioassay approach based on the suppression of urease activity, ammonium ISE is utilised as a transducer. The lowest Cd detection limit was attained at 1.0 µg/l. The ready-made biosensor was used on samples of unfortified and fortified acid-

extracted milk. For Cd, the linear range of detection is l0µg–1mg/l. The designed biosensor has a 65-day shelf life at 40°C and 10% glycerol.

The first "30 cadmium whole-cell biosensors (WCBs)" were created by Jia *et al.* [126] employing various configurations of the host, reporting components, and detection elements. For more examination and engineering, the best-performing "WCB KT-5-R, constituted of CadR and mCherry and using Pseudomonas putida KT2440" as the host, was chosen. The reporter gene dosage was raised, or a positive feedback amplifier was introduced to improve its sensitivity. With an enhanced tolerance to cadmium and a detection limit of 0.01 µM which is the WHO norm, the WCB with the "T7RNAP" amplification module, "p2T7RNAPmut-68", performed the best. When tested with mixed metal ions, it also demonstrated superb specificity for cadmium. This study offered important knowledge for WCB design and demonstrated the effectiveness of circuit engineering. A similar strategy was devised by Hui *et al.* [127]. A biosynthetic gene cluster for indigoidine was combined with a "CadR-based Cd (II)" sensing element to create a visual whole-cell biosensor. Reduced detection background was a result of insufficient indigoidine basal synthesis. "Zn (II), Pb (II), and Hg (II)" caused the whole-cell biosensor to respond slightly, but "Cd (II) caused it to respond robustly (II)". When exposed to Cd (II) during the early exponential phase, the detection limit was 0.049 M, and it was further lowered to 0.024 M during the lag phase. With a nonlinear regression range of 6.25 to 200 M, bioavailable Cd (II) could be measured. A versatile dual-signal output biosensor conducive to diverse detection was successfully constructed and verified by combining indigoidine and mCherry into one biosensing construct. The "indigoidine-based biosensor's capacity to detect Cd (II)" in environmental water samples was confirmed. In quantitative detection, a high-throughput technique based on a 96-well micro plate was proven effective. For rough measurement, naked-eye visible blue in the culture system proved useful. It could be used as a field-deployable biosensor for environmental cadmium pollution surveying due to its simplicity of use, low cost, and minimal equipment requirements. Elcin *et al.* [128] proposed a different strategy. Here, a sensitive and very precise bacterial bio reporter was created to meet the demand for a quick and simple measurement of cadmium. Based on the expression of green fluorescent protein under the control of the zntA gene promoter of heavy metal resistance determinant, "Escherichia coli MG1655 (pBR-PzntA)" was

constructed and characterized as a fluorescence-based whole-cell cadmium bioreporter strain. After 3.5 hours of induction in a specified medium, the created bioreporter found cadmium at a concentration of 5 µg/L. An inorganic phosphate-limiting defined medium was used, and after 1.5 hours the cadmium detection limit was increased to 2 µg/L. Bioreporter cells generated at various growth stages showed drastically variable cadmium sensitivity. The highest level of fluorescence performance was achieved for early exponential growth phase cells. Compared to a variety of other heavy metals, this cadmium bioreporter was more sensitive and selective to cadmium ions. It was only exposed to cadmium at concentrations that meet drinking water quality standards. In P.” putida KT2440”, Zhang *et al*. [129] created a “Cd^{2+} WCB” with a negative feedback amplifier. According to the slope of the linear detection curve, which serves as a measure of sensitivity, the reporter Cherry's output signal was significantly enhanced by WCB with a negative feedback amplifier, producing 33% higher sensitivity than in a comparable WCB without the negative feedback circuit. Although WCB may produce a detectable signal in a significantly shorter period of time due to the enhanced output signal amplification, a quick response is more desirable for real-world field applications. The "World Health Organization standard" for cadmium in drinking water is 27 nM (0.003 mg/L), yet the WCB showed enhanced Cd^{2+} tolerance and a lower detection limit of 0.1 nM, a extraordinary 400-fold improvement over the WCB without the negative feedback circuit. Furthermore, compared to other metal ions, the WCB showed extraordinarily high specificity for Cd^{2+}, generating signals that were 17.6 to 41.4 times weaker than those generated by Cd^{2+}. Since the "WCB negative feedback amplifier" created in this work fits the Cd2+ detection necessities with very high sensitivity and specificity, it can be concluded that genetic negative feedback amplifiers are outstanding tools for enlightening WCB performance.

2.4.6 Immunobiosensors

Immunobiosensors rely on the highly specific interactions between antibodies (or antigens) and their corresponding target molecules. Antibodies are proteins produced by the immune system in response to the presence of specific antigens (foreign substances).

An “immuno-biosensor” is a type of biosensor that uses an antibody or antigen as a bioreceptor to detect the creation of an “immunocomplex” Zhang *et al*. [130]. Analytical methods known as immuno/bioassays make use of the precise molecular recognition that

occurs between antibodies and antigens or between specific biomolecules and receptors. They are incredibly helpful for assessing materials with complicated matrices because to characteristics like high selectivity and low sample consumption. However, immuno/bioassays need lengthy (multi-step) procedures, typically including numerous incubation and washing phases. These are time-consuming and could lead to serious mistakes. Automated immuno/bioassay systems are quite helpful since they can make these procedures easier and quicker. Hartwell and others [131] Immunoassays can also be divided into groups according to the various labelling and detecting techniques employed.They could be categorized as chemiluminescence immunoassay, enzyme-linked sorbent immunoassay, or radioisotope immunoassay, for instance. Based on the format of the stationary phase, such as micro-plate, bead, membrane, and capillary immunoassays, immunoassay techniques can also be categorized. There are several configurations for immune biosensors, which operate on the basis of immunological specificity and measurement based on amperometric or potentiometric biosensors. One of them is an antibody that has been immobilised and can directly bind to an antigen.

b) A free second antigen can bind to an immobilised antibody that binds to an antibody.

c) An immobilised antigen and an antibody can be partially released by competing with a free antigen. In order to test for two "Salmonella species (S. gallinarum and S. pullorum) in eggs and chicken meat", Fei *et al*. [132] developed a sandwich immunoassay. According to research, the detection limit for both species was "3.0x 10^3 CFU/ mL", and a linear response to the Salmonella species was obtained in the concentration "range of 104 -109 CFU/ mL". Xu *et al.* [133] investigated immunosensors using screen-printed interdigitated microelectrode (SP-IDME) transducers. The immunosensor was capable of specifically identifying E. coli O157:H7 and "S. Typhimurium" in pure culture samples between 102 and 106 CFU/mL, according to the heir results. The limits of detection for the two bacteria were "2.05 103 CFU/g and 1.04 103 CFU/mL", respectively, accordingto e. "Cadmium selective polymeric membrane microelectrode (Cd-ISE)" was utilized by Silva *et al*. [134] as a transducer to find "S. Typhimurium in milk". The two bacteria in theculture samples were "2.05 × 103 CFU/g and 1.04 × 10^3 CFU/mL", respectively. Silva *et al*. [134] used "cadmium selective polymeric membrane microelectrode (Cd-ISE)" as a transducer to detect "S. Typhimurium" in milk. The detection limit was found to be 2 cells per 00 µL. Their study indicated an average total test duration of 75 minutes for the identification of

"S. Typhimurium" in milk samples. Bekir *et al.* [135] created "immunosensors that recognize" stress and revive bacteria. In their work, they were able to create a stable and repeatable immunosensor with a sensitivity of 15 k/decade and a detection limit of 101 CFU/mL for S. aureus concentrations ranging from "101 -107 CFU/mL. E implied that both stressed and unstressed bacteria" exposure resulted in a low variation in the "immunosensors response (± 10%)".

CHAPTER 3

Methods and Materials

3.1 Overview

The research objectives, methodology and materials for the proposed research work are represented in this chapter. These goals are appropriately formed to handle the research gap recalled with a comprehensive literature survey of the intended research area. The different Methodological steps are also explained to conduct the proposed research objectives. These strategies have indeed been sufficiently implemented to address the research gap identified. The materials and methodology utilized to help attain the specified research objectives are also highlighted.

3.2 Research gaps

From the literature review, these are some principle findings which are recorded for future research directions and to identify the research gap.

1. Despite many merits, all the analytical chemical methods require a laboratory, instruments and skilled operators; hence expensive.
2. Due to the large size, these methods are unsuitable for online and on-field detection of heavy metals from the milk.
3. Design and development of optical Biosensors are one of the most popular areas of research which will satisfy the need for industrial automation in terms of miniature, fast in response and low in cost.
4. Despite many advantages, such as being small in size, good detection limits and higher sensitivity, optical biosensors are found to have minimal utilization in detecting cadmium contamination in milk.

3.3 Problem Formulation

From substantial literature surveys and research gaps, we can put the hypothesis that there is still a research gap in the development of an optical biosensor for the detection of heavy metal ions in milk. Many analytical methods are available for heavy metal detection but are expensive due to certain limitations, such as the requirement of laboratories, instruments, and skilled operators. Also, these methods are unsuitable for online and on-field detection

of heavy metals from the milk due to their large size. Therefore, there is still an unmet social and clinical need to have precise, portable and accurate biosensors which will satisfy the need for industrial automation in terms of miniature, fast response and low cost. Hence, our primary objective behind this research topic is

Development of Optical Biosensor and its application for detection of Cadmium ions in milk.

3.4 Specific Research Objectives

Specific objectives of the proposed research topic are:

1. Screening of Biocomponent for characterization of Bio-assay principle for detecting cadmium ions.
2. Immobilization of biocomponent by polyacrylamide gel, calcium alginate and hydrosol gel approach.
3. Proximity of selected immobilized bio component to the optical transducer for developing optical biosensor and its applications to develop methods to monitor cadmium in the milk.
4. Validation of developed optical biosensor by existing /conventional method.

3.5 Research work plan

To carry out the proposed research, Initial phase is dedicated to the extensive literature survey to identify research gaps in order to develop a novel approach for development of optical biosensor for cadmium detection in milk. In second phase novel method is implemented for extraction and purification of urease enzyme. In next phase, immobilization of purified enzyme is done with sol-gel technology. And finally, design and validation of optical biosensor with the target milk samples are performed. The developed biosensor results is compared with existing techniques. The work plan undertaken is divided into four subsections which are as given below

1. Extensive literature survey
2. Extraction and purification of bio component enzyme.
3. Immobilization of purified enzyme using sol-gel techniques.
4. Validation of advanced optical biosensors by analytical methods.

The graphical abstract of the research work plan is illustrated in Figure 3.1

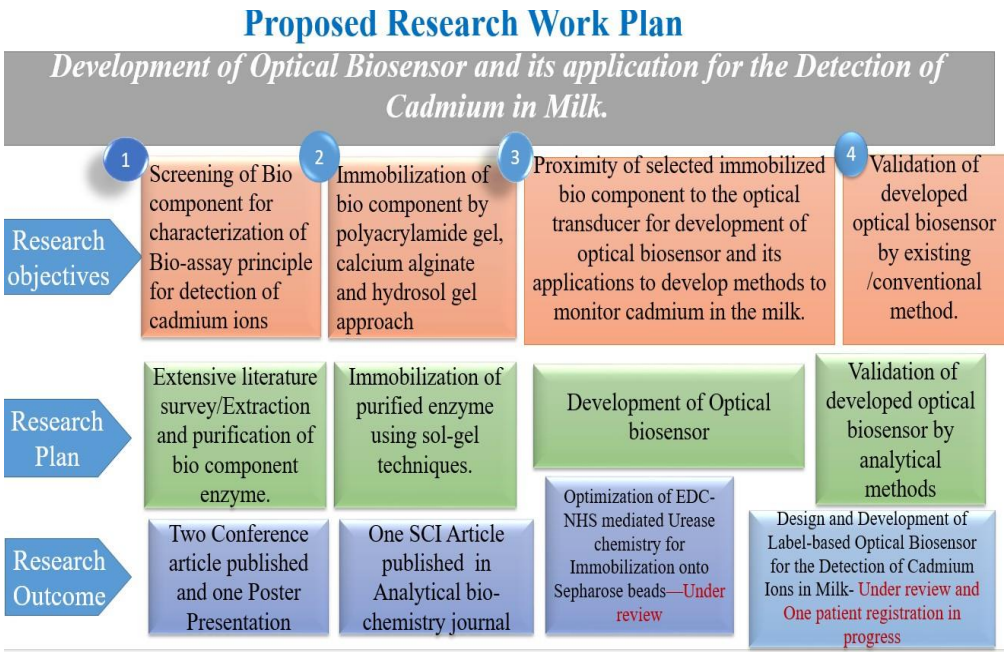


Figure 3.1 Graphical abstract of the proposed research work plan describes the research objectives, execution of research and its outcomes.

