Immobilization of enzyme in Metal Organic Frameworks (MOFs) for enhanced activity, stability, and reusability

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CERTIFICATE

This is certified that the thesis entitled "Immobilization of enzyme in Metal Organic Frameworks (MOFs) for enhanced activity, stability and reusability" submitted by Tanu Priya Sood (Roll No. 302201016), a postgraduate student of the Department of Biotechnology in partial fulfillment for the award of the degree of Masters of Science at Thapar Institute of Engineering and Technology, Patiala, Punjab 147004, India, is a record of student's own work carried out under my supervision and guidance. This report has not been submitted for the award of any other degree or certificate in this institute or any other university or institute.

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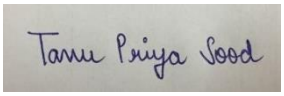
DECLARATION

I hereby declare that the work presented in the dissertation entitled "Immobilization of enzymes in Metal Organic Frameworks (MOFs) for enhanced activity, stability and reusability" in partial fulfilment of the requirement for the award of the degree of Master of Science in Biotechnology, Department of Biotechnology, Thapar Institute of Engineering and Technology (TIET) Deemed to be University, Patiala, is a genuine record of my own work during the period from January 2024 to June 2024, under the supervision of Dr. Jyotsana Mehta, Department of Biotechnology, TIET. The matter incorporated in this thesis has not been submitted to any other university or institute for the award of any degree in India or abroad.



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DEDICATION

I may not have enough words to express my gratitude to my Late grandma, Smt. Pushpa Devi Sood who has always been a source of strength for me. In honor of my dear grandmother, whose love sustained and guided me throughout my life I dedicate this thesis to her, whose everlasting love, wisdom, and support always provided me with fortitude and motivation throughout my academic pursuit. She has inspired me with her tales of perseverance and endurance, showing me the importance of dedication and hard work. Her unwavering support has helped me through both my life's setbacks and successes. There are no words to express how much I appreciate all that she has done.

ABBREVIATIONS

PXRD	Powder X-Ray Diffraction
FTIR	Fourier Transform Infrared Spectroscopy
DLS	Dynamic Light Scattering
SEM	Scanning Electron Microscope
ZIF-8	Zeolitic imidazolate framework-8
MOF	Metal organic framework
BET	Brunauer-Emmet-Teller
UV-Vis	Ultraviolet-Visible Spectrophotometry
MeIm	Methyl imidazole
HRP	Horseradish peroxidase
H_2O_2	Hydrogen Peroxide
TMB	3,3',5,5' – Tetramethylbenzadine
ABTS	2,2'- Azino-bis (3-ethylbenzothiazoline -6- sulfonic acid)
BSA	Bovine Serum Albumin
BCA	Bicinchoninic Acid
pH	Potential of Hydrogen
µg	Microgram
µl	Microliter
ml	Milliliter
mg	Milligram
nm	Nanometer
mU/ml	Milliunits/ml
MOI	Method of immobilization

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ABSTRACT

For a long time, enzymes have been exploited as catalysts for diverse processes. Despite the numerous advantages of biocatalysis, the utilization of enzymes for catalyzing processes necessitates certain environmental conditions that are sometimes incompatible with the structure and function of enzymes. Nevertheless, these challenges might be surmounted via enzyme immobilization onto solid substrates. Immobilization can enhance the stability, biocatalytic activity, and repeated usability of enzymes, leading to improved performance and increased commercial feasibility. Metal organic Frameworks (MOFs) have emerged as efficient substrates for immobilizing enzymes, attributed to their high surface area, porosity, stability, biocompatibility, and capability of providing micro-protective environment for enzymes. In this work, Zeolitic imidazolate framework-8 (ZIF-8) MOF was employed for *in-situ* immobilization of commercially viable horse radish peroxidase (HRP) enzyme considering the low-cost and ambient synthesis of selected MOF. For this, ZIF-8 and HRP@ZIF-8 were synthesized under ambient conditions using co-precipitation method followed by extensive characterization. UV-Visible and Fourier transform infrared (FTIR) spectroscopy, Brunauer-Emmet-Teller (BET) surface area analysis, along with SEM imaging were used for structural and functional characterization of synthesized ZIF-8 and HRP@ZIF-8. The presence of UV absorption peak around 280 nm related to proteinaceous enzyme and amide bond stretching at 1643 cm^{-1} in FTIR spectra of HRP@ZIF-8 indicated towards the successful immobilization of enzyme onto MOF. The X-ray diffraction (XRD) studies revealed that crystalline structure of ZIF-8 remained intact even after the enzyme immobilization with some bulges observed on the structure of HRP@ZIF-8 in scanning electron microscopy (SEM) imaging as compared to ZIF-8. Next, the enzymatic activity, stability, and reusability of HRP after immobilization onto ZIF-8 were assessed. The encapsulation of the enzyme within the MOF resulted in a notable enhancement of both its activity and stability. The immobilized HRP demonstrated a 46.53% increase in catalytic activity as compared to the free form and enhanced reusability, with 81% intact residual activity after five cycles. The explored biocatalytic platform also demonstrated improvement in operation stability and no significant leaching of enzyme from MOF. Therefore, enzyme-MOF based biocatalytic platforms have a wide scope & commercial applicability in various fields for industrial, biomedical, pharmaceutical and environmental processes.

CHAPTER 1

INTRODUCTION

The term "enzyme" was introduced by German scientist Wilhelm Kühne.The root of the word originates from the Greek terms "en" (which means 'inside') and "zume" (which means 'yeast') (Brena et al., 2013). Enzymes are known as biocatalysts because they catalyse the rate of a reaction by binding to the substrate molecule via decrease in the activation energy (Lewis & Stone, 2021). Structurally, they display primary, secondary, and tertiary structures, and those with several polypeptide chains possess quaternary structures (Brena et al.,2013). Enzymes are chains of amino acids joined together in one or more polypeptides. The projected worth of the global industrial enzymes market in 2023 was USD 7.42 billion, with an expected rise to USD 7.53 billion by the end of 2024.

Horse radish peroxidase (HRP), an enzyme that contains heme and uses hydrogen peroxide to oxidize a diverse range of organic and inorganic molecules. HRP is found in abundance in roots of horseradish, scientifically known as *Armoracia rusticana* which is a resilient perennial plant grown in moderate climates worldwide, primarily for its nutritional value derived from its roots. HRP structure comprises of polypeptide chain consisting of 308 amino acid residues. The structure of HRP enzyme consist of heme group which is responsible for the enzyme's peroxidase activity and structure also consists of glycosylation sites and disulfide bonds which play a significant role in enhancing the stability of enzyme. HRP is commonly used in Enzyme Linked Immunosorbent Assay (ELISA) and Western blotting because it produces a visible signal with chromogenic or chemiluminescent substrates. Moreover, it is used in biosensors, bioremediation, protein and nucleic acid diagnostics etc. Because it is less harmful to the environment than chemical bleaches, HRP finds commercial and industrial usage in the bleaching of fabrics. It is possible to bleach paper pulp with HRP without using chlorine.

Due to their exceptional biocatalytic properties, enzymes are widely utilized in several industries, including food, pharmaceuticals, biosensors, energy, and medicine. Although enzymes catalyze reactions with excellent specificity under ideal environmental conditions, but their use is greatly limited by the surrounding environmental factors, particularly the harsh and unpredictable conditions encountered during bioprocessing (Nadar & Rathod, 2019). Generally, enzymes are explored in two distinct forms: soluble and immobilized. Several issues with using soluble enzymes that prevent their full potential, include their short shelf-life, fragility, lack of reusability, heightened susceptibility to denaturing chemicals, and

unfavourable operational storage stability (Hu et al., 2018). One possible way to get rid of a lot of these unwanted restrictions associated with soluble enzymes is to use immobilized enzymes (Mardani et al., 2018).

Enzyme immobilization is a crucial process that involves fixing enzymes onto support materials, allowing them to retain their catalytic abilities for repeated and continuous use (Brena et al., 2013). It has been observed that the thermal stability, chemical stability, and storage stability of enzymes is significantly enhanced after immobilization (Tosa et al., 1966; Li et al., 2020). Additionally, immobilization increases the enzyme reusability which further decreases overall cost of commercial enzymatic processes (Sheldon et al.,2013; Mussolini et al.,2005). Adsorption, covalent coupling, entrapment, encapsulation and cross-linking are the most frequently used methods for immobilizing enzymes (Brady & Jordaan, 2009; Li & Dong, 2020). There are few concerns with respect to enzyme immobilization, for example, possibility of leaching, desorption of enzymes and choice of immobilization carrier or substrate (Illanes,2011; Hartmann & Kostrov, 2013). The carrier must be biocompatible, easily accessible at a low cost, provide protection against enzyme degradation and inert (Mohamad et al., 2015; Mateo et al., 2007).

The use of nanomaterials as immobilization substrates for enzyme fixation is an attractive and cost-effective approach. Nanomaterials possess a remarkable surface area-to-volume ratio, attributed to their minuscule size, porosity and diverse functionalization which provides a multitude of binding sites for enzyme immobilization (Ansari et al.,2012; Hwang et al.,2013). Enzymes immobilized onto nanomaterials exhibit enhanced stability, reusability, and catalytic efficiency, making them indispensable in bio catalysis, biosensing, drug delivery, and environmental pollutant remediation. Utilizing nanomaterials offers a cost-effective solution for immobilizing enzymes in industrial applications, as these materials enhance reusability and greatly reduce enzyme wastage (Nadar & Rathod, 2019). Different nanomaterials for example, magnetic nano particles, carbon nanotubes, graphene oxide, mesoporous silica, to mention a few have been utilized to immobilize enzymes (Rusu et al.,2022).

In recent years, there has been a significant interest in using Metal-Organic Frameworks (MOFs) for immobilizing enzymes. Crystalline inorganic-organic hybrid materials known as MOFs are composed of metal ions, organic ligands and exhibit a diverse array of unique properties (Wang et al., 2019). These materials have distinct features, including adjustable pore size, surface area, excellent chemical and thermal stability. MOFs can adapt their morphology

and functioning to suit specific applications, unlike zeolites, silica, and carbonaceous materials (Mehta et al., 2016). Also, the metal ions and organic ligands found in MOFs play a crucial role as co-factors, boosting the catalytic activity of the immobilized enzyme. Enzyme-MOF composites have been demonstrated to exhibit remarkable advantage in improving enzymes' reusability and catalytic efficiency (Xia et al., 2020). The varied combinations of metal ions and organic linkers can be used to synthesize MOFs to optimize the immobilization of enzymes. The metal ions and organic ligands present in MOFs may also act as co-factor and enhance the activity of immobilized enzyme. (Silva et al., *2022*). Different methods are employed to fix enzymes in the metal-organic framework, including in-situ encapsulation, covalent attachment, surface functionalization, and co-precipitation (Xia et al., 2020). Enzymes have been incorporated into the pores, onto the surface or within the structure of MOFs.