3.6 Research methodology

Optical biosensors have numerous merits over traditional analytical methods because they promote the direct, real-time and label-free detection of multiple biological and chemical substances. The proposed research addresses the design and development of an optical biosensor for detecting toxic metal ions, i.e., Cadmium (Cd), in dairy and raw milk. The proposed methodology is divided into three steps:

1. Screening of Biocomponent
2. Immobilization of Biocomponent
3. Development of Optical Biosensor
4. Validation of Optical Biosensor

A generalized block diagram of the research methodology is depicted in Figure- 3.2

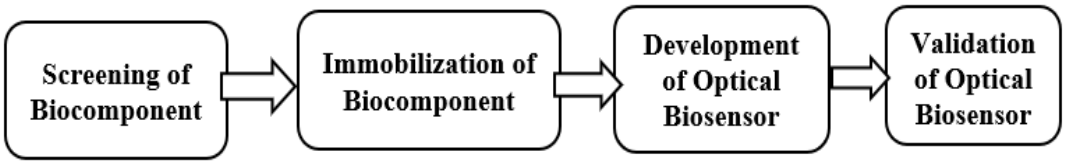


Figure 3.2 Block diagram of the proposed research work depicts the research methodology for extraction, purification and immobilization of biocomponent which is further used for analysis of cadmium in milk sample using developed optical biosensor.

3.6.1 Screening of Biocomponent

This section describes the screening of the Biocomponent step in the proposed research work, basically the Purification and Extraction of urease enzyme from Jack bean meal. It involves processes like urease isolation and precipitation, Polyacrylamide gel electrophoresis, Purification by Anion–Exchange chromatography**,** urease activity assay and Zymography analysis.

3.6.1.1 Materials used for Screening of Biocomponent

Screening of Biocomponent uses reagent grade chemicals, including a Jack Bean source in powdered from LOBA CHEMIE PVT. LTD, Mumbai. Phenol–sodium nitroprusside solution contains 0.05g sodium nitroprusside and 1 g phenol in 100 ml distilled water, which is stored in an amber bottle. In addition to the above, experimental analysis uses alkaline hypochlorite: 3.56 g Na_2HPO_4 and 1 ml sodium hypochlorite in 100 ml distilled water.

3.6.1.2 Urease isolation and precipitation

Initially, extraction of urease from a jack bean source (Canavalia ensiformis) was performed using 50mM phosphate buffer at pH 7.5 and further contact with 50mM β-mercaptoethanol. The centrifugation of the extraction solution was done at 6000rpm for 15 min. Further, Ammonium sulphate and Cold acetone (- 20°C) were added to the supernatant solution, followed by incubation at four °C for one hour [136]. The required sample was obtained in precipitated form after centrifugation at 6000 rpm for 15 min. The obtained samples are converted into a pellet and dissolved in 550 mM phosphate buffer at pH 8.0 for further processes.

3.6.1.3 Polyacrylamide gel electrophoresis

In the second step, the proposed method uses the most common and popular approach, i.e., SDS-PAGE. It works on separating biological macromolecules (proteins or nucleic acids) according to their electrophoretic mobility. SDS-PAGE will denature and divide the oligomeric form into monomers, showing bands representing their molecular weights. These readily available bands can be used to locate and access the protein purity. In the suggested procedure, SDS PAGE was carried out using a Mini Four gel Bio-Rad electrophoresis equipment at room temperature and under decreasing circumstances [137].

Further, the 5% stacking and 8% resolving gel separated the protein mixture. The support of the Laemmli buffer system was employed to prepare the samples, and electrophoresis was carried out for one hour and thirty minutes at a constant voltage. A prestained protein identifier from a pure gene was injected to the proposed sample in order to measure the mol wt. of the protein [138]. The gel is destained until the bands become visible after being submerged in 0.25percent Coomassie blue solution for ten minutes following electrophoresis.

3.6.1.4 Purification by Anion Exchange chromatography

Anion exchange chromatography is operated for preparative and analytical objectives. The merit of this method is that it can split an extensive range of molecules, from amino acids and nucleotides to large proteins. After SDS-PAGE, the proposed method uses Anion exchange chromatography to separate molecules selected on their net surface charge [139]. The proposed sample is loaded on a pre-equilibrated DEAE – Sepharose column with 50 mM phosphate buffer at pH 8.00. The urease was eluted by a step gradient of NaCl (50-500 mM) concentration, prepared in a 50 mM phosphate buffer pH 8.0. After collecting the fractions, protein concentration is measured using Bradford assay [140] and checked for urease activity.

3.6.1.5 Urease Activity Assay

The Urease Activity Assay provides a straightforward process for evaluating urease activity in biological and environmental samples. In this assay, the breakdown of urea by urease catalyses the production of ammonia. Carter et al [141].'s description of the urease chemical reaction is outlined in equation 3.1.

$$\underset{\text{Urea}}{(NH_2)_2CO} + \underset{\text{Water}}{2H_2O} \longrightarrow \underset{\text{Carbon dioxide}}{CO_2} + \underset{}{H_2O} + \underset{\text{Ammonia}}{2NH_3} \qquad (3.1)$$

The Urease Activity Assay kit provides a simple and direct procedure for measuring urease activity in biological and environmental samples. In this assay, urease catalyzes the hydrolysis of urea resulting in the production of ammonia. The ammonia is determined by the Berthelot method resulting in a colorimetric product measured at 670 nm, proportionate to the urease activity present in the sample. The entire Biocomponent screening procedure is depicted in the Figure. 3.3

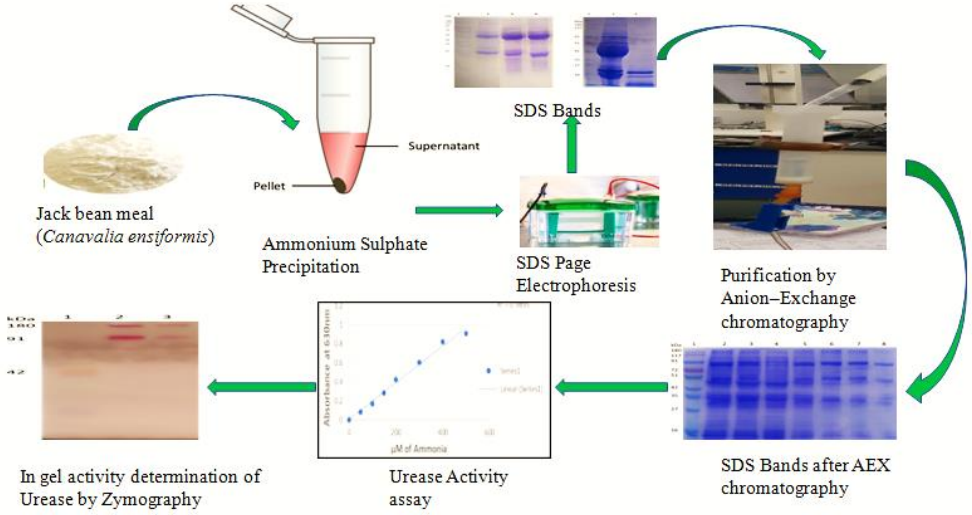


Figure 3.3 Screening procedure of the Biocomponent involving extraction of protein from JB meal,its precipitation using $(NH4)_2SO_4$ precipitation technique. Purification of urease using Ion exchange chromatography and determination of urease activity assay.

For detecting urease activity, the suggested method uses the Phenol-Hypochlorite Assay. 500 µl of 50 mM urea are added to 100 µl of sample in the process. A 30-minute incubation period at 37 °C in a water bath is given to the reaction mixture. Response was terminated by transferring 50 l of the combination to a different tube containing 500 µl each of alkaline hypochlorite solution and phenol-sodium nitroprusside solution [142]. 30 minutes were then spent incubating the solution at room temperature. A spectrophotometer is used to measure the colour complex's absorbance at 630 nm in comparison to a blank. Results are contrasted against a standard curve made with $(NH_4)_2SO_4$ to determine accuracy. According to Senthil *et al*. [143], 1 unit of urease activity is defined as the amount of enzyme that releases 1 mg of ammonia from urea per minute under the specified assay conditions.

3.6.1.6 Zymography analysis

Zymography is a specialized laboratory technique used to study the activity of enzymes, particularly proteases and matrix metalloproteinases (MMPs), in a biological sample. The proposed method uses a fast, novel and economical way to validate urease activity known as Zymography. As per Li P *et al*. [144], Zymography is classified into three types:

Gel Zymography, in-situ Zymography, and Vivo Zymography. This proposed research uses

In-gel Zymography. An SDS-PAGE-based enzyme assay used polyacrylamide gels to increase the gel's local pH level by urease's ureolytic activity. This activity results in the purple-red coloured band after incubating the polyacrylamide gel with urea. The fundamental merits of this method are that the urease activity is concrete for catalytically active urease, even in crude preparations. This method is more sensitive than existing methods, with rapid, economic and fast responses. Further can be used to demonstrate the presence of different charge isoforms of urease.

In this proposed method, polyaramids gel electrophoresis at 8% non – denaturing condition was carried out Bio–Rad unit. After electrophoresis, the gel solution was washed with distilled water for 10 min. After the first wash, the gel solution is washed three times with 0.01M and 0.05 M acetate buffer at pH 6 for 15 min each. After this, the gel solution was immersed in 5M urea solution and incubated at 37^{o} C for 5 min without shaking. The gel was transferred to a 1.5 % sodium nitroprusside solution and kept for 5 min. A 200µl of freshly prepared 1M β- mercaptoethanol was added immediately, and the gel was gently shaken until a dark purple-red colour band developed [145].

3.6.2 Immobilization of Biocomponent

This section describes urease immobilization with EDC-NHS cross-linked sepharose beads. The Characterization of Immobilize urease to observe the effect of coupling time at different pH and, finally, stability and reusability study of Biocomponent.

3.6.2.1 Materials used for Immobilization of Biocomponent

For immobilization of Biocomponent, purified urease is used, 1-Ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC), N-Hydroxy succinimide(NHS), Hydroxyethyl-piperazine ethane-sulfonic acid (HEPES), all are purchased from Sigma Aldrich, Sodium Carbonate, Sodium bicarbonate, Monosodium phosphate, Disodium phosphate, Sodium chloride, Tris-HCl, Coupling buffer (0.1 M Phosphate, pH- 7.5, 8, 8.5), PBS (50 mM Phosphate, 150 mM NaCl, pH7.4).

3.6.2.2 Preparation of EDC-NHS cross-linked sepharose beads and % Immobilization

Commercially available matrix 15ml of Sepharose 2B slurry was taken in a beaker. The storage solvent (30% ethanol) was removed after settling the resin slurry [146-147]. Further, the slurry was washed with distilled water to eradicate the storage solvent. Then 5 ml of the slurry was taken in a 15 ml tube, and 10 ml of coupling buffer was added and

washed twice with the same buffer to ensure the resin was wholly equilibrated with the coupling buffer. 5mM EDC and 22mM of NHS were kept ready for solubilization. Purified Urease extracted from jack bean, reported in Varsha *et al*. [148], was weighed as per required concentrations (10 mg/ml), dissolved in coupling buffer (of respective pH), and kept ready for the coupling reaction. The washed resin was taken in a 2 ml centrifuge tube. EDC and NHS were dissolved in 1 ml of distilled water and immediately added to activate the resin. After this step, dissolved urease was immediately added to the activated slurry for covalent coupling [149] of enzyme onto the Sepharose 2B matrix. Then the reaction was allowed to agitate at room temperature for 15 mins and 30 mins to determine the optimum coupling of urease onto the matrix. After the coupling time, the resin was allowed to settle down, and the supernatant was collected as a post-coupling supernatant. Then the resin was blocked with 1M Tris pH 8.0. The immobilized urease resin was washed five times with 0.05 M Phosphate buffer saline (PBS) pH 7.5 to remove non-specifically bounded enzyme. All these washed-out products were collected and assayed for urease activity to determine the uncoupled enzyme, which will, in turn, help in determining the % immobilization. Further, % Immobilization was calculated by using the equation-1

$$\% \text{ Immobilization} = \frac{(\text{Initial enzyme units} - \text{Unbounded enzyme units})}{\text{Initial enzyme units}} \times 100 \qquad (3.2)$$

Initial enzyme units refer to the total amount of enzyme units added, and unbounded enzyme units refer to the unbound enzyme units obtained from washes. The difference gives the total bounded enzyme units.

EDC-NHS cross-linking is a chemical method used to covalently link two molecules together, typically proteins or peptides, for various research and medical applications. EDC-NHS stands for "1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride" and "N-hydroxysuccinimide." These chemicals are commonly used in combination to facilitate the formation of stable amide bonds between amino groups (NH_2) and carboxyl groups (COOH) on molecules. EDC-NHS cross-linking is selective for primary amines and should be used with caution to avoid non-specific cross-linking. Additionally, the reaction is often carried out in a slightly acidic pH range (around pH 5.5 to 6.5) to maximize reaction efficiency. A graphical representation of the procedure for preparing EDC-NHS cross-linked sepharose beads and urease immobilization isrepresented in Figure 3.4.

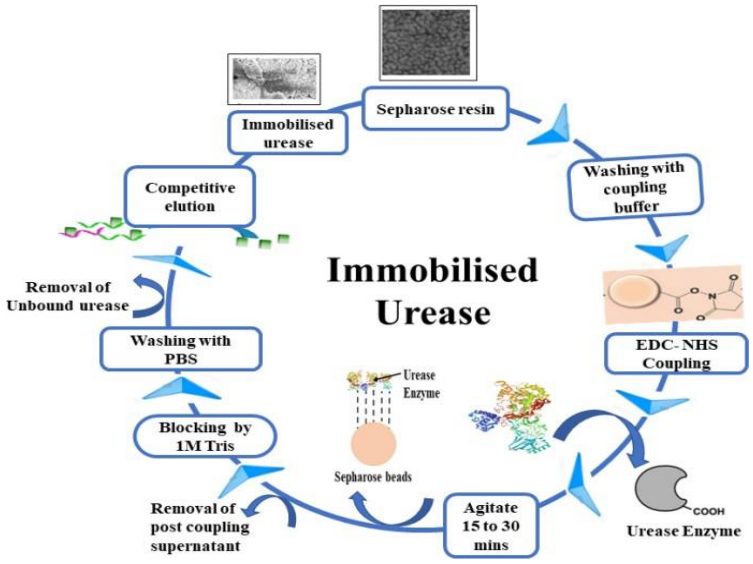


Figure 3.4. Immobilization process of urease enzyme using sepharose beads as the matrix. Activation of beads surface via EDC -NHS coupling. Immobilization of urease on activated sepharose beads.

3.6.2.3 Characterization

A Fourier transform infrared spectrum (Perkin Elmer FTIR model) was used to evaluate all modification stages done on sepharose beads, beginning before EDC-NHS and ending after EDC-NHS activation and urease immobilization. FESEM-EDX was used to visualize the morphological appearance of the manufactured sepharose beads before activation by EDC-NHS (Normal beads) and urease immobilised beads (Field Emission Scanning Electron Microscopy with Energy Dispersive X-Ray Spectroscopy). The investigated beads were covered in urease enzyme, and imaging using 20kV potentials was done at magnifications ranging from 10,000 to 40,000.

Instruments and Equipment

UV-Visible spectrophotometer (Model: Lab India 3000+), pH meter (Model: EuTech), Field Emission Scanning Electronic microscope (FE-SEM), Energy Dispersive X-Ray Spectroscopy (EDX).

3.6.2.4 Effect of pH and coupling time

The coupling reaction was performed at different pH and coupling times, viz. pH 7.0, 7.2, 7.5, for 15 mins and 30 mins. After the reaction, the beads were washed and checked for urease activity. Furthermore, urease activity analyses for free and encapsulated urease were carried out at pH 7.5.

3.6.2.5 Urease activity assay

The purpose of the urease activity assay is to investigate if the enzyme is still active after immobilization; hence, we have measured the urease activity using phenol–hypochlorite assay [150-151]. The ability of urease enzyme to promote urea hydrolysis in response to the following reaction was examined to better understand urease activity. Equation 2 describes the chemical reaction of urease [152-153].

$$(NH_2)_2CO + 2H_2O \longrightarrow CO_2 + H_2O + 2NH_3 \qquad (3.3)$$

Urea Water Carbon dioxide Ammonia

The proposed method uses phenol–hypochlorite assay for urease activity detection. The reaction contains 500 µl of sample and 500 µl of 50 mM urea. The reaction mixture was incubated at 37°C for 30 min in the water bath. The reaction was stopped by transferring 50 µl of the mixture to another tube containing 500 µl of phenol–sodium nitroprusside solution and 500 µl of alkaline hypochlorite solution, as reported in our previous publication [148]. After this, the solution was incubated at room temperature for 30 minutes. The absorbance of the colour complex was measured at 630 nm against a blank ina spectrophotometer. Obtained results were compared to a standard curve prepared with $(NH_4)_2SO_4$. [154] under the above assay conditions, Senthil *et al*. [143]. Calculate one unitof urease activity as the amount of enzyme liberating 1 mg of NH_3 from urea per minute [155] the residual urease units were estimated to obtain % Immobilization.

3.6.2.6 Stability study

An enzyme's stability is defined by its capacity to retain its active structural conformation despite disruptive forces, such as an increase in temperature, pH and, to some extent, by the presence of the substrate [156]. To increase the stability of enzymes, many research articles have immobilized the enzyme on a substrate [157], as the immobilized enzyme hasa higher resistance against thermal denaturation than the free enzyme [158]. Thus, in our research, a small amount of immobilized urease, i.e., 500 µl, was taken, and the activity assay was carried out using phenol–hypochlorite assays. Further, the solution was incubated at room temperature for 30 min, and the absorbance of the colour complex was measured at 630 nm against a blank in a spectrophotometer. The resin was washed with PBS [159] and stored at 4°C for further use. The same cycle was repeated with fresh immobilized urease after one week for one month.

3.6.2.7 Reusability study

Enzymes act as a catalyst which binds to a substrate and catalyzes the reaction. Further, the enzyme is released, unchanged, and can be used for another reaction. A small amount of im-mobilized urease was taken to examine the reusability of the immobilized urease, i.e., 500 µl, and the activity assay was carried out. The reaction with urea was completed, and the supernatant containing reaction products was separated and taken for further reaction and spectroscopic analysis. The resin was washed with PBS and stored at 4°C for further use. The same cycle was repeated with the stored immobilized urease until the activity was observed to be reduced. As can be observed, the activity slowly decreased upon completing the 6^{th} reuse cycle. Thus, enzyme immobilization, mainly through covalent bonding, is capable of maintaining the conformation of the three-dimensional structure of the protein macromolecules from being damaged or even significantly altered during the repeated performance of the enzyme.

3.6.3 Development of optical biosensor

This section will describe the development of optical biosensors, which consist of Milk sample preparation, Hydrolysis of urea using urease enzyme, Inhibition study of urease enzyme by Cadmium, Extraction of Cadmium from milk sample and finally, development and validation of optical biosensor. Figure 3.5 illustrates the different stages for development of optical biosensor for cadmium detection in milk.

3.6.3.1 Materials for optical biosensor development

The development of optical biosensors typically involves the selection of specific materials and components to construct a device capable of detecting biological molecules with high sensitivity and specificity. Here are some key materials and components commonly used in optical biosensor development: This section will describe the different components and materials used to develop the proposed optical biosensor. The list of components and chemicals as given below:

Immobilised urease, Phenol Red Indicator (0.1g phenol red ,0.1NaOH, ethanol), PBS (50 mM Phosphate, 150 mM NaCl, pH7.4), UV-Visible spectrophotometer Lab India 3000+), pH meter (EuTech), ICMP, the proposed biosensor kit including microprocessor, optical fiber,display, cuvette, light source and Power supply.

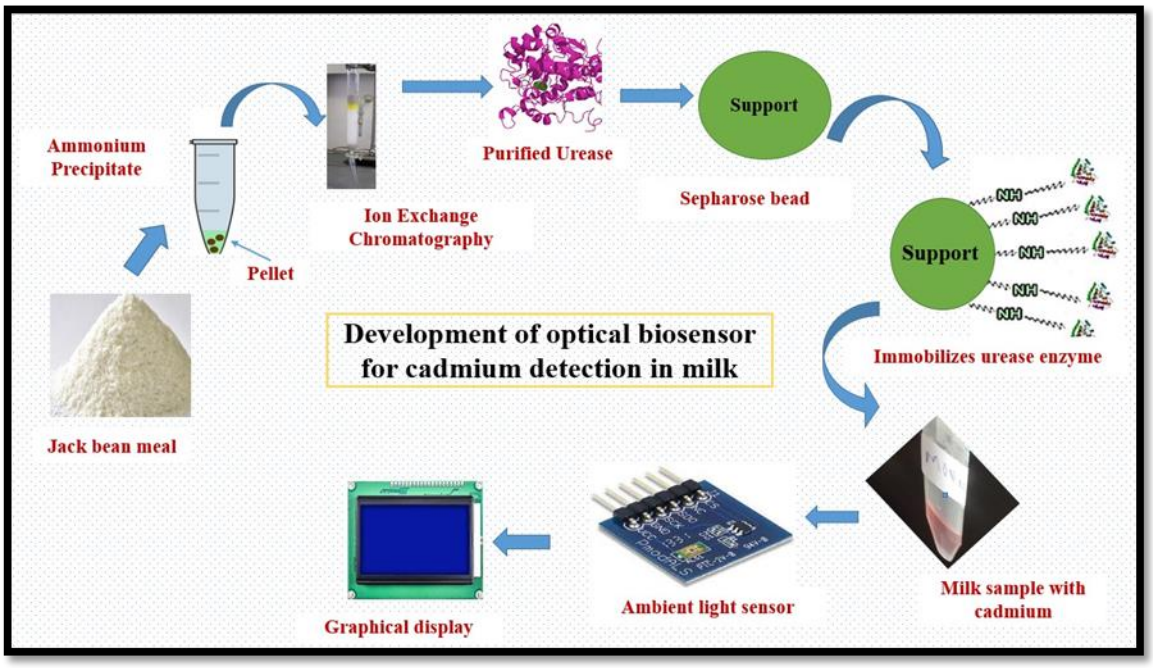


Figure 3.5 A schematic prototype of the proposed optical biosensor which represents different steps of extraction and purification of urease enzyme. Immobilization of enzymeon sepharose beads and detection of cadmium in milk sample by the developed optical biosensor.

3.6.3.2 Development and validation of proposed optical biosensor

The proposed research work is completed in three steps. The first step is spectrophotometric analysis of milk samples. In the second step the same milk samples are validated with ICP-MS. The final step includes development of Optical Biosensor and analyses of tested milk samples.

3.6.3.3 Hydrolysis of urea using urease enzyme

To perform hydrolysis of urea, 5ml of Immobilized urease slurry as reported in our previous research work by Varsha *et al*. [148] was taken in a 15ml tube. Storage phosphate buffer was removed after settling down the resin slurry to make 250ml of the bead. Varying concentrations of 0.5M urea (1ml to 4ml) were prepared in 50mM phosphate buffer pH 7.2, and 200µl of phenol red indicator was added to the resin. Similarly, the blank sample was prepared containing urea and phenol red indicator without immobilized urease. All solutions were incubated at RT for 30min. The absorbance of solutions was determined using U.V. spectrophotometer. The baseline was set with 2ml of phosphate buffer pH 7.2.

After the wavelength scan, 555 nm was found to be λmax therefore, the absorption was studied at 555 nm.

3.6.3.4 Inhibition study of urease enzyme by Cadmium

For the inhibition study of the urease enzyme by cadmium, 1 ml of immobilized urease slurry was taken in a 15 ml tube. Storage phosphate buffer was removed after settling down the resin slurry to make 500 µl of the bead. 1ml of different concentration of Cd (5µg/l to 10mg/l) was added and incubated at RT for 30min. After the reaction, time slurry was allowed to settle down, and the supernatant was collected and decanted. 2ml of urea and 200µl of phenol red indicator were added to the resin and incubated for 30min. The absorbance of all solutions was determined using the developed optical biosensor.

3.6.3.5 Extraction of Cadmium from milk sample

Acid extraction of Cd2+ from milk samples was achieved by adding 2-3 drops of HNO_3 acid in 10 ml of milk sample followed by centrifugation at 2000rpm for 30 min at 20°C. The supernatant was taken, and pH was brought back to pH 7.0. 1ml of milk extract was added to 1ml of urease and incubated for 30min. Further, 2ml of urea and 200µl of phenol red indicator was added to the resin and incubated for 30min. The reliability of the developed biosensor was checked with the spiked sample by the standard addition method. Spiking was made by adding 1ml of 10µg/l Cd to 1ml of acid-extracted milk sample and repeating the steps as explained for milk extract. The absorbance of all solutions was determined using the developed optical biosensor.

3.6.3.6 Development of optical biosensor

In this work, we have designed an optical biosensor suitable for On-field detection of the cadmium that must be portable and operator-independent. It works on the principle of absorption of light. Absorption of light transition in any media depends upon two assumptions a) Absorption of light transition is directly proportional to the concentration of the solution of the sample used for the experiments b) The absorbance is directly proportional to the length of the light path, which is equal to the width of the cuvette [160]. As per Beer-Lambert Law,

$$A=\epsilon lc \qquad \textbf{(3.4)}$$

where ‘A’ is absorption, ‘l’ is the length of the light path, ‘c’ is the concentration of the

solute, on and the constant 'ϵ' is called molar absorptivity or molar extinction coefficient and is a measure of the probability of the electronic transition. The molar extinction coefficient is not fixed as most protein extinction coefficients (ε %) range from 4.0 to 24.0. Therefore, although any given protein can vary significantly from ε percent = 10 %, the average for a mixture of many proteins will likely be approximately 10. The basic building block diagram of the proposed optical biosensor is shown in Figure 3.6.

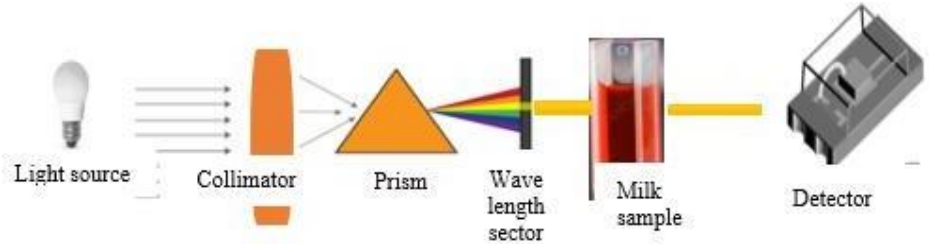


Figure 3.6 Basic block diagram of the proposed optical biosensor which is based on the principle of absorption of light and its measurement by the detector.