Zeolitic imidazolate framework-8 (ZIF-8) is a highly significant MOF synthesized using Zinc Nitrate Hexahydrate and 2 methyl imidazole. In a report, an X-shaped ZIF-8 has been synthesized and used to immobilize Rhizomucor miehei lipase (RML) through encapsulation. The morphological structure of ZIF-8 underwent a noticeable change after immobilization and RML@ZIF-8 exhibited a remarkable 26-fold increase in its activity when compared to free lipase. The immobilized RML@ZIF-8 exhibited reusability with the conversion yield being consistent at 84.7% even after 10 cycles. (Adnan et al., 2018). In another study, penicillinase, a specific form of β-lactamase has been immobilized into ZIF-8 via a self-assembly method. The results showed that the catalytic activity of the enzyme increased significantly in encapsulated form as compared to free form. (Yang et al., 2021). Horseradish peroxidase (HRP)has also been attached to the functionalized surface of ZIF-8 (Wang et al., 2019). The so formed HRP@ZIF-8 biocomposite not only showed long-term stability, but also exhibited enhanced resistance to high temperatures and alkaline conditions (Wang et al., 2019).

In the present thesis, ZIF-8 MOF which can be synthesized at ambient conditions in ambient conditions has been explored for in-situ encapsulation of HRP enzyme within the structure of MOF. For this, MOF and HRP-MOF composite have synthesized separately under similar conditions followed by structural characterization using UV-Visible spectroscopy, FTIR spectroscopy along with X- ray diffraction and surface area analysis. Furthermore, enzymatic activity, reusability and stability of immobilized HRP has been assessed and compared to that of free HRP.

CHAPTER 2

REVIEW OF LITERATURE

2.1 ENZYME IMMOBILIZATION

Enzymes are macromolecules and serve as natural biocatalysts for biological processes due to which they have extensive applications in several industries (El-Shishtawy et al., 2023; Hou et al., 2016). In the commercial application enzymes are used in soluble form (Zhang et al., 2022). However, the widespread commercialization of enzymes in soluble forms is hindered due to limited reusability leading to economic unfeasibility. Furthermore, it is vital to tackle the issue of preserving the structural stability of enzymes during biochemical reactions under harsh conditions (Sirisha et al., 2016). Enzymes, however, have limits since they have a tendency to undergo structural changes when exposed to adverse environmental circumstances (Liang et al., 2020). Nevertheless, the performance of the soluble enzyme in free form can be easily influenced by several factors in the reaction system, such as elevated temperature, organic solvents, and pH levels. Additionally, they frequently have unstable operation stability over the long term and a problematic recovery procedure, which raises processing costs and hinders their extensive industrial uses (Bin et al., 2020). In this regard enzyme immobilization is attractive alternative which involves confining enzyme molecules to a solid matrix or support (Brena et al., 2013; Kawaguti et al., 2006). The use of immobilized enzymes eliminates several unwanted constraints of instability and limited reusability (Tosa et al., 1966; Li et al., 2020). The different techniques for immobilization of enzymes are covalent binding, adsorption, cross linking, entrapment and encapsulation to name a few (Li & Dong, 2020). Immobilized enzymes securely attach to a support matrix or surface, resulting in a stable and reusable enzyme system. Enzyme immobilization improves the stability, efficiency, and specificity of enzymes and simplifies the process of separating and recovering the enzyme once it has catalyzed the reaction (Liang et al., 2020). Enzymes that are immobilized have been widely utilized in several industrial sectors, including manufacturing food and drinks, medicines, and biofuels (Boudrant et al., 2020; Alharbi et al., 2023).

2.1.1. METHODS OF ENZYME IMMOBILIZATION

(a) Adsorption:

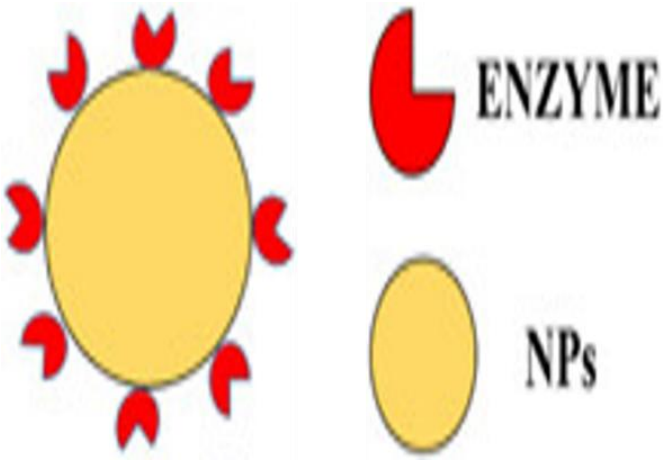

Figure 2.1 Adsorption of enzyme onto surface of substrate (Anboo et al.,2022)

Enzyme adsorption onto insoluble supports is a well-established and straightforward method with extensive applications which involves physical attachment of enzyme onto solid substrate/adsorbent illustrated in Figure 2.1 (Spahn&Minteer,2008). Choosing the right support for enzyme immobilization is a crucial step. It is important for the support to have a large surface area and be compatible with the enzyme being used (Bilal et al., 2024). There are several commonly used adsorbents, for enzyme immobilization include activated carbon, silica gel, cellulose, chitosan, and various synthetic polymers. Successful adsorption is dependent on the presence of specific active groups on the carrier material that promotes the stable interaction between enzyme and carrier.

(b) Entrapment:

Enzymes can be permanently trapped within lattice-like support materials using a technique known as entrapment which is irreversible immobilization as depicted in Figure 2.2 (Singh, 2009). This process helps prevent the leakage of enzymes as enzymes are trapped within the support matrix using methods such as microencapsulation (Wadiack & Carbonell, 1975) or gel/fibre entrapment (Bernfeld & Wan, 1963). Hydrogels and sol-gel matrices are efficient substrates for entrapment. Different types of interactions and forces are involved in entrapment of enzyme into the MOF such as Hydrogen bonding, coordination bonds, Van der Waals forces, electrostatic interactions to name a few (Grosová et al., 2007; Wu et al., 2015; Cui et al., 2012)

Figure 2.2 Entrapment of enzyme into the structure of MOF (Anboo et al., 2022)

(c) Cross linking:

In this method of enzyme immobilization, linkages are formed between the molecules of the enzymes by using multifunctional chemical compounds known as organic linkers shown in Figure 2.3. This approach does not require any carrier. As a result, three-dimensional crosslinked aggregates are formed which are completely insoluble in water (Maghraby et al., 2023). Chemical crosslinking occurs within molecules (intramolecular crosslinking) or between different molecules (known as intermolecular crosslinking). The activity of crosslinked enzymes depends on the number and positions of formed (Gao et al., 2010).

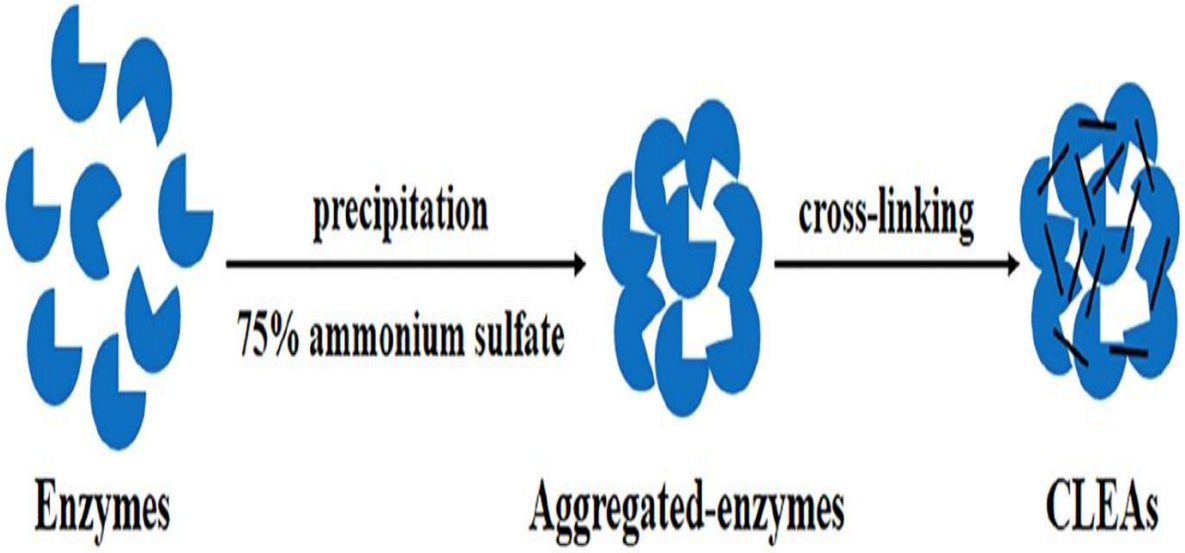

Figure 2.3 Cross linking of enzyme forming Cross linked Enzyme Aggregates (Zucca et al., 2022)

(d) Covalent Binding

Enzymes can also be immobilized onto solid substrates via formation of covalent bonds between the enzyme molecules and the support material. This method has a significant advantage due to the strong bonding between enzymes and support materials which prevents any leaching of enzymes into the solution. Support materials can also be functionalized to improve the stability of immobilized enzymes as shown in Figure 2.4 (Fu et al.,2011). The support material and enzyme are mixed and subjected to ideal conditions (pH, temperature, buffer composition) to promote the formation of covalent bonds such as amide, ether, thioether, or carbamate bonds (Maghraby et al., 2023; Hartmann M, Kostrov X, 2013). The immobilization of enzyme onto or within the support matrix highly depends on the carrier. The carrier should be biocompatible, available at low cost, inert, and protect enzyme from degradation (Mohamad et al., 2015).