3.6.3.7 Practical Implementation

The proposed optical biosensor, cadmium specific, is designed to observe the behaviour of the urease enzyme when there is cadmium content inside the milk. The milk sample, which consists of cadmium, is kept under the observation of an ambient Light Sensor. Ambient light sensors play a crucial role in enhancing user convenience, energy efficiency, and the overall performance of optical biosensor. An ambient light sensor, also known as a light-dependent resistor (LDR), photoresistor, or photodetector, is an electronic component that is sensitive to the intensity of light in its surrounding environment. It is used in various applications to measure and respond to changes in ambient light levels. The ambientlight sensor (TEMT6000X01) is a silicon NPN epitaxial planar phototransistor in a miniature transparent 1206 package for surface mounting. It is sensitive to visible light, much like the human eye, and has a peak sensitivity of 570 nm. The ambient light sensor has a bandwidth range of 440 to 800 nm. Figure 3.7 depicts the schematic design of the optical biosensor, bandwidth range, and photographic representation of the actual sensor kit. Depending upon the colour composition of the sample under trial, the number of protons transferred from the light source to the ambient sensor is calibrated in terms of µg/lof cadmium content.

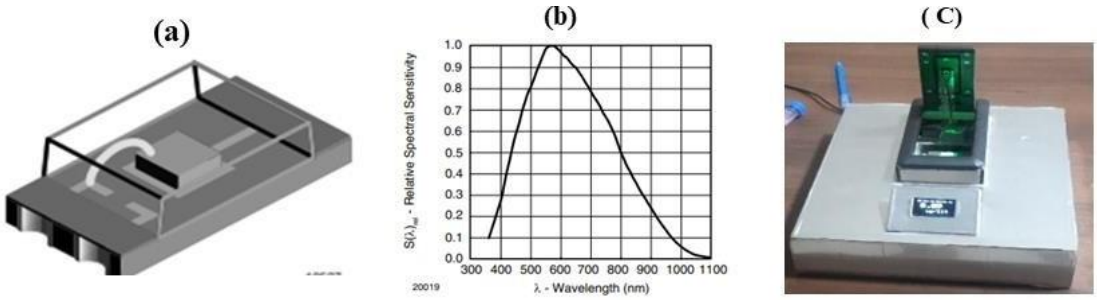


Figure 3.7 Schematic design of the optical biosensor (a) Physical view of sensor which the ambient light sensor (TEMT6000X01) (b) Bandwidth within the range of 440 to 800nm (c) Implementation view of the prosed biosensor.

Block diagram of the proposed optical biosensor is depicted in the Figure 3.8, which consists of five blocks: Ambient Light Sensor (TEMT6000X01), ATMEGA 328P Microcontroller (CPU), Ambient Light Source, Graphic LCD display and power supply block. The ambient sensor output is given to the o microcontroller for further calibration, and the final result is displayed with Graphics LCD. A 5V DC 500mA power supply drives the whole experimental setup.

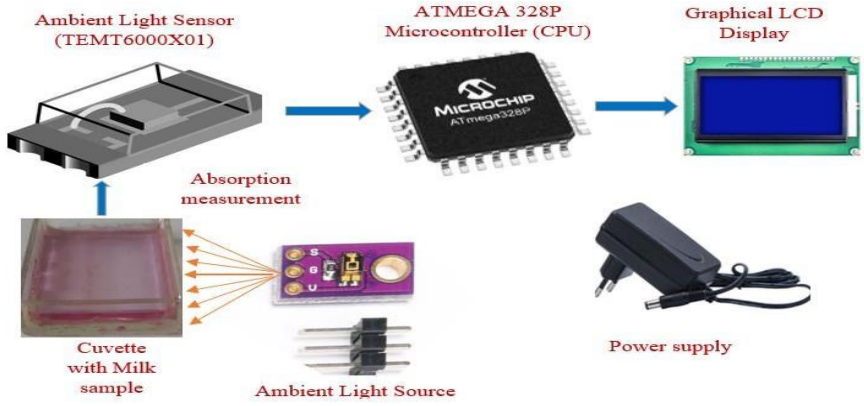


Figure 3.8 Block diagram and basic building blocks of the proposed optical biosensor which consists of five blocks: Ambient Light Sensor (TEMT6000X01), ATMEGA 328P Microcontroller (CPU), Ambient Light Source, Graphic LCD display and power supply.

3.6.3.8 Validation by Spectrophotometer

The absorbance of all solutions was determined using a U.V. spectrophotometer. The baseline was set with 2ml of phosphate buffer pH 7.2. After the wavelength scan, 555 nm

was found to be λmax. Therefore, the absorption was studied at 555 nm.

3.6.3.9 Validation by ICP-MS

(ICP-MS) is a fundamental study technology qualified to detect most of the periodic table of elements at milligram (mg) to nanogram (ng) levels per litre. In the proposed research, different milk samples are tested with this technique and results obtained from this method are used as standard readings for further calibration.

CHAPTER 4

Results and Discussion

4.1 Overview

This chapter elaborates on the experimental results and multiple extractions and purification processes used for urease enzyme extraction to achieve the proposed objectives. It also depicts the immobilization techniques used to retain the urease enzyme on the support. It emphasizes attention on the innovative approach used to detect cadmium in milk using a portable optical biosensor. These results are further validated with analytical methods, and the outcome of the proposed approach is discussed. This chapter is further divided into four subsections:

1. Outcomes analysis of extraction and purification of urease enzyme
2. Results analysis of immobilization of urease enzyme on the support
3. Result from analysis of detection of cadmium metal in milk sample using proposed Optical Biosensor
4. Validation results of the proposed optical biosensor using a Spectrophotometer and ICP-MS

4.2 Outcomes analysis of extraction and purification of urease enzyme

The proposed method extracts the urease enzyme from jack bean meal, which involves aqueous extraction, and salt precipitation followed by desalting resulting in concentrated target protein subjected to ion exchange purification. The eluent of this step consists of a purified fraction of the urease enzyme. Denaturating PAGE and activity assay has been used as an analytical tool to detect Urease post-purification steps. In step 1, the fractional acetone and ammonium precipitation have been performed to analyze urease precipitation after 40% and 60% for acetone and 40%, 60%, and 80% for ammonium concentration. Proteins were separated using 8% SDS PAGE stained with CBB staining under reducing conditions. Electropherograms describes the concentration phenomenon that occurred for urease after successive concentration of acetone and ammonium sulphate precipitation.

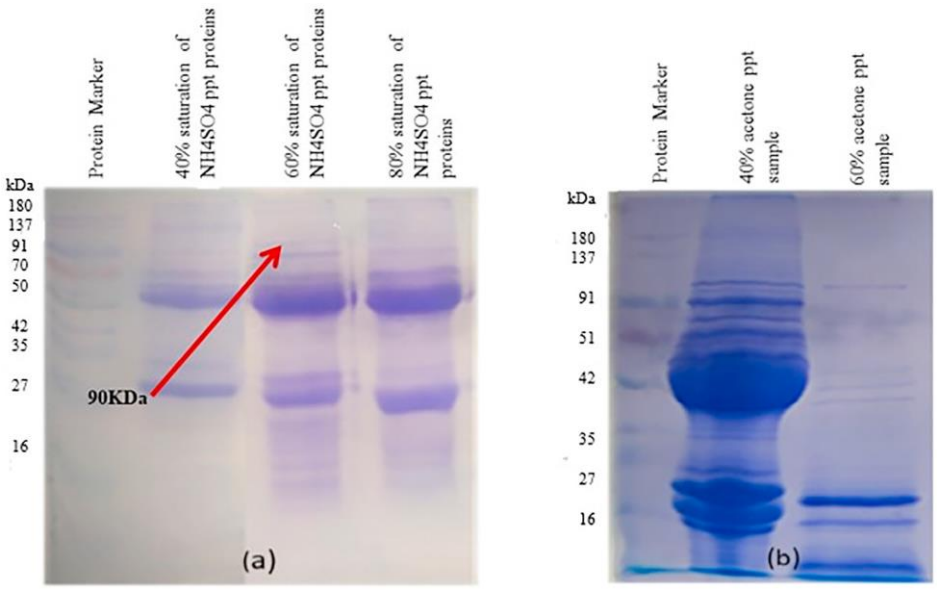


Figure 4.1 Schematic representation of analysis of urease after (a) ammonium sulphate and (b) acetone precipitation, showing band of urease with molecular weight of 90KDa at 60% solution of(NH4)2SO4 precipitated protein.

In Figure 4.1. (a), Lane 1 shows the prestained protein marker; lane 2, 3 and 4 contains 40%, 60% and 80% saturation of ammonium sulphate precipitated proteins. In Figure. 4.1. (b), Lane 1 shows the prestained protein marker, lane 2 and 3 show 40% and 60% of acetone precipitated samples. Compared to acetone, ammonium sulphate has precipitated and concentrated a significant amount of urease. The band's intensity corresponding to 90 kDa was significantly better than that of the post-acetone precipitation method; thus, effective extraction of urease from jack bean meal was achieved at 60% ammonium concentration. Similar results were obtained by Balasubramanian *et al*. [33], in which ammonium sulphate concentration has been considered optimum, which is further validated by the X-ray diffraction post acetone precipitation technique. In step 2, the precipitated sample is prepared for ion-exchange chromatography by desalting a dialysis membrane with a molecular weight cut of 10 kDa and 10 mM phosphate buffer (pH 8.0) as an exchange buffer. Among various process parameters of chromatographic purification, binding buffer pH is a critical parameter as the ionic charge density on the molecule changes as per the pH of the buffer. The current study focuses on the effect of binding buffer pH on the separation of urease from the jack bean meal. Thus, ion-exchange chromatography was carried out at pH 7, 7.5and 8.0 to purify urease extract. The total enzyme units and % purification is depicted in Table 4.1.

Table 4.1 Purification of urease extracts by anion exchange chromatography at pH 7.0, 7.5 and 8.0 showing highest purification of 99.71% obtained at pH8.0

Sr No	Samples	Total Enzyme Units		
		AEX at pH 7.0	AEX at pH 7.5	AEX at pH 8.0
1	Loading Sample	132.211	139.053	138.00
2	Flow through	19.930	26.947	29.930
3	Flow through binding buffer	9.404	16.772	8.800
4	Elution 1(50ml NaCl)	12.695	11.853	9.853
5	Elution 2 (100ml NaCl)	13.011	9.958	24.126
6	Elution 3 (150ml NaCl)	26.274	41.432	22.484
7	Elution 4 (250ml NaCl)	10.589	8.800	13.221
	Output Units	**91.902**	**106.140**	**137.6**
	% Purification	**69.51**	**76.33**	**99.71**

The urease was eluted by a step gradient of NaCl (50–250 mM) concentration, prepared in a 50 mM phosphate buffer pH 8.0. Maximum elution of enzyme units was observed at pH 8.0 with 99.71% purification of urease enzyme. Figure 4.2 depicts the elution profile of urease after AEX separation at different pH. As shown in Figure 4.2, elution 3 exhibited the highest ammonia release amongst all binding pH, which depicts urease's elution at 150 mM NaCl concentration. The urease binding was significantly higher at pH 8.0 than at near-neutral pH among three different pH studies.

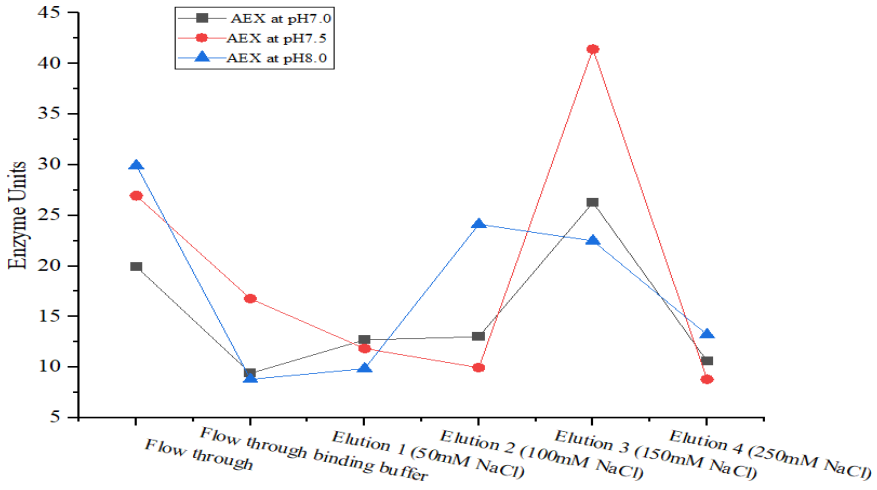


Figure 4.2. Graphical representation of elution profile of urease after AEX separation by a stepgradient of NaCl (50–250 mM) concentration at different pH

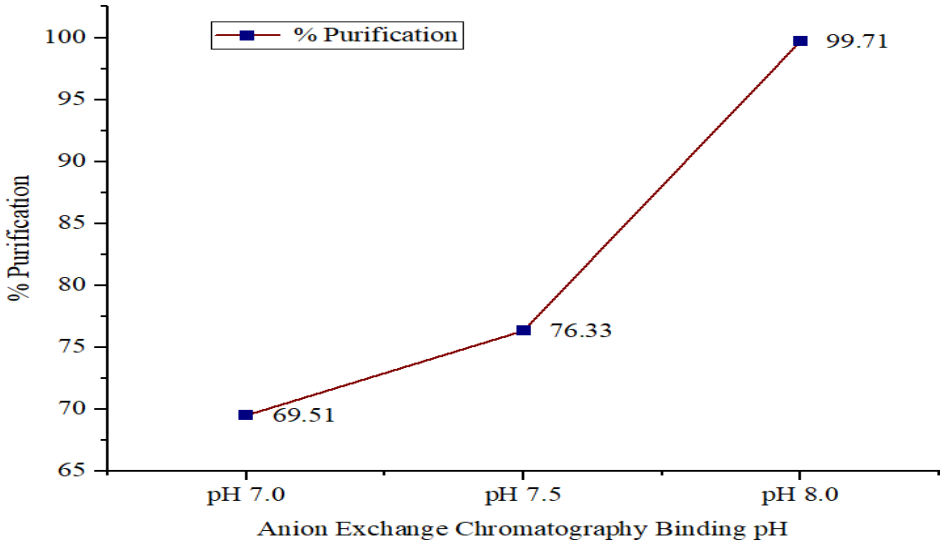


Figure 4.3 Percentage purification of elution profile of urease after AEX separation at different pH showing highest purification obtained at pH 8.0

The purification was effective and increased with increased pH towards the alkaline range, as seen in Figure 4.3. This phenomenon can be correlated with the increase in negative charge density on urease and depicting the amino acid content. The elution extracts (step 3) obtained after anion exchange chromatography at different pH were further used to perform an 8% SDS- PAGE analysis stained with CBB staining under reducing conditions. The extraction and purification of urease enzyme from a biological source can be a multi-step process involving several techniques to obtain a highly purified and active enzyme preparation. The choice of purification techniques may vary depending on the source of urease and the specific requirements of your research or application. Additionally, consider factors like pH, temperature, and enzyme stability during the purification process to maintain urease activity. SDS-PAGE is used for various purposes, including protein separation and quantification, assessment of protein purity, estimation of molecular weight. Monitoring protein expression and purification. The effect of pH on the purification of urease enzyme can be significant because urease activity is pH-dependent. Urease catalyzes the hydrolysis of urea to ammonia and carbon dioxide, and its activity is influenced by the pH of the surrounding environment.