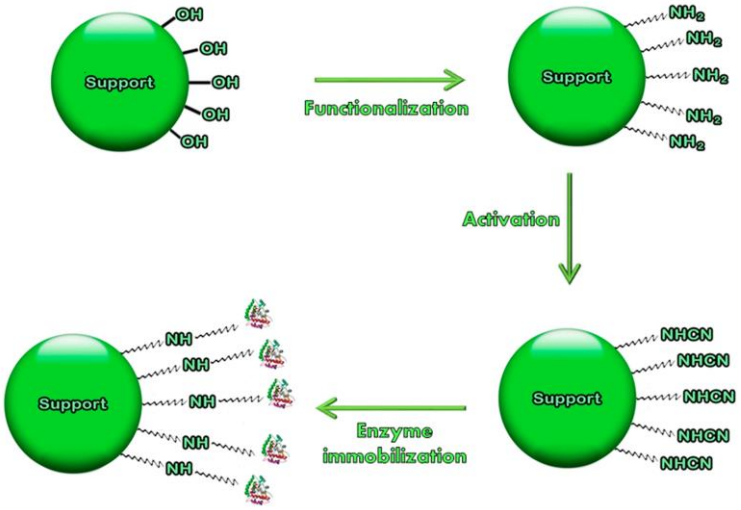

Figure 2.4 Surface functionalization for covalent attachment of enzyme (Zucca et al., 2014)

2.2 Nanomaterials for enzyme immobilization

Nanomaterials serve as flexible surfaces for fixing enzymes, providing distinct benefits such as large surface area, programmable characteristics, and tailored functioning. Enzymes immobilized on nanomaterials show improved stability, reusability, and catalytic efficiency, making them essential in bio catalysis, biosensing, drug delivery, and remediation of environmental pollutants (Xia et al., 2020). Nanomaterials have a high surface area-to-volume ratio which is due to their small size, which makes way for a large number of binding sites for enzyme immobilization. The large surface area supports effective use of enzymes and facilitates improved catalytic activity. They provide customizable physical and chemical characteristics, such as size, shape, surface chemistry, and porosity. These characteristics can

be meticulously designed to enhance enzyme-substrate interactions, improve stability, and modify the catalytic environment for particular goals. Nanomaterials consist of a diverse array of substances, such as metals (e.g., gold, silver), metal oxides (e.g., titanium dioxide, iron oxide), carbon-based materials (e.g., graphene, carbon nanotubes), polymers, and hybrid materials. Various nanomaterials, including magnetic nanoparticles (MNPs), surfaces made of silica, nanocomposites of graphene oxide, or surfaces coated with polymers, might affect the efficiency of an enzyme. Each material offers certain advantages and can be chosen based on the preferred method of immobilization and specific requirements for usage. Nanomaterials can be used to immobilize enzymes to improve their stability and reusability (Anboo et al., 2022). This allows for repeated cycles of catalytic activity without encountering a significant reduction in activity. Using nanomaterials makes immobilization of enzymes cost effective for industrial applications as reusability of these materials decreases the wastage of enzyme to a significant level (Nadar & Rathod, 2019).

2.3. Metal-Organic Frameworks (MOFs) as enzyme immobilization substrates

2.3.1. Introduction to MOFs

The last twenty years have seen a massively increased interest in synthesizing metal-organic frameworks (MOFs) because of the potential to create a wide range of visually pleasing structures that may be very useful for applications in several sectors involving porous materials (Norbert Stock and Shyam Biswas,2012). It wasn't until 1995, that the term MOF became prominent (Yaghi et al., 1995). MOF-5 and HKUST-1, two of the most studied MOFs today, had their syntheses reported in 1999 (Li et al., 1999; Shen et al., 2021). In 2002, Férey et al. documented MIL-47 (Karin Barthelet et al., 2001), a nonflexible porous MOF, and MIL-53/ MIL-88 (Baumann et al., 2019), a flexible porous MOF. The zeolitic imidazole frameworks (ZIFs) family of chemicals has also been expanded to include compounds based on imidazolates in 2002 (Tian et al., 2002). Some examples Different types of MOFs synthesized are shown in Table 1.

MOFs are composed of two primary constituents: (a) Metal centers: These are commonly metal ions or metal clusters that function as the central points of the framework. (b) Organic linkers: These are organic molecules that join the metal centers, creating the boundaries of the framework (Sun et al., 2021). Structure of MOF is illustrated in Fig.2.5. MOFs exhibit a wide range of structural variation due to the incorporation of several metal centers and organic linkers. By altering these constituents, the dimensions, configuration, and characteristics of the

MOFs may be adjusted to suit certain uses (Syzgantseva et al., 2019; Kustov et al., 2019). The metal centers and biological linkers combine to form a three-dimensional structure (Mancuso et al., 2020).

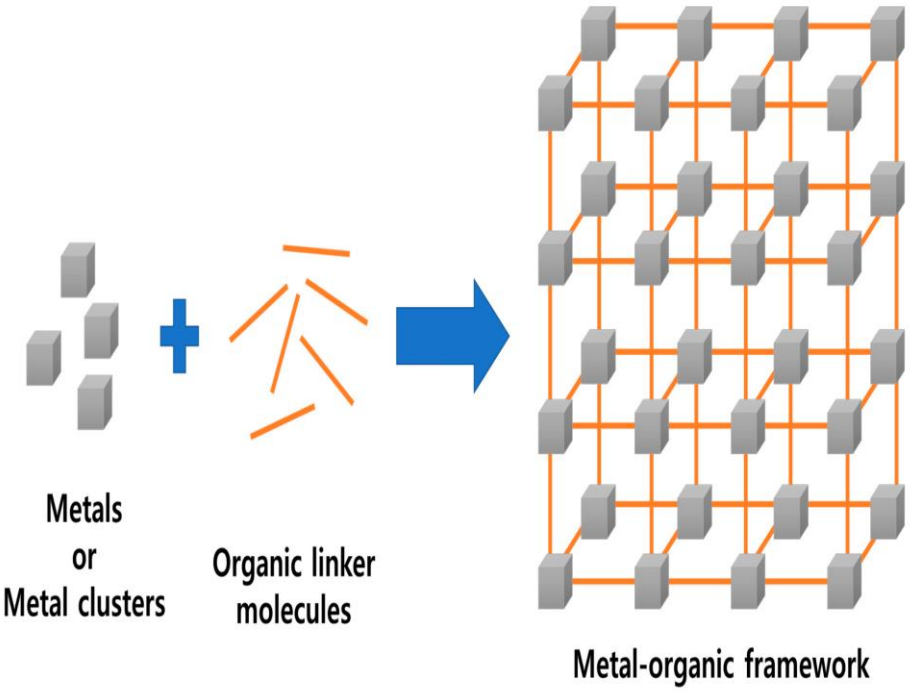

Figure 2.5 Structure of MOF (Heo DY et al., 2020)

2.3.2. Synthesis of MOFs

MOFs are synthesized via several techniques; each possessing distinct benefits and uses. Most prevalent techniques of MOF synthesis are:

(a) Hydrothermal Synthesis

This technique utilizes elevated temperatures and pressures in solvent to generate MOFs through chemical synthesis. This approach is commonly employed to create MOFs that exhibit exceptional crystallinity and stability (Chen et al., 2019).

(b) Microwave assisted synthesis

Microwave-assisted synthesis is a fast and effective technique for synthesizing MOFs. Microwave radiation is utilized to expedite the synthesis process, hence decreasing the duration needed for crystallization. In this technique microwaves are used to generate MOFs with elevated surface areas and pore volumes (Furukawa et al., 2013).

(c) Co precipitation method

Co-precipitation is the process in which metal ions and organic ligands are precipitated together at the same time. This technique is frequently employed to create MOFs with precise compositions and frameworks at ambient conditions, if necessary (Mahreni and Ristianingsih, 2020).

(d) Mechanochemical synthesis

Mechanochemical synthesis is a process that utilizes mechanical energy to create MOFs. Metal precursors and organic linkers are ground in a ball mill without solvents. High-energy milling at a specific speed and duration is applied to the mixture. This approach is efficient and sustainable. This technique is advantageous for generating MOFs with elevated surface areas and can be executed at ambient temperature (Rowsell & Yaghi, 2004)

(e) Sono chemical synthesis

Sono chemical synthesis is a method that utilizes ultrasonic radiation to generate MOFs. This technique is renowned for its rapid crystallization and environmentally friendly nature. (Mahreni and Ristianingsih, 2020)

(f) Electrochemical synthesis

Electrochemical synthesis is a process that utilizes electrical currents to synthesize MOFs. This technique is valuable for generating Metal-Organic Frameworks (MOFs) with precise structures and compositions.

Table 1. Examples of some metal organic frameworks

Metal Organic Framework	Metal Ion	Organic Linker	References
ZIF-8	Zn^{2+}	2- Methylimidazole	(Wang et al., 2020)
ZIF-67	Co^{2+}	2- Methylimidazole	(Duan et al., 2022)
MOF-5	Zn^{2+}	1,4-benzodicarboxylate (BDC)	(Li et al., 1999)
MIL-53	Al^{3+}	1,4-benzodicarboxylate (BDC)	(Baumann et al., 2019)
HKUST-1	Cu^{2+}	1,3,5-benzene tricarboxylic acid (H_3BTC)	(Shen et al., 2021)

UiO-66	Zr^{2+}	1,4-benzodicarboxylate (H_2BDC)	(Liu et al., 2015)
MOF-74	Bimetallic Zn/Mg	2,5-dihydroxy-1,4-bezenedicarboxylic acid (DHBDC)	(Rosi et al., 2005)

2.3.3. MOFs as efficient substrates for enzyme immobilization

In order for a material to be considered acceptable as a support, it must have high surface area, diverse functionalization along with the ability to maintain the highest achievable degree of enzyme activity, while simultaneously providing protection to the enzyme and allowing for its reuse in practical applications. Several support materials, such as graphene oxide (GO), carbon nanotubes (CNTs), mesoporous silica (MPSs), and metal-organic frameworks (MOFs), have been used to immobilize enzymes (Alshawafi et al., 2018; Nadar et al., 2018). Some examples of the different MOFs explored for immobilizing enzymes are summarized in Table 2.

MOFs possess distinct characteristics that render them very suitable for enzyme immobilization (An et al., 2019). For instance, large surface area, diverse functionality, and customizable pore diameters of MOFs allow for efficient, stable, and high enzyme loading (Ahmed et al., 2019). In addition, MOFs can protect enzymes against adverse circumstances, such as elevated temperatures, abnormal pH levels, and organic solvents, which are known to cause enzyme degradation. (Ahmed et al., 2020). Moreover, varied functionality can be incorporated in the structure of MOF for facilitating stable enzyme immobilization.
Majorly, three methods are employed for enzyme immobilization into MOFs

(a) Attachment of enzyme onto surface of MOFs

Enzymes are attached onto the surface of MOFs via covalent binding and physical adsorption depicted in Figure 2. 6. Therefore, chemical reagents are required to functionalize the surface of MOFs and mediate the binding between the enzyme and functional group of MOF surface. In 2015, Liu and his colleagues immobilized Lipase in UiO-66 a Zirconium based via Surface attachment.