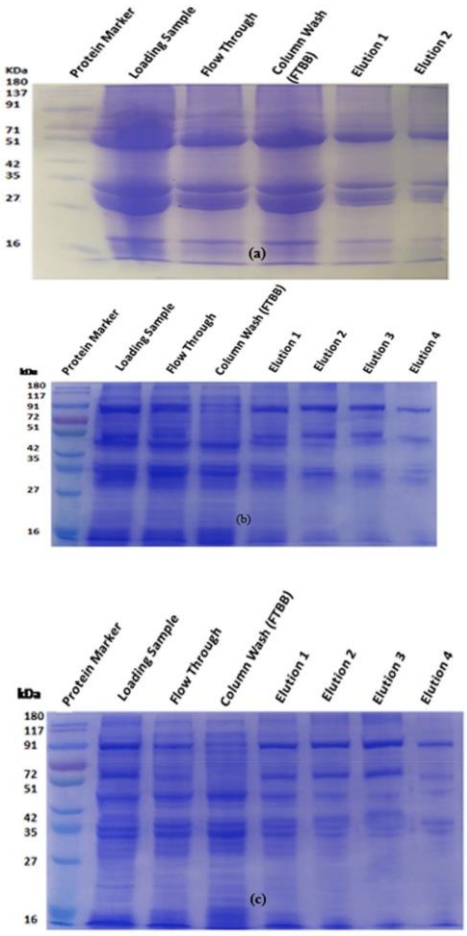


Figure 4.4 Schematic representation of purification of urease extracts using anion exchange chromatography at (a) pH 7.0, (b) pH 7.5, (c) pH 8.0.

Figure 4.4 describes the electropherograms of urease extracts using anion exchange chromatography at pH 7. 0. In Figure 4.4 (a), lanes 1 shows the prestained protein marker, lanes 2, 3, 4, 5, and 6 show the loading sample flow-through, column wash (FTBB), elution 1 and elution 2 samples respectively. In Figure 4.4, (b) lane 1 shows the prestained protein marker, lane 2, 3, 4, 5, 6, 7, and 8 shows the Loading sample flow-through, column wash (FTBB), elution 1, elution 2, elution 3, and elution 4 samples respectively. In Figure. 4.4 (c), lane 1 shows the prestained protein marker, lanes 2, 3, 4, 5, 6, 7 and 8 show the loading sample, flow-through, column wash (FTBB), elution 1, elution 2, elution 3, and elution 4 samples respectively. Lane 2 and lane 8, when compared for protein bands; fewer bands indicate the removal of nonspecific proteins from the jack bean protein mixture leaving behind purified urease observed at 90 kDa.

Hence Figure. 4.4 shows the AEX chromatography of urease at binding pH 7.0, 7.5 and

8.0, respectively, as urease bearing anionic charge at slightly alkaline pH. Hence, diethylaminoethane (DEAE) Sepharose has been used for the separation. As per the amino acid content of urease, the charge density on the protein at pH 8.0 is more. Thus, the binding to the anion exchanger is stronger than lower pH, and the ionic bonding to the column deciphers the specificity of chromatographic procedures. Higher % recovery indicates the specific binding of urease onto chosen chromatographic media at the pH value where urease bears a high charge density. The eluent ion concentration used in the chromatography is appropriate to desorb the bound urease off the column and aids in purification with significant specificity. Thus, the current chromatography procedure is an effective method for purifying urease from jack bean meal compared to literature methods. In step 4, the eluted fractions were assessed for urease activity using phenol-nitroprusside dependent ammonia release assay. Unit of ammonia released is proportionately equal to the unit activity of the urease enzyme. Enzyme activity assay are predominately performed to identify the presence or quantity of a specific enzyme in an organism, tissue, or sample. The different methods used to measure include spectrophotometry, fluorescence, and radiolabeling. In our research, urease activity determines phenol–hypochlorite assay as presented in Tables 2-5 [142]. In this assay, 100 µl of sample and 500 µl of 50 mM urea were incubated at 37 ◦C for 30 min in the water bath. 50 µl of this mixture was transferred to another tube containing 500 µl of Phenol – sodium nitroprusside solution and 500 µl of alkaline hypochlorite solution. Further, the solution was incubated at room temperature for 30 min, and the absorbance of the colour complex was measured at 630 nm against a blank in a spectrophotometer. The results obtained are depicted in Tables 4.2, 4.3 and 4.4. Urease activity detection using the phenol-hypochlorite method is a common biochemical assay for quantifying the activity of the enzyme urease. Urease is an enzyme that catalyzes the hydrolysis of urea to produce ammonia and carbon dioxide. This method is based on the measurement of the ammonia produced by urease activity and involves a colorimetric reaction. The absorbance reading obtained from the spectrophotometer is used to calculate the urease activity. A standard curve can be generated using known concentrations of ammonia or a urea standard to convert the absorbance reading into urease activity units (e.g., micromoles of ammonia produced per minute).

Table 4.2 Urease activity detection using phenol–hypochlorite using anion exchangechromatography at pH 7.0

Sr No	Samples	AEX at pH 7.0		
		Absorbance at 630nm	µM of Ammonia released	U/ml
1	Loading Sample	0.756	396.63	13.221
2	Flow through	0.116	59.79	1.993
3	Flow through binding buffer	0.056	28.21	0.940
4	Elution 1(50 mM NaCl)	0.123	63.47	2.116
5	Elution 2 (75 mM NaCl)	0.125	64.25	2.152
6	Elution 3 (100 mM NaCl)	0.126	65.05	2.168
7	Elution 4 (150 mM NaCl)	0.252	131.37	4.379
8	Elution 5 (250 mM NaCl)	0.103	52.95	1.765

Table 4.3 Urease activity detection using phenol – hypochlorite assay of urease extracts usinganion exchange chromatography at pH 7.5.

Sr No	Samples	AEX at pH 7.5		
		Absorbance at 630nm	µM of Ammonia released	U/mL
1	Loading Sample	0.795	417.16	13.905
2	Flow through	0.156	80.84	2.695
3	Flow through binding buffer	0.098	50.32	1.677
4	Elution 1(50 mM NaCl)	0.115	59.26	1.975
5	Elution 2 (75 mM NaCl)	0.139	71.89	2.396
6	Elution 3 (100 mM NaCl)	0.168	87.16	2.905
7	Elution 4 (150 mM NaCl)	0.097	49.79	1.660
8	Elution 5 (250 mM NaCl)	0.086	44.00	1.467

Table 4.4 Urease activity detection using phenol – hypochlorite assay of urease extracts usinganion exchange chromatography at pH 8.0

Sr No	Samples	AEX at pH 8.0		
		Absorbance at 630nm	µM of Ammonia released	U/mL
1	Loading Sample	0.79	414.53	13.82
2	Flow through	0.101	51.89	1.73
3	Flow through binding buffer	0.085	43.47	1.45
4	Elution 1(50 mM NaCl)	0.095	48.74	1.62
5	Elution 2 (75 mM NaCl)	0.08	40.84	1.36
6	Elution 3 (100 mM NaCl)	0.561	294.00	9.80
7	Elution 4 (150 mM NaCl)	0.26	135.58	4.52
8	Elution 5 (250 mM NaCl)	0.078	39.79	1.33

After comparison of urease activity at pH 7.0, 7.5 and 8.0, maximum urease activity was

obtained at pH 8.0 at a concentration of 100 mM NaCl concentration containing 9.80Units/ml of urease enzyme. Obtained results were compared to a standard curve prepared with Na_2HPO_4 presented in Table 4.5. The ammonia concentration was varied within a range of 50µM–500µM, and the absorbance obtained at 630 nm are depicted in Table 4.5

Table 4.5. Calibration of standard (NH4)2SO4 using phenol – hypochlorite assay showing Final ammonia concentration (µM) and absorbance at 630nm

Sr No	$(NH_4)_2 SO_4$ Standard (µL)	Final Ammonia concentration (µM)	Absorbance at 630nm
1	100	500	0.91
2	80	400	0.82
3	60	300	0.635
4	40	200	0.495
5	30	150	0.286
6	20	100	0.167
7	10	50	0.085
8	-	0	0

The increase in urease activity with the increase of ammonia concentration is a common phenomenon observed in enzymatic reactions involving urease. Urease is an enzyme that catalyzes the hydrolysis of urea into ammonia and carbon dioxide. the increase in urease activity with the increase of ammonia concentration is a consequence of the enzyme's function and the principles of chemical equilibrium. As ammonia concentration rises, the reaction equilibrium shifts in favor of ammonia production, promoting higher urease activity to maintain the equilibrium. This phenomenon is a key characteristic of the enzymatic hydrolysis of urea by urease. The increase of urease activity with the increase of ammonia concentration is graphically represented in Figure 4.5

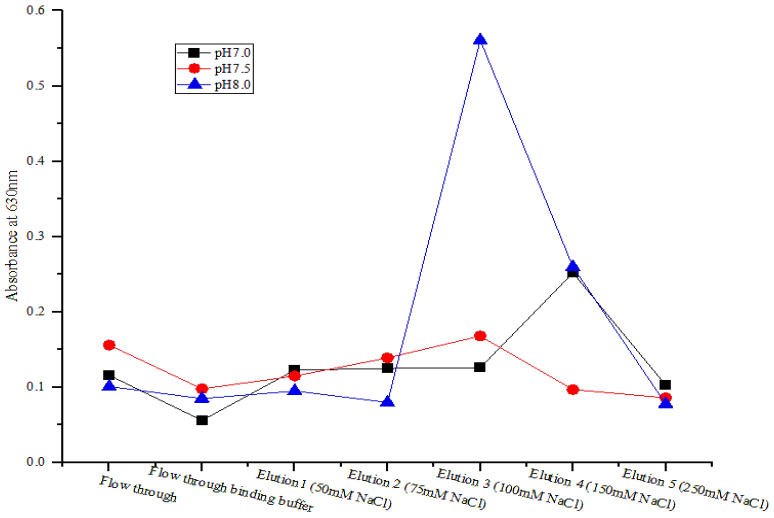


Figure 4. 5 Schematic representation of the variation of absorbance at 630nm at different pH of proposed sample.

The maximum value of absorbance at 630 nm is obtained at pH 8.0 where elution of urease was carried out at 100 mM NaCl concentration containing 9.80 units/ml of urease enzyme.

4.2.1 Specific activity and fold of purification of urease

Specific Enzyme Activity is the number of enzyme units per ml divided by the protein concentration in mg/ml. The fold of purification is calculated by measuring the ratio of specific activity before purification and after purification. Results are represented in Table 4. 6

Table 4.6. Protein concentration and activity analysis of urease showing specific activity and %Foldactivity of anion exchange purified urease.

Sr No	Samples	Protein mg/ml	Activity U/ml	Specific activity U/mg	% Fold
1	Crude	0.112	1.6	14.29	-
2	Ammonia precipitated	0.36	13.8	38.33	2.68
3	AEX purified	0.09	15.63	173.67	12.16

Thus, the activity of anion exchange purified urease in comparison with crude and ammonia precipitated urease is found to be highest is depicted in Figure. 4.6.

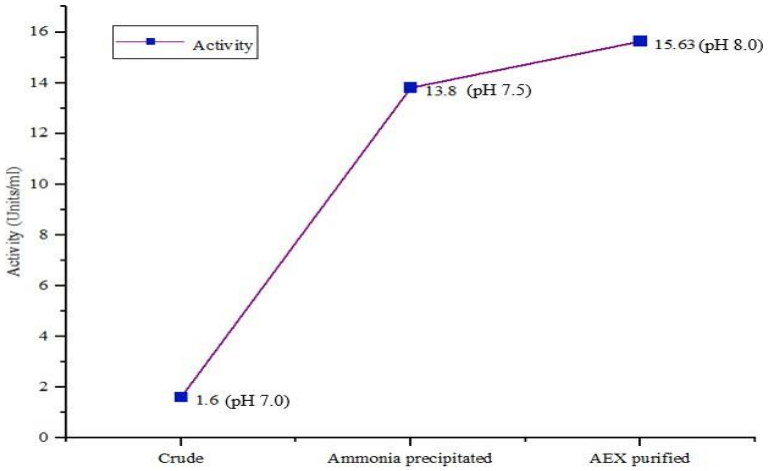


Figure 4.6 Comparison of urease activity of crude, ammonia precipitated, and AEX purified showing highest activity of anion exchange purified urease.

4.2.2 In-gel activity determination of urease by Zymography

The Zymography technique is a powerful tool for obtaining the orthogonal data that affirms the molecular weight and the enzyme activity [50,51]. The presence of purified and the pink-coloured band confirms active urease post-purification at 90 kDa. The presence of a band at the top of the gel corresponding to 180 kDa resembles the dimer of the Urease enzyme in its active form. SDS-PAGE (8%), activity stained under reducing conditions showing activity of purified urease post-anion exchange chromatography at binding pH 8.0. is shown in Figure 4. 7.

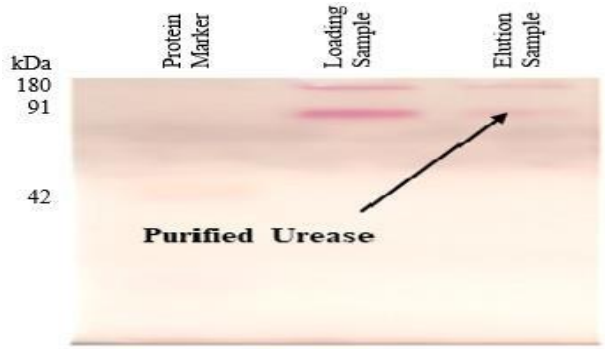


Figure 4.7 The activity of purified urease post-anion exchange chromatography at pH 8.0. The pink-colored band at 90 kDa confirms the presence of purified and active urease post-purification.

Lane 1 shows the prestained protein marker, and lanes 2 and 3 show the loading sample and elution samples, respectively. The presence of purified and active urease post-purification is confirmed by the pink-colored band at 90 kDa. The band at the top of the gel corresponding to 180 kDa resembles the dimer of the urease enzyme in its active form. In this proposed research, to address above mentioned issues, ammonium sulphate precipitation and anion exchange chromatography [146] are proposed to extract and purify urease enzymes from jack bean source. The merits of ammonium sulphate precipitation method are its excellent properties like readily available, low cost, highly soluble, relatively low density, and helps to stabilize protein structure. Various urease activity assays are recorded in the literature [130], but this article proposes phenol-hypochlorite assay to determine urease activity due to a simple and reliable analytical method. Correspondingly this method provides better stability at different pH and temperatures. The proposed method is comparable with different state-of-the-art methods. However, due to variations in techniques, input units, and experimental conditions, a direct comparison of the proposed method with other state-of-art methods is not possible.

This section compares the proposed method with other existing methods depicted in Table 4.7. Khodadadi *et al*. [30] proposed the acetone-precipitation method to extract urease enzyme from the jack bean source. SDS-PAGE [137] is used to analyze the urease's molecular weight and Nessler's reagent assay to determine urease activity [142]. This proposed combination resulted in a low percentage of purification (56%) and specific activity (117 U/mg). Our proposed method outperforms this in terms of a high recovery rate (99.71%) and specific activity (173.67 U/mg). From the above-obtained results, it is clear that the ammonium sulphate precipitation provides far better results than the acetone-precipitation method, and the phenol-hypochlorite assay resulted in high specific activity compared with Nessler's reagent assay. Another method directly comparable to our method is the Weber *et al*. [31] method used to extract and crystallize urease enzyme from jack bean source. The authors suggested three different precipitation methods for purification in this approach: Poly (Ethylene Glycol), Li_2SO_4, and NaCl as precipitates. The comparative

analysis of three methods shows that Poly (Ethylene Glycol) has provided the better specific activity of 160 units/mg. The fundamental limitation of this method is that the purification rate is not provided for comparison. To reduce time-consuming steps and purification costs, Tekiner *et al*. [32] proposed a novel method of immobilized metal affinity chromatography using Cu^{+2} chelated poly (hydroxyethyl methacrylate-N- methacryloyl-(L)-histidine methyl ester) [PHEMAHCu^{+2}]–based cryogenics investigationfor purification of urease enzyme. The specific activity of this method is processed with anammonia assay kit (Sigma, USA). This purification process has recorded 88% purificationbut consists of less specific activity of 8.82 units/mg. Zusfahair *et al*. [35] followed a similarapproach as proposed by Khodadadi *et al*. [30], in which acetone precipitation is used at different concentrations. Then urease activity was determined by Nessler's reagent assay. This method recorded 428.59 Units/mg specific activity, but electrophoresis analysis resulted in four polypeptides with a molecular weight of about 15, 17, 35, and 55 kDa, which is significantly less. The fundamental advantage of the proposed method is that it stands good in terms of percentage purification, specific activity and molecular weights atdifferent pH. Validation of molecular weight and activity of urease is performed using Zymography tool. The stability analysis at different temperatures is under study.

4.3 Immobilization of Urease Enzyme

The proposed research addresses a novel method of immobilizing urease using 1-Ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC), NHS crosslinking approach. In the proposed method, sepharose beads were activated EDC/NHS amine coupling by preparing the sample under test and blank, as depicted in Table 4.7.

Table 4.7 Surface activation of sepharose beads using 1-Ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC); and N-Hydroxy succinimide (NHS) . Coupling of Urease active sepharose beads and activity analysis of immobilized urease.

Sample	Sepharose Bead volume	EDC	NHS	Urease	Final volume	Activity (U/ml)
Test	1000 µl	2 mM	5 mM	1 mg/ml	2000 µl	154.17
Control	1000 µl	0	0	1 mg/ml	2000 µl	12.5

Further, the purified urease immobilization was performed at pH 7.0, 7.2 and 7.5 at 15 mins and 30 mins, as illustrated in Figure 4.8.

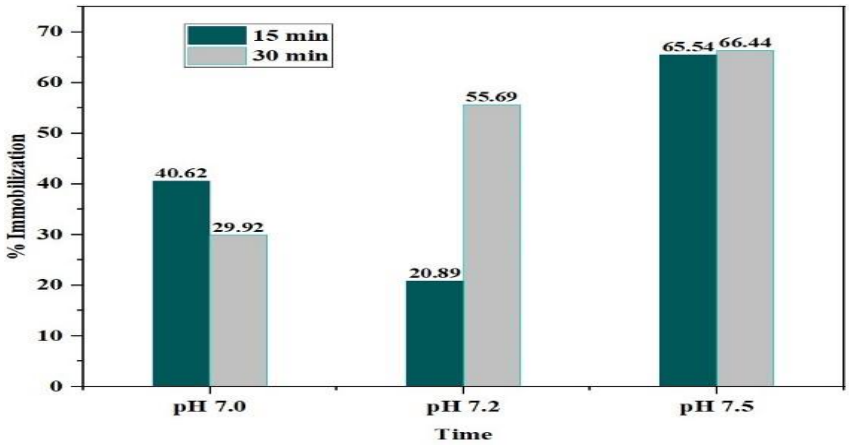


Figure 4.8 % Immobilization of purified urease and activated sepharose beads at pH (7.0, 7.2, 7.5) for 15 mins and 30 mins for optimum coupling of urease onto the matrix. Maximum % Immobilization is observed at pH 7.5 and duration of 30min. Beyond pH 7.5 the enzyme gets denatured and decrease the stability.

The effective immobilization was observed when the coupling conditions were slightly alkaline and longer coupling duration. Although EDC crosslinking is effective under acidic pH, incorporating NHS effectively conjugates with primary amines under slightly alkaline conditions. Not much significant difference in % immobilization is observed at pH 7.5. Thus, a shorter coupling time was selected as it provides an immediate immobilization product. The enzyme activity assay itself estimated the immobilization extent. Hence the values obtained were of active moieties immobilized on the surface.

4.3.1 Characterization of immobilized urease

Characterization of immobilized urease refers to the process of analyzing and assessing the properties and performance of urease enzymes that have been immobilized onto a solid support or within a matrix. Immobilization is often carried out to enhance the stability and reusability of enzymes in various biotechnological and industrial applications. Here are some common methods and parameters for characterizing immobilized urease: The immobilization of urease [161] was tested using FTIR spectroscopy using PerkinElmerFTIR model. The samples tested were before activation by EDC-NHS, & post-immobilization of urease, which is displayed in Figure 4.9.

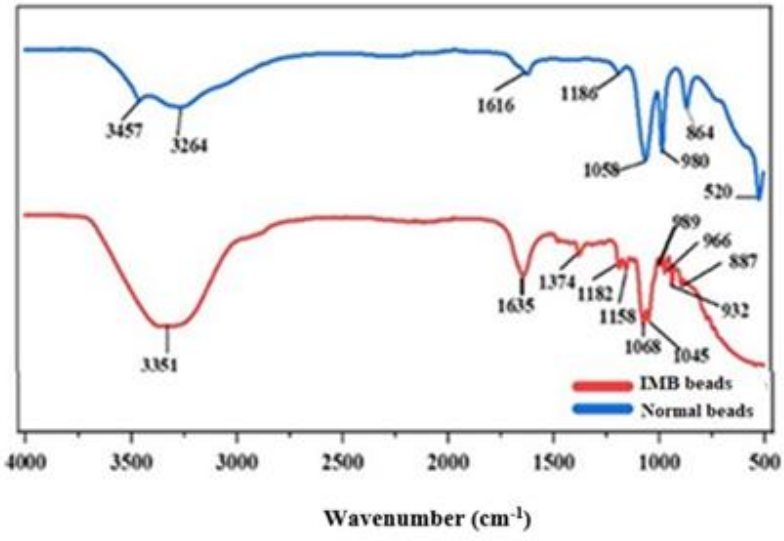


Figure 4.9 The Fourier Transform Infrared (FTIR) spectra of sepharose beads (a) Normal beads before activation by EDC-NHS; (b) Urease Immobilized on activated sepharose beads.

The binding of urease onto EDC-NHS-activated agarose particles was analyzed by FTIR spectroscopic analysis. Figure 4.9 represents the spectra of Normal beads and Immobilized urease on activated sepharose beads. The amide I and II regions, i.e., 1600 -1800 cm^{-1} and 1470-1570 cm^{-1}, respectively,in the spectra, were checked for the difference in the before and after immobilization of theurease. Amide I is the most intense absorption band in proteins. The peak at 1616 cm^{-1} is seen in the amide I region, primarily due to C=O stretching vibrations in combination within-plane NH bending vibrations of the peptide backbone, which is a random coil band. Asa protein moiety, urease consists of amide bonds absorbed in the IR regions. Amide A has more than 95% absorption due to the N-H stretching vibration. It has wave numbers between 3475 and 3150 cm^{-1} for hydrogen bond lengths between 2.69 to 2.85 Å amide. Peaks of Amide A are visible in the spectra at 3457 cm^{-1} and 3264 cm^{-1}, representing secondary amides. Amide II is located in the 1470 and 1570 cm^{-1} regions and is more complex than amide I. Amide II derives mainly from in-plane N-H bending (40-60% of thepotential energy). Amides III and V are very complex bands dependent on the details of theforce field, the nature of side chains and hydrogen bonding, as seen in the spectra with peaks obtained at 1058 cm^{-1} and 980 cm^{-1}. Therefore, these bands are only of limited use for extracting structural information [162].

4.3.2 Surface Morphology

It is the most powerful and efficient tool used for material characterization, as the dimensions of the material are sinking daily as we divert towards nanotechnology. It is helpful for the morphological studies of the samples like size and shape of the object, texture or pores, pores, etc. In this research work, we used SEM [163, 164] analysis to investigate and identify the morphology of the normal and immobilized beads. The SEM images of the immobilized beads before activation by EDC-NHS and post-immobilization of urease are presented in Figure. 4.10.