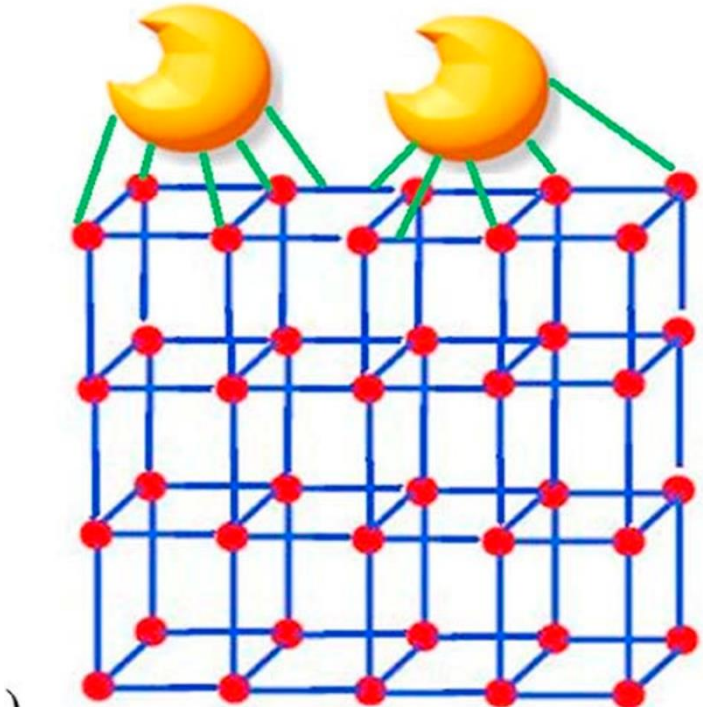

Figure 2.6 Attachment of enzyme onto surface of MOFs (Mehta et al., 2016)

(b) Diffusion of enzyme into the pores of MOF

Enzymes permeate the pre-formed MOF's pores from a solution by diffusion shown in Figure 2.7. Immobilization results from either being physically bound or having weak interactions within the pores. Since most microporous MOFs have pore diameters lower than 2 nm, enzymes smaller than this dimension can only penetrate the pores. In 2012, Chen and his colleagues immobilized Microperoxidase 11 enzyme in Terbium meso-MOF via diffusion into the structure of MOF (Chen et al., 2012).

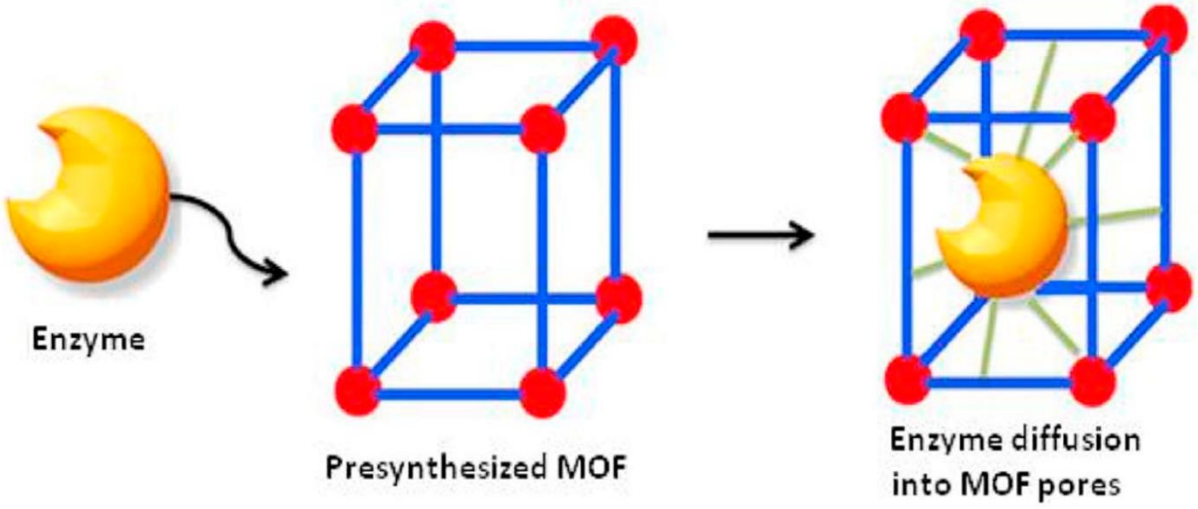

Figure 2.7 Diffusion of enzyme into the pores of MOF (Mehta et al., 2016)

(c) *In-situ* enzyme encapsulation within MOF

Since most of the MOFs have pore diameters smaller than the size of enzymes, it is necessary to use different approaches to broaden the range of MOF-enzyme platforms. Therefore, a new method of enclosing large enzymes within the structure of MOFs using *in-situ* encapsulation during MOF synthesis itself has been reported illustrated in figure 2.8. In 2018, Patil and his colleagues immobilized Laccase enzyme in ZIF-8 via in – situ approach using a one pot synthesis method by Combining a solution of zinc acetate, 2-methylimidazole, and laccase at room temperature (Patil et al., 2018).

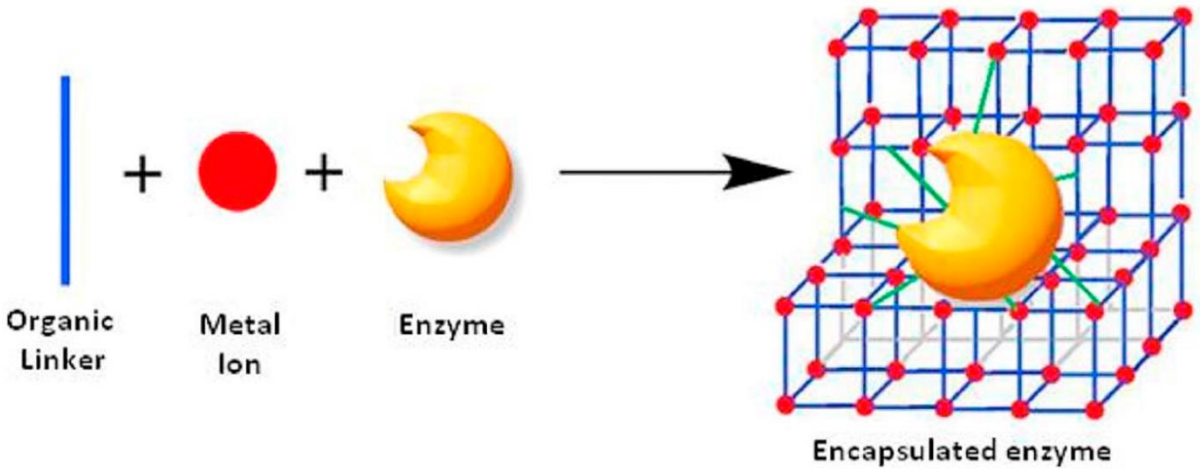

Figure 2.8 *In-situ* enzyme encapsulation within MOF (Mehta et al., 2016)

There are limitations associated with pore diffusion and surface attachment. Pore diffusion allows immobilization of only those enzymes which are smaller than the pore size of the MOFs. Therefore, the technique is restricted to very small number of commercially important enzymes as majority of enzymes are larger in size when compared to pore size of MOFs. On the other hand, surface immobilization requires chemical reagents in large amounts for MOF surface functionalization and binding of enzymes with the MOF. *In situ* encapsulation overcomes the limitations associated with pore diffusion and surface immobilization. However, this technique necessitates the use of MOFs which can be synthesized at ambient conditions so as to protect enzymes to be encapsulated from harsh conditions.

Due to its highly gentle synthesis conditions, the ZIF-8 is the first and most versatile material to be employed for immobilizing enzymes in their original location. In 2014, Lyu et al. first documented the incorporation of cytochrome c (Cyt c) into a ZIF-8 framework via combining zinc nitrate hexahydrate, 2-methylimidazole, and polyvinylpyrrolidone (PVP) modified Cyt c in methanol. The presence of embedded Cyt c had no impact on the shape and crystalline

structure of ZIF-8. However, the conformational changes brought on by the methanol incubation of Cyt c in MOF resulted in an exposed heme group, and the immobilized Cyt c showed a 10-fold increase in apparent activity compared to free Cyt c (Lyu et al., 2014).

In another report, Horseradish peroxidase (HRP) has been densely incorporated into the spacious cavities of nanometre-sized MOF, PCN-333(Al) to enhance signal strength in enzyme-linked immunosorbent assay (ELISA). The catalytic efficiency of the enzyme-labelled antibody complex, which was produced using nanometre-scale PCN-333(Al), remained high. The V_m and K_{cat} values for the substrates 3,3',5,5'-Tetramethylbenzidine (TMB)-H_2O_2 were determined to be 4.84×10^{-5} mM/s and 4.84×10^{4} min^{-1}, respectively. The developed platform HRP@PCN-333 showcased a signal amplification approach for a colorimetric detection of human prostate-specific antigen (PSA) with very low detection limit of 6 pg/mL (S/N=3) (Sun et al., 2021). Considerably higher loading of enzyme markers, better enzyme stability and usage along with an easier way to prepare enzyme-labelled antibodies are the key features of this approach.

A Fe_3O_4/Fe-MOF core-shell structure with hierarchical pore diameters has been grown using solvothermal synthesis. The Fe_3O_4/Fe-MOF material combining magnetic properties with a hierarchical porosity has been explored to support chloroperoxidase (CPO) and horseradish peroxidase (HRP). The immobilized CPO/HRP demonstrated a notable increase in stability when exposed to high temperatures along with enhanced reusability. Also, with 94.1% of its activity retained even after being reused 10 times. Furthermore, CPO/HRP- Fe_3O_4 /Fe-MOFs showed high efficacy in decomposing organic pollutants, such as isoproturon and 2,4-dichlorophenol, in simulated wastewater, achieving complete destruction within a few 15 minutes. The findings showed that the substrates' diffusion resistance on the substrate was less than that in a bulk solution due to their initial concentration in proximity to enzymes on the hierarchically porous support resulting in greater specificity and binding affinity than the free enzymes (Gao et al., 2020).

X-shaped ZIF-8 has also been synthesized and was employed to immobilize Rhizomucor miehei lipase (RML) for generating renewable fuel. The morphological structure of ZIF-8 changed following immobilization with observable change in surface area and pore size before and after immobilization. RML@ZIF-8 showed a 26-fold increase as compared to free lipase with an optimal conversion yield of 95.6% to produce biodiesel from soybean oil. The immobilized enzyme's reusability data showed conversion yield stayed at 84.7% of its starting

activity even after 10 cycles. Since ZIF-8 is easily synthesised and immobilizing RML onto ZIF-8 is responsible for the stability and excellent performance of the immobilized enzyme (Adnan et al., 2018).