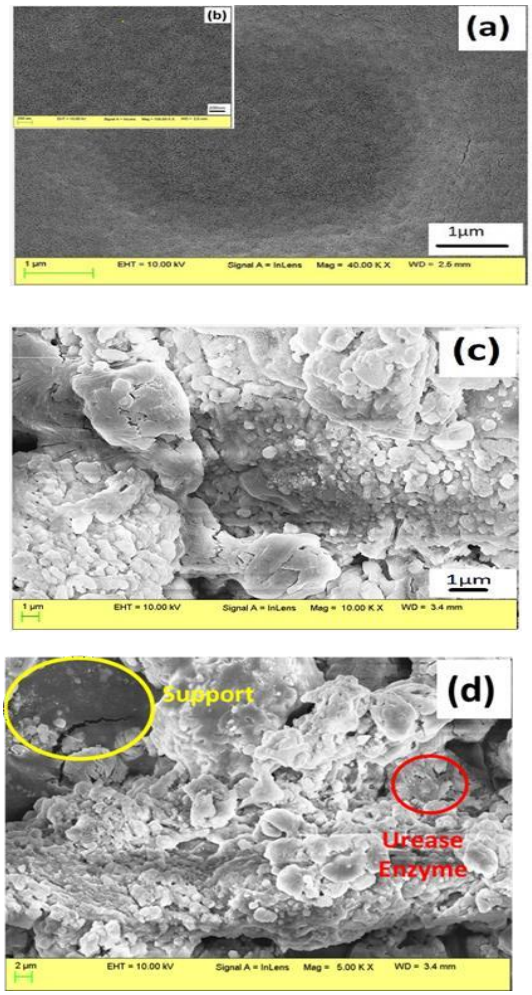
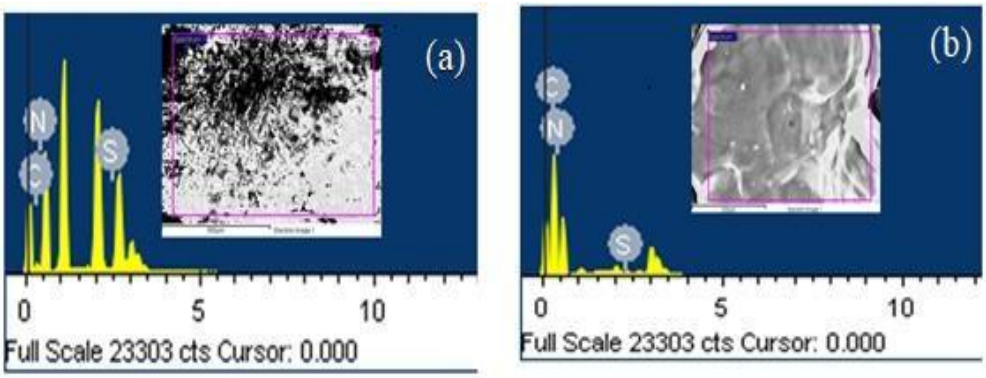


Figure 4.10. The SEM images of the immobilized beads (a, b) Normal sepharose beads before activation by 1-Ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC); N-Hydroxy succinimide (NHS); (c) Activated sepharose beads post-immobilization of urease (d) Immobilized urease enzyme on EDC-NHS activated Support (Sepharose beads) highlighted in red colour area is identified as a black colour shadow on white colour background of support.

As can be seen, the surface morphology of the examined beads exhibited apparent changes during the performance of each step. Figure. 4.10 (a), and Figure 4.10 (b) shows the normal beads. Morphology at 40.00 K X and 100.00 KX magnification respectively, indicating the used normal bead is highly smooth and homogeneous. The absence of troughs and valleys is necessary for the efficient immobilization of beads. At the same time, the morphological structure of the immobilized beads [165-166] at 10.00 K X magnification is clearly shown in the SEM images in Figure. 4.10 c. The following SEM images show the successful immobilization of the urease enzyme on sepharose beads material using EDC- NHS coupling. Figure. 4.10 d represents the surface morphology of the urease immobilized on the support. It clearly shows the surface of the support, which is not immobilized by the urease under study, whereas the urease enzyme uniformly immobilizes the remaining part of the support [167]. This demonstrates that the urease enzyme has been successfully immobilized.



Element	Weight%	Atomic%
C K	-11.27	-13.33
N K	112.09	113.69
S K	-0.82	-0.36
Totals	100.00	

Element	Weight%	Atomic%
C K	-240.58	-460.92
N K	342.08	562.00
S K	-1.51	-1.08
Totals	100.00	

Figure 4.11 The Energy Dispersive X-Ray Spectroscopy (EDX) spectra of (a) support Urease and (b) urease enzyme Immobilized on Support. The results of both spectrums shows that the weight % of C, N and S is increased after immobilization of the urease enzyme onto the beads.

EDX analysis was carried out to verify the surface immobilization of the urease enzyme onto the support. Different areas were focused during the EDX measurement, and the corresponding peaks are shown in Figure. 4.11. Figure 4.11 (a) represents Spectrum 1; the values were (-11.27, 112.09, and -0.82 measured in weight % and -13.33, 113.69 and -0.36 in atomic % for C, N and S, respectively, while Figure. 4.11(b)represents, Spectrum 2; the values were (-240.58, 342.08, and -1.51, measured in weight % and -460.92, 562 and -1.08 in atomic % for C, N and S, respectively. Comparing the results of both spectrums shows that the weight % of C, N and S is increased after immobilizing the urease enzyme onto the beads.

4.3.3 Stability study

The amount of activity that is maintained after storage is a crucial issue that could impact how well enzymes are applied. In order to determine the stability of immobilized urease, phenol-hypochlorite activity assay was used. In this test, 500 µl each of sample and 50 mM urea were incubated in a water bath at 37 ^{o} C for 30 minutes. 50 µl of this combination was transferred to another tube containing 500 µl of Phenol - sodium nitroprusside solution and 500 µl of alkaline hypochlorite solution. A spectrophotometer was used to compare the colour complex's absorbance at 630 nm to a control after the solution was incubated at room temperature for an additional 30 minutes. [168-169]. The outcomes are displayed in (Table 4.8).Thus, it is seen from (Figure 4.12) that the stability has been enhanced by urease immobilization on the redesigned solid support beads. After every seven days, the immobilized ureases by sepharose beads maintained approximately 66% of its initial activity and retain the activity to 67% after three weeks [170-171].

Table 4.8 Determination of Activity of Immobilized urease enzyme for stability study. Stability study of % Immobilized enzyme for four weeks at 37ºC for 30min. Every week the immobilized ureases by sepharose beads seems to maintain approximately 66% of its initial activity and retain the activity to 67% after three weeks.

Duration	Absorbance at 630nm	Total Units (U/mL)	% Immobilization
Initial Activity	0.48	158.75	69.23
Week 1	0.37	110.00	66.34
Week 2	0.35	105.33	67.80
Week 3	0.36	109.13	66.86
Week 4	0.36	106.04	67.43

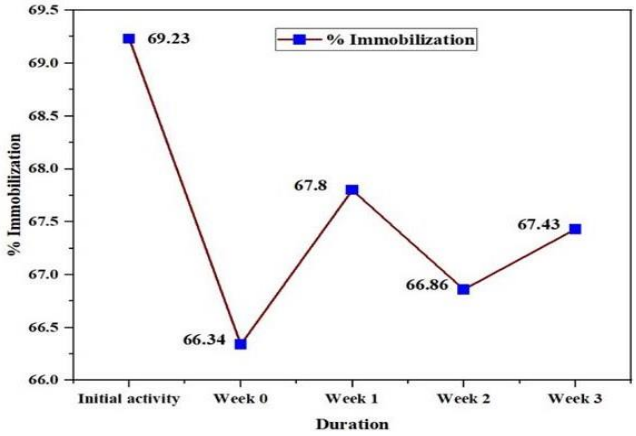


Figure 4.12 Stability study of % Immobilized enzyme for three weeks at 37oc for 30min. Every week the immobilized ureases by sepharose beads seems to maintain approximately 66% of its initial activity and retain the activity to 67% after three weeks.

It is observed from Figure 4.12 that urease immobilization onto the modified solid support beads has enhanced the stability. After every 7 days, the immobilized ureases by sepharose beads maintained approximately 66% of their initial activity and retained the activity to 67% after three weeks.

4.3.4 Reusability study

Immobilized beads have a property that makes it simple to remove them from the substrate, allowing the enzyme to be reused for additional processes. The results of the 15-minute reusability tests on the immobilized urease at pH 7.5 are shown in Table 4.9. The table shows that the activity was initially 69.3%, and that it gradually reduced after each use until it was minimal, or 0.98%, after six uses

Table 4.9 Determination of Activity of immobilized urease enzyme for reusability study. The reusability studies of the immobilized urease at pH 7.5 for 15 min. Initially the activity observed was 69.3% and after every use the activity slowly decreased to 0.98% after six uses.

Duration	Absorbance at 630nm	Total Units (U/mL)	% Immobilization
Initial Activity	0.48	158.75	69.3
Week 1	0.24	57.50	57.4
Week 2	0.19	35.00	34.9
Week 3	0.14	15.42	15.8
Week 4	0.11	4.17	4.3
Week 5	0.05	0.90	0.98

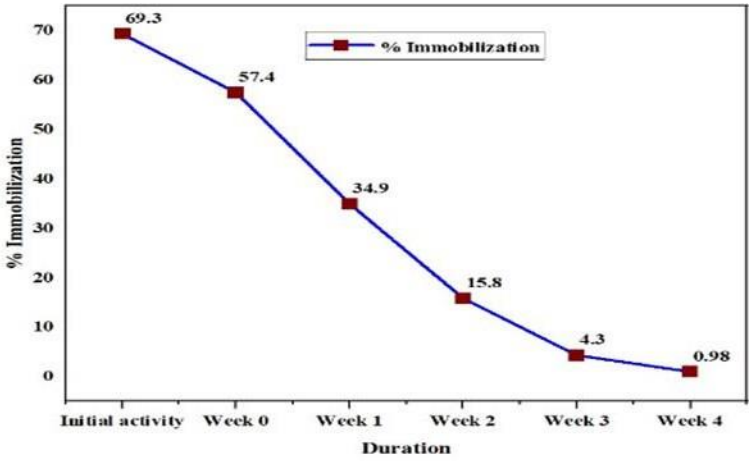


Figure 4.13 Reusability study of % Immobilized enzyme for six cycles at 37° C for 15min to investigate the activity units after six cycles which decreased from 69.3% to 0.98% after six cycles.

To investigate the stability, the time-dependent % variation of immobilization of urease enzyme is shown in Figure 4.13. As can be observed in Figure 4.13, initially, the activity observed was 69.3%, and the activity slowly decreased after every use, and there was negligible activity, i.e., 0.98% after six uses.

4.4 Result analysis of the proposed optical biosensor

The urease activity is the most crucial parameter in developing the proposed optical biosensor. The activity analysis of the urease enzyme (5ml) at different urea concentrations is performed at absorption at 555nm. Obtained readings reveal that urease activity varies linearly with different urea concentrations. The results of this experiment are highlighted in Table 4.10

Table 4.10 Urease activity on varying concentrations of urea varies in linearity. Absorbance increases as the concentration of urea increases.

Sr No	Concentration of Urea in ml	1ml	2ml	3ml	4ml
		Absorbance at 555nm			
1	Blank	0.26	---	---	---
2	Sample	0.34	0.52	0.63	0.70

Enzyme inhibition studies are an important area of biosensor research since these investigations have found biosensors which is helpful in various food industry applications. Inhibitors of urease can be broadly divided into two major classes: Active site-directed

(substrate-like) and Mechanism-based directed. In the proposed research work, we have used an active site-directed substrate, i.e., cadmium, to perform an enzyme inhibition study. Results of enzyme inhibition study of proposed research is presented in Table 4.11.

Table 4.11 % Inhibition of urease enzyme by Cadmium spectrophotometrically. As theconcentration of cadmium in in milk samples increases, %Inhibition also increases in linearity.

Sr. No	Samples	Absorbance at 555nm	Blank - Sample	%Inhibition
1	Blank	0.1246	---	----
2	Raw Cow milk (R.C)	0.5769	0.4523	21.59
3	Packed Buffalo milk (W.B)	0.5659	0.4413	22.01
4	Packed Cow milk (A.C)	0.5666	0..4420	21.99
5	Packed Buffalo milk (G.B)	0.5711	0.4465	21.81
6	Packed Cow milk (G.C)	0.5702	0.4456	21.85
7	Packed Cow milk (W.C)	0.4792	0.3546	26.00
8	Spiked-packed Buffalo milk (G.B.M)	0.4248	0.3002	29.33
9	Raw Buffalo milk (R.B)	0.4002	0.2756	31.13
10	Spiked Raw milk (R.C.M))	0.3791	0.2545	32.86

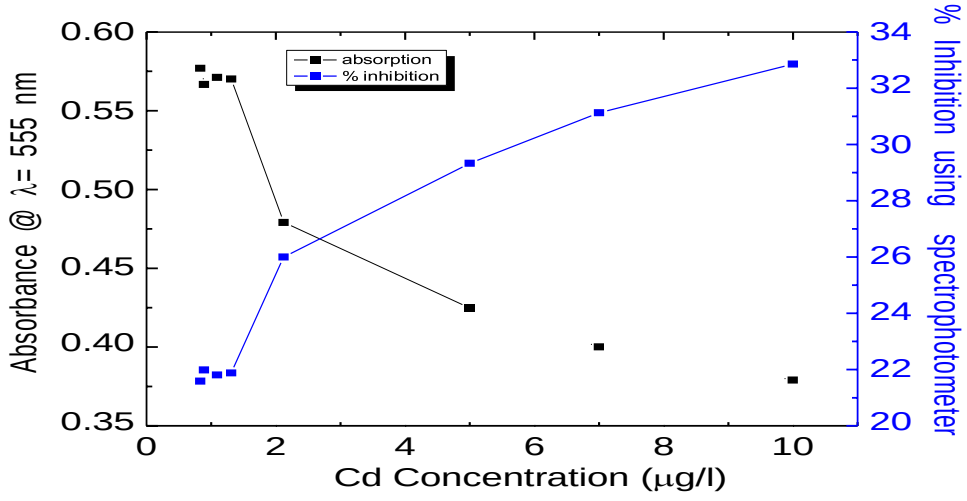


Figure 4.14 Absorption and Inhibition variation with respect to the cadmium concentration. It is seen that as the %Inhibition increases as the cadmium concentration increase above 2 µg/l.