In another report, Yang and his colleagues immobilized penicillinase, a specific type of β-lactamase, into ZIF-8 using a self-assembly approach to facilitate the degradation of penicillin antibiotics. The results demonstrated that the catalytic activity and stability of the encapsulated enzyme against elevated temperatures, organic solvents, and enzyme inhibitors dramatically improved when compared to that of the free enzyme. It has been reported that the Zn (II) ion on ZIF-8 frameworks create a complex with the target molecule, weakening the bond of the four-membered β-lactam ring in the penicillin molecule resulting in increased enzyme's degradation efficiency (Yang et al., 2021).

Similarly, HRP@ZIF-8/DNA hybrids have been developed as a fluorescence quencher and used to detect Hg^{2+} and phenol down to nanomolar range (Wang et al., 2019). In addition to demonstrating long-term stability, the produced HRP@ZIF-8 biocomposite significantly exhibited increased resistance to high temperatures and alkaline conditions.

In 2018, Gascón et al. immobilized Laccase onto semi-crystalline Fe-BTC MOF material within 10 minutes, using conditions compatible with biological systems. The biocomposite developed exhibited a two - fold increase in activity post immobilization with no significant leaching of enzyme in the reaction mixture. The robust π···π interactions between the enzyme molecule and the organic ligand enhanced the enzyme's retention within the MOF (Gascón et al., 2018)

Molina et al. recently investigated the immobilization of laccase from the fungus *Myceliophtora thermophila* on NH_2-MIL53 (Al) MOF via *in situ* and post-synthesis procedures. The Lac@NH_2-MIL-53(Al) demonstrated a 100% immobilization efficiency, with high efficiency to eliminate 98% bisphenol A (BPA) from water in under 3 minutes. The developed composite outperformed the free enzyme in terms of effectiveness (Molina et al., 2021).

In another study by Nadar et al., biomineralization approach has been used to immobilize lipase inside ZIF-8 after activating it with proline. The lipase-proline MOF produced showed a 135% higher catalytic activity and four times thermal stability. In terms of reusability, the lipase-proline MOF exhibited 72% residual activity after six cycles (Nadar et al., 2019).

Table2 Different enzymes immobilized in MOFs

MOF	Metal Ion	Enzyme	MOI	Improved attribute	Application	References
ZIF-8	Zn^{2+}	HRP	*In-situ*	Increased thermal stability and resistance to organic solvents	Biosensors and pharmaceutical	(Chulkaival sucharit et al., 2015)
ZIF-90	Zn^{2+}	Catalase	*In-situ*	Retained activity in the presence of protease *proteinase K*	Degradation of hydrogen peroxide	(Shieh et al., 2015)
HKUST-1	Cu^{2+}	Lipase	Surface attachment	90% of enzymatic activity still retained after 10 cycles	Reaction rate increased 17 times	(Cao et al., 2016)
Uio-66	Zr^{2+}	Lipase	Surface attachment	Retention of activity after 35 days	Warfarin s	(Liu et al., 2015)
MIL-100-Fe	$Zn^{2+,}$ Fe^{3+}	GOx	Surface attachment	Significantly reduced sensitivity together with increased response time values	Detection of electrocatalytic glucose	(Patra et al., 2015)
PCN-888	Al^{3+}	GOx+ HRP	Pore diffusion	It showed enhanced tolerance to degradation of trypsin	-	(Lian et al., 2016)

2.4 HRP as model enzyme for immobilization onto MOF

HRP is a heme containing enzyme that uses hydrogen peroxide to oxidize a diverse range of organic and inorganic molecules. Horseradish peroxidase isoenzyme C constituted of a single polypeptide chain of 308 amino acid residues. HRP C consists of two distinct metal centers: iron (III) protoporphyrin IX, commonly known as the 'heme group', and two calcium atoms. Both components are necessary to maintain the overall structure and proper functioning of the enzyme. The heme group is firmly attached to the protein by coordination with a histidine residue. Horseradish peroxidase, specifically HRP C, is utilized as a reagent in organic synthesis and biotransformation. It is also employed in linked enzyme assays,

chemiluminescent assays, immunoassays, and the treatment of waste waters. Structure of HRP is depicted in Figure 2.9.

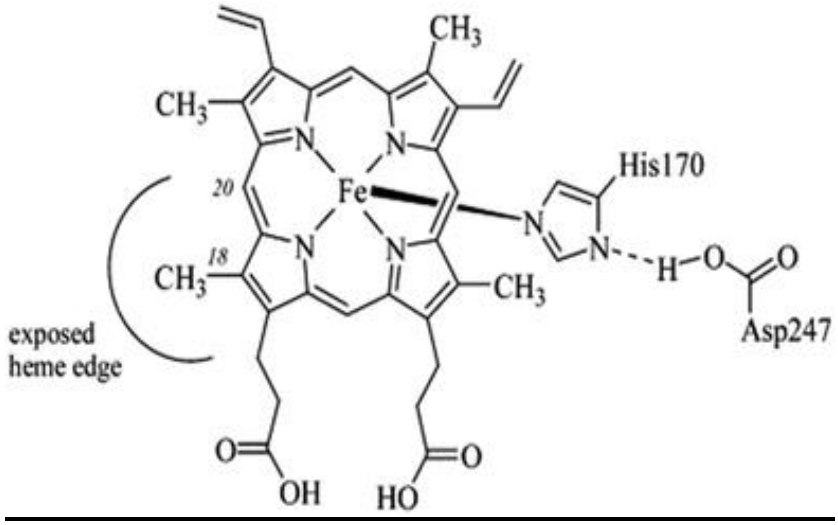

Fig.2.9 Structure of HRP (Bergund et al.,2002) .

The immobilization of horseradish peroxidase (HRP) onto metal-organic framework (MOF) structures improves the enzyme's stability, reusability, and activity making it appropriate for a wide range of applications.

HRP-MOF composites hold considerable promise in industrial biocatalysis, environmental remediation, and biosensing applications. These composites enhance the effectiveness and sustainability of catalytic reactions in industrial processes. Additionally, they can be utilized to break down contaminants and detoxify waste streams in the environment. Moreover, the increased stability and efficiency of HRP when fixed on MOFs results in composites used as sophisticated biosensors, facilitating the precise and selective identification of diverse chemicals in clinical diagnostics and environmental monitoring.

CHAPTER 3

RESEARCH GAPS, HYPOTHESIS, AND OBJECTIVES

3.1 RESEARCH GAP

On the basis of extensive literature review following research gaps have been found:

- The extensive research is needed for improving the activity, stability and reusability of enzymes important for industrial use at low cost.
- Immobilization of commercially viable Horseradish peroxidase (HRP) enzyme onto substrates via simple method without involving chemical agents is less explored.

3.2 HYPOTHESIS

MOFs are attractive alternatives as enzyme immobilization substrates due to high surface area, tunable porosity, diverse functionalization, extended stability and ease of synthesis. Moreover, the metal ions and organic ligands present in the structure of MOF are reported to enhance the enzymatic activity. The enzymes are immobilized onto MOFs via diffusion into pores, surface attachment or *in-situ* encapsulation. The diffusion of enzymes into MOFs is restricted only those enzymes which have size smaller than the pore size of MOFs. Also, the surface attachment requires large number of chemical reagents which makes process costly and denaturing for enzymes. Therefore, in the present thesis in-situ encapsulation has been chosen as the method to immobilize HRP enzyme into the structure of ZIF-8 at ambient conditions during the synthesis of MOF itself.

Therefore, the present thesis hypothesizes that "**The use of MOFs exhibiting higher surface area, porosity and stability as substrates for *in-situ* immobilization of commercially viable enzymes results in enhanced activity, stability, and reusability of enzymes for efficient applicability in various sectors at low cost.**"

3.3 OBJECTIVES

The objective of the present thesis are as follows:

1. Synthesis and characterization of ZIF-8
2. Immobilization of HRP onto ZIF-8 and characterization of HRP@ZIF-8 composite
3. Evaluation of enzyme activity, stability and reusability after immobilization on ZIF-8

CHAPTER 4

MATERIALS AND METHODS

To fulfil the objectives of the presented study, the experiment has been divided into three steps:

- **STEP 1**
  - ✓ Synthesis of ZIF-8 MOF via co-precipitation method
  - ✓ Characterization of synthesized ZIF-8 using spectroscopic and microscopic techniques along with X-ray diffraction and BET
- **STEP 2**
  - ✓ *In-situ* encapsulation of the HRP enzyme within the structure of ZIF-8
  - ✓ Structural characterization of the HRP@ZIF-8
- **STEP3**
  - ✓ Quantitative estimation of HRP immobilized onto ZIF-8
  - ✓ Enzymatic activity determination after immobilization
  - ✓ Stability and reusability studies at different pH and temperature

4.1. MATERIALS

All the used chemicals were of analytical grade and are mentioned below

Table 3. Materials used in the study along with their suppliers

S.NO.	MATERIAL	SUPPLIER
1.	Zinc nitrate hexahydrate	Sisco Research Laboratories
2.	2 methyl imidazole	Loba Chemie Pvt. Ltd.
3.	Potassium Dihydrogen phosphate	Sigma-Aldrich
4.	Dipotassium hydrogen phosphate	Sigma-Aldrich
5.	Copper (II) sulphate pentahydrate 4% solution	HI media
6.	Bicinchoninic Acid (BCA)	TCI Chemicals
7.	Bovine Serum Albumin	Sigma-Aldrich
8.	Amplite^{R} Colorimetric Peroxidase Assay Kit.	AAA Bio quest

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4.2. PREPARATION OF REAGENTS

4.2.1 Preparation of Phosphate buffer saline:

Solution of KH_2PO_4 (acidic) was prepared by dissolving 8.48 g of Potassium dihydrogen phosphate in 100 ml of distilled water likewise a solution of K_2HPO_4 (basic) was prepared by dissolving 6.8 g of Potassium hydrogen phosphate (KH_2PO_4) in 100 ml of distilled water. Then the acidic solution was added to the basic solution (K_2HPO_4) of and pH was set at 6.9.

4.2.2 Preparation of BCA reagent:

Reagent was prepared by dissolving 1 ml of 4% Copper (II) sulphate pentahydrate solution, in 49 ml of Bicinchoninic Acid (BCA) solution.

4.2.3 Preparation of HRP Substrate:

250 µl of DMSO was added to Amplite^R Blue substrate to make 100X Amplite^R substrate stock solution.

4.2.4 Preparation of H_2O_2 Solution:

H_2O_2 Solution was prepared by dissolving 2.3 µl of H_2O_2 was added to 97.7 µl of buffer.

4.3. SYNTHESIS OF ZIF-8:

- Solution A was prepared by dissolving 0.450 g of $Zn(NO_3)_2.6H_2O$ in 30 ml of ultrapure water.
- Solution B was prepared by dissolving 1.950 g of 2 MeIm in 30 ml of ultrapure water.
- Solution A was added to Solution B, a milky white sediment started to appear on mixing and the mixture was stirred magnetically for 24 h at room temperature. After 24 h the sample was centrifuged at 6000 rpm for 8 min.
- Precipitate was collected and washed with distilled water and ethanol 3 times respectively.

- Pellet was vacuum dried overnight at 60˚C.

Figure 4.1. Synthesis of ZIF8

4.4 SYNTHESIS OF HRP ENZYME IMMOBILIZED ZIF-8

HRP immobilized ZIF-8 was synthesized by using the protocol similar to the one used in synthesis of ZIF-8 along with the addition of HRP enzyme solution. The synthesis procedure was performed in ambient conditions i.e. at room temperature, 1mg/ ml of enzyme was added to the ZIF-8.

- Solution A was prepared by dissolving 7.44 mg of Zn $(NO_3)_2.6H_2O$ in 500µl of ultrapure water.
- Solution B was prepared by dissolving 2.05 mg of 2-MeIm in 500µl of ultrapure water.
- 1ml HRP solution of concentration 1 mg/ml was prepared by dissolving 1mg of enzyme in 1ml of PBS.
- HRP solution was added to the solution of 2-MeIM. The solution was mixed for 5 minutes.
- HRP – 2-MeIM solution was added to the Zn $(NO_3)_2.6H_2O$ solution, a milky white sediment started to appear on mixing and the mixture was stirred magnetically for 24 h at room temperature. After 24 h the sample was centrifuged at 6000 rpm for 8 min.
- Supernatant was collected to estimate the quantity of HRP immobilized into MOF.
- Precipitate containing pellet was collected and washed with distilled water and ethanol 3 times respectively.
- Pellet was vacuum dried overnight at 30°C.

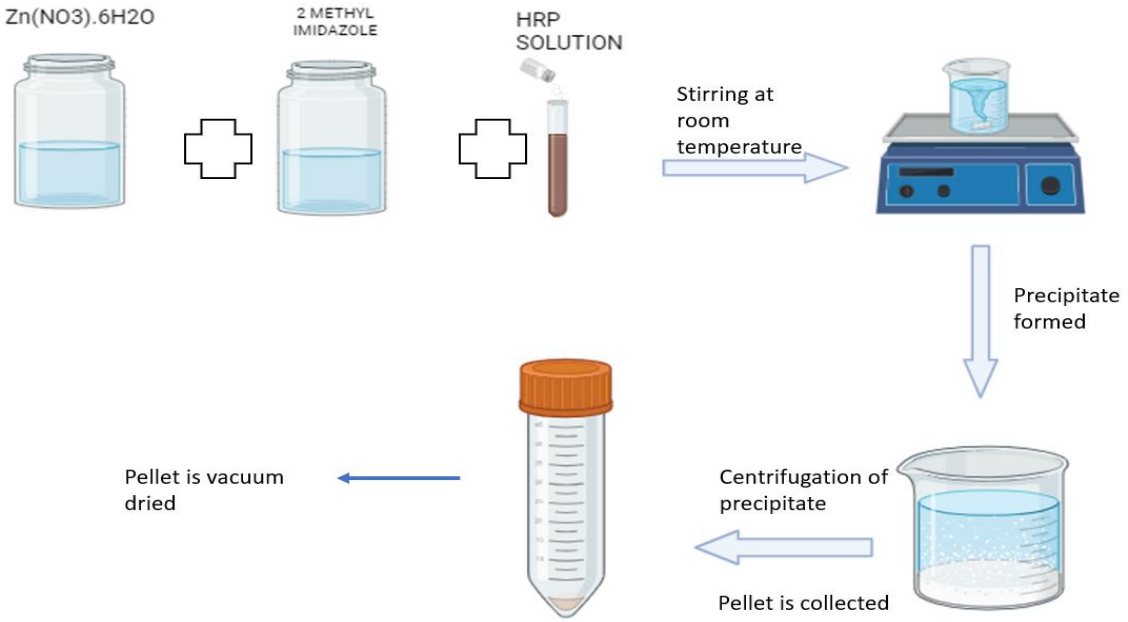


Figure 4.2. Schematic representation of Synthesis of HRP@ZIF8 composite

4.5. ESTIMATION OF HRP IMMOBILIZATION ON ZIF-8

Quantitative estimation of HRP was done using BCA assay

- Reagent was prepared by dissolving 1 ml of 4% Copper (II) sulphate pentahydrate solution, in 49 ml of Bicinchoninic Acid (BCA) solution.
- BSA was used as standard. Stock solution of 2mg/ml of BSA was prepared by dissolving 20 mg BSA in 10 ml distilled water. A standard curve of BSA was prepared. Different concentration of 25, 125, 250, 500, 1000, 1500, 2000 µg/ml of BSA solution were prepared from it, using

$$C_1V_1=C_2V_2$$

C_1 = concentration of stock solution C_2 = concentration of solution to be prepared

V_1 = volume of stock solution required V_2= total volume of 1X solution to be prepared

Table 4. Calculations and absorbance of different BSA standards analyzed using BCA assay

Concentration (µg/ml)	Volume of BSA (µl)	Volume of distilled H_2O (µl)	BCA Reagent (ml)			Absorbance (O.D.) at 562 nm
25	12.5	987.5	4	Vortexed thoroughly	Incubate at 37˚C for 10 minutes	0.0094
125	62.5	937.5	4			0.066
250	125	875	4			0.11
500	250	7500	4			0.186
1000	500	500	4			0.322
1500	700	300	4			0.476
2000	1000	-	4			0.596

The encapsulation efficiency of the MOF i.e. the amount of enzyme loaded onto the MOF was calculated by quantifying the residual amount of HRP in the supernatant using UV-Visible spectroscopy and calculating the concentration of enzyme in supernatant from BCA calibration curve equation. The quantity of the active enzyme enclosed within the ZIF-8 structure was assessed (Mehta et al., 2019). The supernatant was collected after washing pellet and the concentration in the supernatant was quantified by performing BCA assay (Smith et al., 1985).

Amount of enzyme immobilized onto the MOF surface =

Initial amount of enzyme taken for immobilization − Amount of enzyme left in the supernatant after immobilization

4.6 CHARACTERIZATION TECHNIQUES

4.6.1 UV-vis Spectroscopy

UV absorption spectrum of the MOF before and after immobilization of enzyme provides information about the continued existence and sustainability of the enzyme within the MOF and interaction of enzyme within the MOF.

4.6.2 Powder X-rays Diffraction (XRD)

X-ray diffraction (XRD) is a non-invasive method used to ascertain the crystal structure of a substance. The XRD patterns can be fitted to yield some crucial information, including the

material lattice parameters, crystal orientation, stress in crystalline areas, and secondary phases in the sample.

4.6.3 Fourier Transform Infrared Spectroscopy (FTIR)

Infrared spectroscopy (IR) involves the interaction of a sample with electromagnetic radiation in the infrared region of the spectrum. The absorption of radiation is influenced by the chemical groups present in the sample. The absorption pattern across the infrared spectrum is a unique identifier for organic material. Additionally, infrared spectra can be obtained by reflecting the IR beam off the surface of a sample.

4.6.4 Scanning Electron Microscopy (SEM)

SEM provides detailed images of the surface morphology of MOFs. SEM images reveal the topographical features of MOFs, including porosity, surface texture, and the presence of defects or irregularities.

4.6.5 DLS Analysis

It helps to figure out the size distribution and aggregation condition of the enzyme-MOF composites. Alterations in the size distribution before and during the addition of enzymes can indicate effective immobilization.

4.6.6 BET Analysis

The Brunauer-Emmett-Teller (BET) theory is commonly used to determine the surface area of porous materials before and after immobilization. A decrease in surface area and pore volume after enzyme immobilization suggests that the enzyme molecules occupy the pores of the MOF.

Table 5. List of all Characterization Equipment used on the study

S.No.	CHARACTERIZATION	EQUIPMENT
1.	UV-Vis Spectroscopy	SHIMADZU UV1900I
2.	X- Ray Diffraction	Smart Lab, SE model
3.	Fourier Transform Infrared Spectroscopy	SHIMADZU Lab Solutions IR
4.	Dynamic Light Scattering	Malvern Panalytical
5.	Scanning Electron Microscopy	Carl Zeiss Sigma 500 model
6.	Brunauer-Emmett-Teller	Quntachrome^{R} ASiQwin^{TM}

4.7. ESTIMATION OF HRP ACTIVITY POST IMMOBILIZATION

Amplite^{R} kit purchased from AAT Bio quest was used to determine the HRP activity post immobilization

- Most commonly used substrates are ABTS/TMB
- Substrate solution was prepared by dissolving the substrate in DMSO.
- Enzyme solution i.e. HRP solution was prepared by dissolving 15µl of HRP solution into 985 µl of buffer.
- Control: 10µl of substrate, 10µl of H_2O_2 solution was added to 980 µl of buffer.
- For test solution 1: 10µl of substrate, 10µl of H_2O_2 solution, 10 µl of HRP enzyme solution was added to 970 µl of buffer.
- For test solution 2: 10µl of substrate, 10µl of H_2O_2 solution, 10 µl of HRP @ZIF8 supernatant was added to 970 µl of buffer.
- For test solution 3:10µl of substrate, 10µl of H_2O_2 solution, 10 µl of HRP@ZIF 8 pellet solution was added to970 µl of buffer.
- Control and test solutions were incubated for 30 min in cold and dark room.
- Absorbance was noted at 654 nm.

  The enzymatic activity of HRP after encapsulation within the structure of ZIF-8 in terms of specific activity was calculated using the formula,

  $$\text{Specific activity} = \frac{\text{No. of enzyme units per ml}}{\text{Concentration of protein (mg/ml)}}$$

4.8 STABILITY & REUSABILITY OF HRP AFTER IMMOBILIZATION ONTO ZIF-8

After incubation in sodium acetate buffer (0.1 M, pH 5) at various temperatures (30-80° C) for 1 h, the thermal stability of the HRP@ZIF-8 in comparison with free HRP was investigated. Subsequently, the reusability of HRP@ZIF-8 in comparison with free HRP was examined using the same method as activity analysis, and evaluated by residual activity.

The pH stability of HRP@ZIF-8 was measured in different PBS buffer. The HRP and HRP@ZIF-8 biocomposite were subjected to incubation in a pH range of 4 to 9.0 for a duration of 1 hour.

For the thermal stability the HRP and HRP@ZIF-8 biocomposite were stored in a temperature range of 30-80° C. To determine the ideal temperature, the performance of soluble and immobilized HRP was assessed at different temperatures (ranging from 30 to 80° C) using conventional assay procedures.