The graphical representation of variation in the absorbance at 555nm and % Inhibition with Cd concentration is shown in Figure 4.14. Figure 4.14 clearly indicates that as the change in the absorbance at 555nm at low Cd concentration is slightly changed but above the Cd concentration of 2 µg/l it decreases rapidly. Also, it can be observed that the % Inhibition is saturated at low Cd concentration but it increases rapidly when the Cd concentration goes above 2 µg/l.

The percentage Inhibition was calculated using the following formula:

$$\% \text{ Inhibition} = \frac{(\text{Control OD} - \text{Sample OD})}{\text{Control OD}} \times 100 \quad \textbf{(4.1)}$$

where Control OD is absorbance of negative control and Sample OD is absorbance of test sample Absorption and inhibition study shows that as cadmium concentration increases, absorption decreases while % inhibition increases. These results indicates that there is a linear relation between all the quantities and these promising results will further be helpful to develop the optical biosensor.

Table 4.12 Validation results for Cadmium concentration using ICP-MS for varied milk samples under test and spiked milk samples with cadmium.

Sr. No	Samples	Cadmium conc. by ICP-MS (µg/l)
1	Raw Cow milk (R.C)	0.83
2	Packed Buffalo milk (W.B)	0.88
3	Packed Cow milk (A.C)	0.89
4	Packed Buffalo milk (G.B)	1.09
5	Packed Cow milk (G.C)	1.31
6	Packed Cow milk (W.C)	2.12
7	Spiked packed Buffalo milk (G.B.M)	5.00
8	Raw Buffalo milk (R.B)	7.57
9	Spiked Raw milk (R.C.M))	10.00

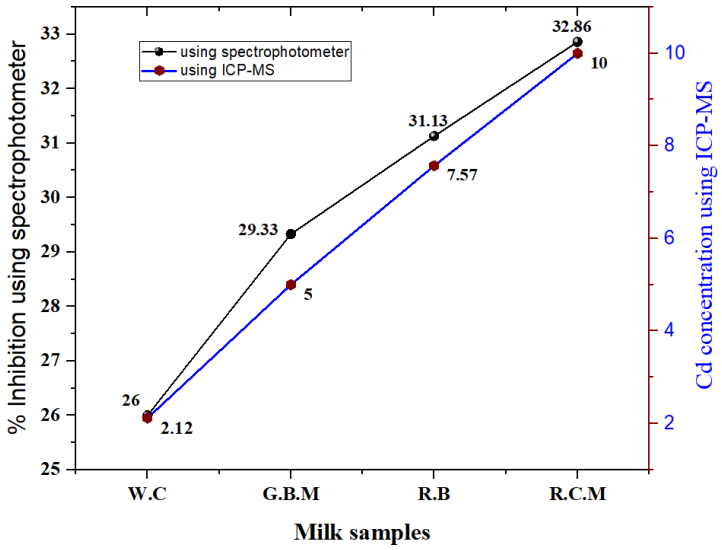


Figure 4.15 shows the calibration curve of cadmium concentration by ICPMS vs % Inhibition using Spectrophotometer. From the figure linearity is observed for Cd concentration and %Inhibition.

The variation in the Cd concentration determined by ICPMS vs % Inhibition using a spectrophotometer extracted from various milk samples viz W.C, G.B.M, R.B and R.C.M is shown in Figure 4.15. These measurements indicate that the lowest concentration of Cd is obtained for the W.C sample, and the highest Cd concentration is obtained for the R.C.M sample. Overall, it can be observed that the Cd concentration determined by ICP-MS vs % inhibition using a spectrophotometer extracted from various milk samples viz W.C, G.B.M, R.B and R.C.M has a linear trend, and both techniques show agreement with each other. These readings indicate that if the sensor is built based on spectroscopic measurements, then it could easily detect Cd.

Inhibition readings are further used to calibrate the optical biosensor. The proposed optical biosensor is initially calibrated with % Inhibition readings of a spectrophotometer and then tested with milk samples readings of optical biosensors are highlighted in Table 4.13

Table 4.13 Validation results for Cadmium concentration using an optical sensor showing absorbance at 555nm and % Accuracy.

Sr. No	Samples	Cadmium conc. by Optical sensor (µg/l)	Absorbance at 555nm	% Accuracy
1	Raw Cow milk (R.C)	--	0.4523	--
2	Packed Buffalo milk (W.B)	---	0.4413	--
3	Packed Cow milk (A.C)	----	0..4420	---
4	Packed Buffalo milk (G.B)	-----	0.4465	---
5	Packed Cow milk (G.C)	-------	0.4456	---
6	Packed Cow milk (W.C)	-----	0.3546	---
7	Spiked packed Buffalo milk (G.B.M) Cd (5 µg/l)	4.80	0.3002	±0.04
8	Raw Buffalo milk (R.B)	7.78	0.2756	±0.027
9	Spiked Raw milk (R.C.M) Cd (10 µg/l)	9.82	0.2545	0.018

Validation of the proposed optical biosensor is done with standard readings of ICP-MS and % accuracy is calculated by the equation 4.2

$$\% \text{ Accuracy} = \frac{\text{Standard reading} - \text{Actual reading}}{\text{Standard reading}} \times 100 \qquad (4.2)$$

The maximum permissible error of optical biosensor is observed in a range of ±0.04

4.4.3 Merits, Limitations and future scope of proposed optical biosensor

During the development of the proposed optical biosensor following are some observations recorded that need to be addressed in future. The fundamental advantage of the proposed research work is it has achieved a lower detection limit as per heightened in Table-6 which is 4.8 µg/L which comes under the toxic range. To reduce the detection limit of less than 4.8 µg/L, modification in the cuvette's light source and work environment is under process and part of future research scope. The stability of immobilized urease enzyme concerning

time duration and temperature is another concern for precisely calibrating the proposed optical biosensor. The cost and complicated procedure of urease extraction and purification is another concern of design needed to be tackled. Our future research direction is to address the abovementioned issues to develop a precise optical biosensor suitable for on-field applications.

Table 4.14. Quantitative analysis of optical detection methods for cadmium detection representingLinear range of detection and biocomponent used.

Reference	**Year**	**Country**	**Milk type**	**Range of detection. (µg/l.)**	**Biocomponent**
Verma *et al.* [115]	2010	India	Raw milk	8.1 Linear Range -0.1 to 10	*Bacillus badius isolate*
Kumar *et al.*[116]	2017	India	Raw milk	10 Linear Range -10-50	Escherichia coli (pAD123) and Escherichia coli DH5α.
Jegadesan *et al*[3]	2018	India	Raw milk	10	----
Proposed method	**2022**	**India**	**Raw and packed milk**	**4.80**	**Urease enzyme with phenol red indicator**

CHAPTER 5

Conclusion and Future Perspectives

5.1 Overview

In summary, the primary objective behind this study is to investigate novel methods for detecting Cadmium metals in milk samples. This study presents many novel approaches in the research study as mentioned below:

1. A novel approach of extraction and purification techniques to achieve maximum % purification and highest specific activity of urease enzyme.
2. A novel approach of immobilization using an EDC/NHS covalent bonding approach
3. A novel approach for designing and developing portable Optical biosensors.
4. This section describes the conclusion and future perspectives of the proposed research work.

5.2 Conclusion

Urease enzymes were extracted from the jack bean meal and treated with the ammonium sulphate precipitation method. The molecular weight of extracted urease was found to be 91 kDa with SDS-PAGE support, and the results were validated with Zymography. The specific activity of the extracted enzyme is found to be 173.67 Units/mg. The desired protein was separated with anion exchange chromatography and obtained a purity of about 99.71%. The current process yields a significant amount of urease compared to other methods available in the literature with 12 folds purification after a single step of chromatography. The orthogonally has been established for the urease enzyme activity with the help of enzyme activity assay and Zymography analysis.

The main objective of this research is to Investigating an efficient approach for immobilizing urease enzymes that is also appropriate for many commercial applications is the main goal of this research. An advanced EDC-mediated surface functionalization approach for covalent immobilizing urease has been optimized to a porous matrix to achieve this task. The urease enzyme was immobilized onto sepharose beads using EDC-NHS coupling in this proposed method. This method maintained approximately 66% of its

initial activity and retained the activity to 67% after three weeks. The characterization of immobilized beads was conducted using FTIR. In the spectra, the amide I and II regions, i.e., 1600 - 1800 cm-1 and 1470 - 1570 cm-1, respectively, were analyzed to evaluate the difference in functional groups present before and after immobilization of the urease. Surface imaging analysis also validated the successful immobilization of the urease enzyme on sepharose beads material using EDC- NHS coupling. Comparing the results obtained via EDX out-comes analysis, it is observed that the weight % of C, N and S is increased after immobilizing the urease enzyme onto the beads. Overall, an actively immobilized urease was obtained using EDC-NHS crosslinking approach, which acts as a rapid and effective immobilization protocol. A minimal literature has been found about EDC-NHS coupling of enzymes onto porous surfaces, hence acting as a novel approach for immobilizing urease enzymes.

Optical biosensors are predicted to have significant research applications in diverse fields like healthcare, food industry and biomedical engineering as they can provide a new analytical tool with miniature size to test various samples for diverse parameters. In the proposed research work, the extracted protein from the jack bean meal is purified by anion exchange chromatography. EDC-NHS coupling with sepharose beads is used to immobilize the urease enzyme. An inhibition study of immobilized urease enzyme is performed in urea and phenol red indicator assay. Spectrophotometer absorption level and Inhibition study are used to calibrate the proposed optical biosensor. The proposed optical biosensor is successfully applied to acid-extracted milk samples. To the best of our knowledge, our proposed optical biosensor shows the lowest limit of Cd concentration available in the literature. The lowest Cd detection limit achieved was 4.0 µg/L with an accuracy of ± 0.04%. The linear range of detection of Cd concentration lies in the field of 4.8µg - 10 µg/L. The biosensor stored at 4^{o} C can detect Cd even after 35 days indicating its excellentsensing stability, which is a bottleneck for the proposed biosensor. However, our future efforts of specific modification will reduce the proposed biosensor's lower detection limit to be suitable for the on-field detection of Cd ions in milk samples.

5.3 Future perspectives

Since minimal literature has been found about EDC-NHS coupling of enzymes onto porous surfaces, hence acting as a novel approach for immobilizing urease enzymes. Due to the encouraging results regarding the specific activity and % purification of extracted urease, the proposed method is suitable for extracting urease enzymes in various industrial applications. If combined with Optoelectric-magnetic nano-systems, the proposed optimized methods can be further adopted to design and fabricate efficient biosensors for detecting urea in physiological and low-level clinical settings. The proposed work is undoubtedly helpful in addressing the issues mentioned above. During the development of the proposed optical biosensor, the following are some limitations recorded that need to be addressed in the future. The stability of immobilized urease enzyme life and temperature has remained another concern for the precise calibration of the proposed optical biosensor. The cost and complicated procedure of urease extraction and purification are added design concerns that need to be tackled. Finally, the following research work is proposed as part of future research scope. To further reduce the detection limit of less than 4.8 µg/L, modification in the cuvette's light source and work environment will be carried out in detail. Our future research direction will not only address the abovementioned issues but also will come up with the development of a precise optical biosensor suitable for on-field applications.

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List of Publications from this Research work

1) Pawar, V. S**,** Bhande, D., Pawar, S. D., Mudila, H., Kaushik, A., & Kumar, A. Investigating purification and activity analysis of urease enzyme extracted from jack bean source: A green chemistry approach. Analytical Biochemistry, 659, (2022)., 114925. https://doi.org/10.1016/j.ab.2022.114925
2) Pawar, V.S., Pardhi, V., Pawar, S.D, Mudila, H., Kaushik, A., & Kumar, A, Development of Low-Cost Optical Biosensor for the Detection of Cadmium Ions in Milk, International Journal of Dairy Technology, Wiley Publication, (2023), Under Review
3) Pawar, V.S., Pawar S.D, Mudila, H., Kaushik, A., S.D, & Kumar, A, Advances from conventional to real time detection of cadmium ions in milk using biosensor techniques, Journal of Biosensors and Bioelectronics, (2023), Under Review.
4) Copyright Granted by IPR for the proposed research work 'Development of Optical Biosensor for the Detection of Cadmium ions in Milk' with Reg No L-123416/2023

List of Other Publications

1) Sood, Y., Pawar, V. S., Mudila, H., & Kumar, A., A review on synthetic strategies and gas sensing approach for polypyrrole-based hybrid nanocomposites. Polymer Engineering & Science, 61(12), (2021),2949-2973, https://doi.org/10.1002/pen.25822
2) Sood, Y., Pawar, V. S., Mudila, H., Influence of Polypyrrole Morphology on Gas Sensing. In Journal of Physics: Conference Series (Vol. 2267, No. 1, p. 012050). IOP Publishing, (2022), https://doi.org/ 10.1088/1742-6596/2267/1/012050.

Conference Publications

1) Pawar, V., Verma, N., & Pawar, S. Overview of Different Milk and Milk Products Contaminant Detections by Biosensors. In Proceedings of the 3rd International Conference on Advances in Science & Technology (ICAST) (2020), https://dx.doi.org/10.2139/ssrn.3571717
2) Pawar Varsha, Viraj Mankar, Shivaji D Pawar, Technical Advancement of Cadmium and Lead Detection Methods and Future Scope for Biosensor in Diary Industry: An Overview. First Online International Conference on "Continuity, Consistency and Innovation in Applied Sciences and Humanities'(ICCIASH), ISSN: 2249-6661. Vol-43, (2020), No.-04 (IV).