Thermal stability was assessed by pre-incubating the reaction mixture at different temperatures (ranging from 30 to 80° C) for 30 minutes before adding the substrate. The mixture was then cooled in an ice bath. The enzyme's activity at the initial time point was considered to be 100%.

Next, immobilized-HRP reusability assays were performed under standard enzyme assay conditions. The reaction product was detected at 654 nm after 30 min. The immobilized -HRP was rinsed with 50 mM Tris-HCl buffer pH 7.0 and removed from the reaction media. The reusability of the ZIF-8/HRP composites was assessed. Subsequently, the samples were centrifugated to retrieve the pellet, which was then reconstituted in the appropriate solvent. A total of 6 use cycles were assessed, with the activity after each cycle compared to the starting activity seen after the first cycle. The original activity was used as a control (100%) to calculate the residual percentage activity after each usage. The reusability was checked for 6 cycles.

4.9 STABILITY AGAINST LEACHING

The HRP@ZIF-8 composite was immersed in a phosphate-buffered saline (PBS) solution for various durations of 1 h, 2 h, 5 h, 12 h, and 24 h. The pellet obtained from centrifugation was collected, and the supernatant was analyzed for the presence of protein in supernatant using BCA assay to check for any leaching of enzyme from the MOF.

CHAPTER 5

RESULTS

5.1 UV-Visible absorption Spectroscopy:

The UV-Vis absorption is employed to assess the electronic structure of a compound (Goyal et al., 2018). 1mg of both ZIF-8 and HRP@ZIF-8 each were taken separately in eppendorf tubes and dissolved in 1 ml of distilled water followed by sonication to obtain a transparent solution. The UV-Vis spectra of the ZIF-8 and HRP@ZIF-8 were recorded within the wavelength range of 200-800 nm. Kaur et al had discussed the maximum peak absorbance of ZIF-8 at 218 nm attributed to n – σ^* transitions (Kaur et al., 2017). However, in composite an additional peak at 286 nm representative of aromatic acids present in proteinaceous HRP enzyme in addition to 218 nm absorption peak of ZIF-8 indicated toward successful encapsulation of enzyme in ZIF-8 (Zhang et al., 2017).

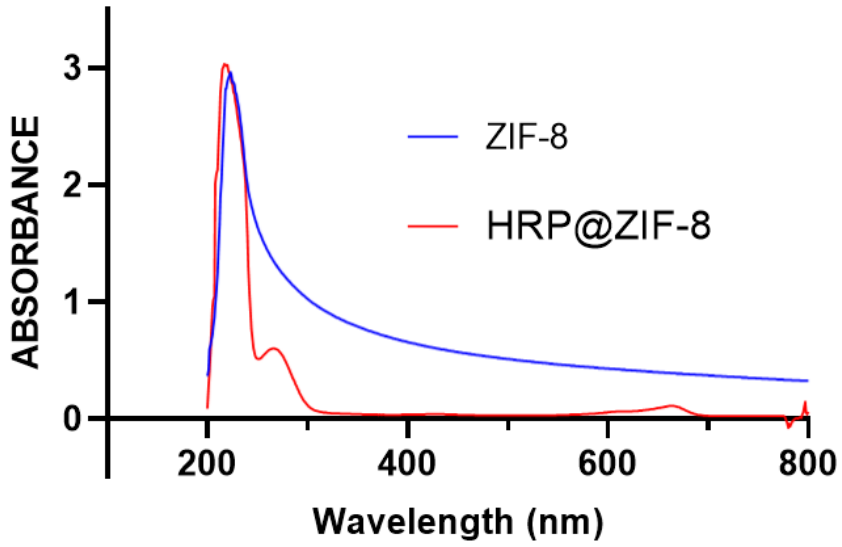


Figure 5.1 UV spectra of ZIF-8 and HRP@ZIF-8

5.2 Fourier Transform Infrared Spectroscopy (FTIR)

The FT-IR spectra shown in Fig.5.2 confirmed the functional groups of powder ZIF-8, and HRP@ZIF-8 composite**.** The FTIR spectra of both samples exhibited a distinct peak at 423 cm^{-1}, indicating towards the presence of Zn-N stretching mode. This suggests that zinc molecules are bonded to nitrogen atoms of 2-MeIM linkers, resulting in the formation of ZIF-8 structure (Xu et al., 2020). The peaks below 800 cm^{-1} correspond specifically to the out of plane bending of the imidazole ring, whereas those in the range of 950–1350cm^{-1} are linked to the in-plane

vibrations (Wang et al., 2018). ZIF-8 also showed characteristic peaks at 1567 and 1397 cm^{-1} corresponding to C=N bond and imidazole ring, respectively (Wang et al., 2018). These same peaks were observed in the FTIR spectrum of HRP@ZIF-8. In addition to these peaks; characteristic peaks of amide bond around 1643 cm^{-1} was also observed depicting the successful encapsulation of HRP in ZIF-8 via amide bond formation (Wang et al., 2019).

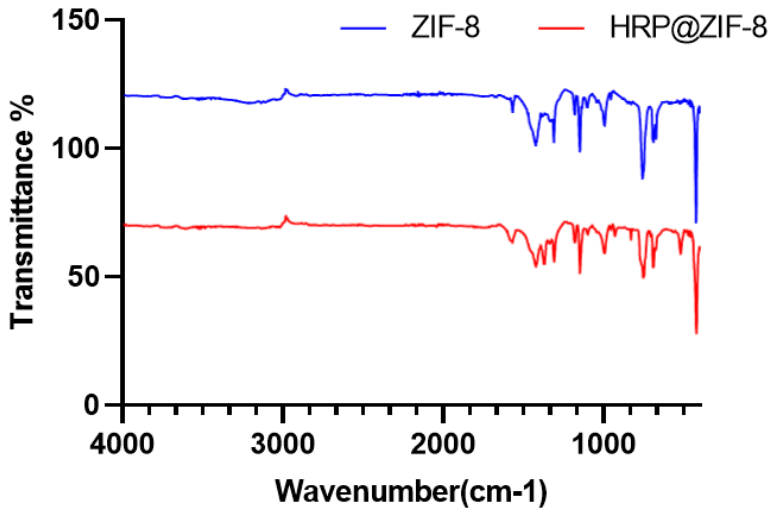


Figure 5.2 FTIR spectra of ZIF-8 and HRP@ZIF-8

5.3 Powder X-rays Diffraction (XRD) and BET Surface area studies

The identification of the phase and crystal structures of the synthesized materials was carried out by using PXRD. The XRD pattern of ZIF-8 and HRP@ZIF-8 are shown in Fig.5.3. The patterns of synthesized ZIF-8 and HRP@ZIF-8 MOF were discovered to be highly consistent with the equivalent patterns reported in the literature. With an index between 5° and 30˚, sharp diffraction peaks of ZIF-8 were observed at 2θ values of 7.44°, 10.36°, 11.04°, 13.8°, 14.9 °, 16.7°, 18.7°, 19.26°, 24.6°, 26.36°, and 29.14° both in ZIF-8 and HRP@ZIF-8 (Li et al., 2020). However, upon HRP encapsulation in ZIF-8, it was observed that the intensity of few typical diffraction peaks of the structural components of ZIF-8 was slightly reduced. However, the crystalline structure of ZIF-8 MOF remained intact even after encapsulation of HRP (Li et al., 2020).

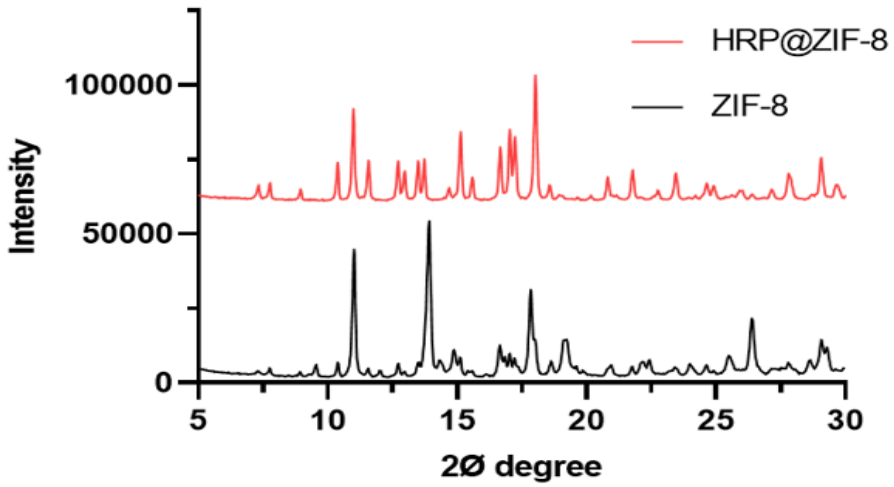


Figure 5.3 XRD plot of ZIF-8 and HRP@ZIF-8

Next, Brunauer-Emmet-Teller (BET) surface area of ZIF-8 and HRP@ZIF-8 was investigated by recording N_2-sorption isotherms at 77K as shown in Fig.5.4. The surface area of ZIF-8 was found to be 789.125 m^2/g which correlates well with the literature (Lai et al., 2016). However, after immobilization of HRP, the surface area reduced to 321.26 m^2/g for HRP@ZIF-8. A decrease in the surface area after the immobilization of HRP confirmed successful immobilization of HRP onto ZIF-8.

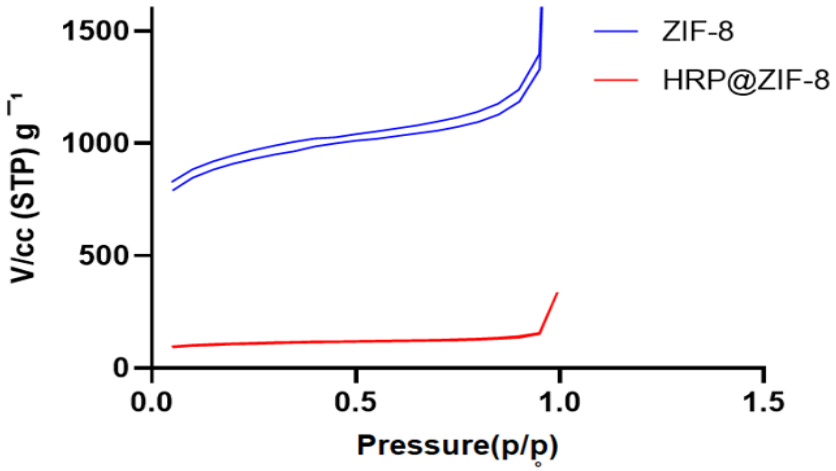


Figure 5.4 N_2 adsorption isotherms for ZIF-8 and HRP@ZIF-8

5.4 Scanning Electron Microscopy (SEM) imaging and Dynamic Light Scattering (DLS) Analysis

The shape, size, and surface morphology of ZIF- 8 & HRP@ZIF-8, were illustrated using SEM imaging as in Fig.5.5. The SEM images revealed that the synthesized ZIF 8 possessed X shaped well defined flower like crucifate structure that corresponds to the previous reports (Adnan et al., 2018) (Cui et al., 2017). However, the SEM images of HRP@ZIF8 showed some irregularities in the basic flower like crucifate structure found in ZIF-8. Despite of irregularities, the overall structure and size of particles remained same in both ZIF-8 and HRP@ZIF-8.

DLS studies were also performed to determine the size of ZIF-8 and HRP@ZIF-8. The increase in size of HRP@ZIF-8 (899.6nm) was observed when compared to ZIF-8 (583.6 nm). Both the samples exhibited PDI below 0.4 indicating structural homogeneity (Zielecka et al., 2011).

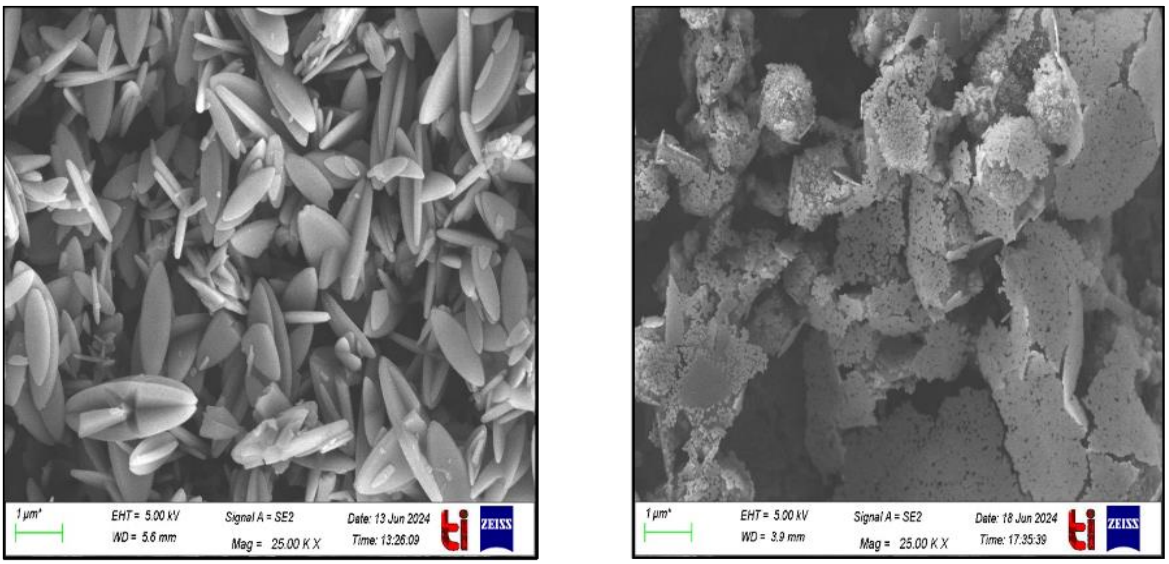


Figure 5.5 SEM images of ZIF-8 and HRP@ZIF-8

5.5 ESTIMATION OF HRP IMMOBILIZATION ON ZIF-8

Estimation of quantity of enzyme loaded onto ZIF-8

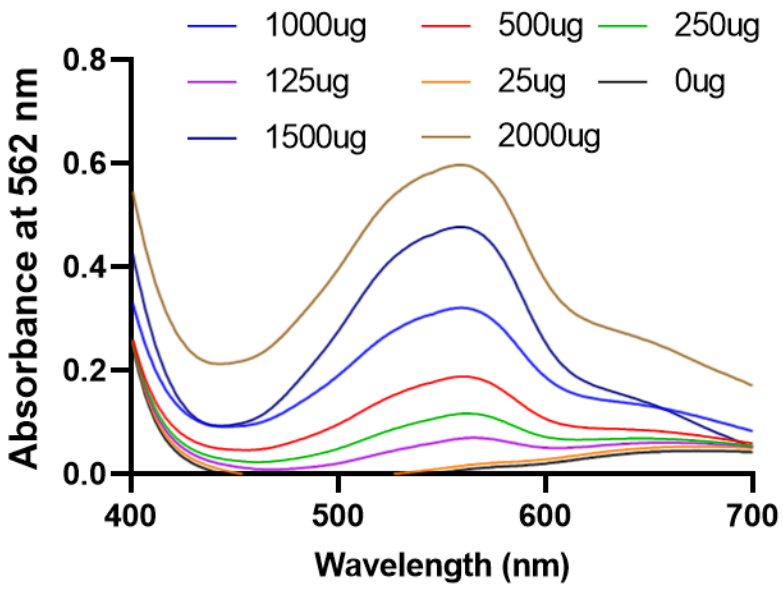
BCA assay was performed to estimate the amount of enzyme loaded onto ZIF-8 using BSA standards. Calibration equation was derived from the graph

$$y = mx + c$$

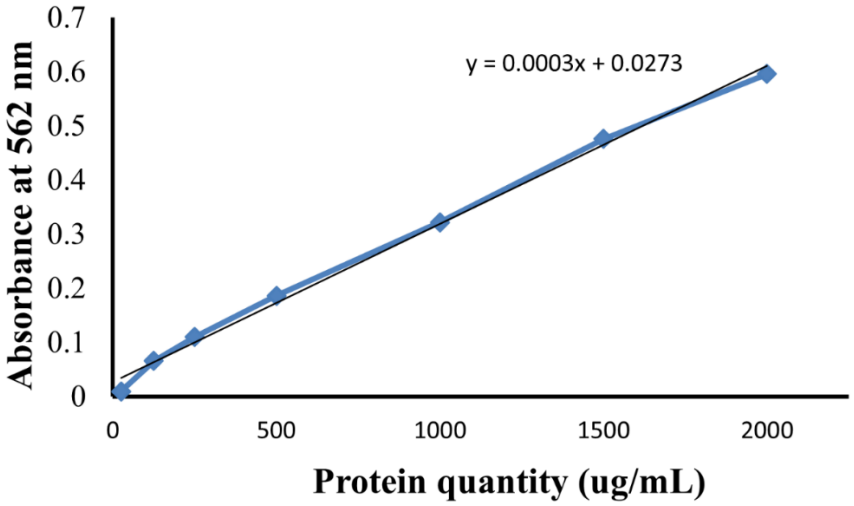
where y = absorbance; x= concentration

Absorbance was observed at 562 nm and plotted against concentration of BSA standards

Amount of enzyme in supernatant was calculated w.r.t BSA standard using BCA Calibration curve equation



(a)



(b)

Figure 5.6 (a) Protein quantity estimation graph w.r.t BSA standards using BCA (b) BCA Calibration curve

BCA Calibration Curve equation, y = 0.0003x+0.0273 eq1 [graph shown in Fig. 5.6(b)]

Absorbance of supernatant at 562 nm was found to be 0.346 [graph shown in Figure 5.7]

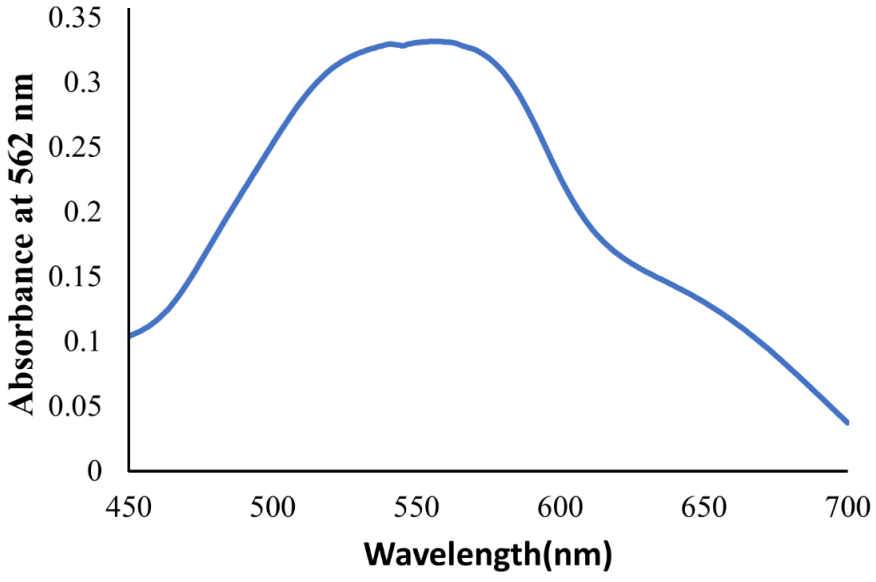


Figure5.7 Estimation of HRP in supernatant of HRP@ZIF-8

By putting the value of absorbance (y) from Fig. 5.7 in equation 1, concentration of enzyme (x) in supernatant was found to be 1mg/ml

The amount of enzyme loaded has been calculated using following formula,

Amount of enzyme immobilized onto the MOF surface = Initial amount of enzyme taken for immobilization − Amount of enzyme left in the supernatant after immobilization

Initial amount of enzyme taken for immobilization= 2 mg

So, the amount of HRP immobilized in ZIF 8 = 1 mg/ml

Also, the overall yield of HRP@ZIF 8 composite = 1.39mg/ml

Therefore, the quantity of HRP enzyme immobilized per unit of weight of composite = 1mg of enzyme / 1.39 mg of composite.

5.6 ESTIMATION OF HRP ACTIVITY POST IMMOBILIZATION

The activity of HRP was measured using Amplite^{R} Blue Colorimetric Peroxidase kit.

For this the standard of HRP enzyme (purchased from Bio quest with mentioned specific activity of 300 units/ml) ranging from 1- 100 mU/ml was prepared and calibration curve was plotted between absorbance and specific activity of HRP standards. The plotted graph is shown in Figure 5.8.

From calibration curve of HRP standards for determination of specific activity, the derived calibration equation was

$$y = 0.002\ x \qquad \text{eq 2 (graph plotted in Fig. 5.7)}$$

Thereafter, equation 2 was used for determining the activity of immobilized HRP enzyme and comparing it with free HRP and analyzing parameters such as stability and reusability.

Absorbance of 0.01 mg/ml HRP solution at 654 nm was found to be 0.560

Corresponding specific activity was 280 mU/ml.

Absorbance of 0.01 mg/ml HRP@ZIF-8 solution at 654 nm was found to be 0.590

Corresponding Specific activity was calculated to be 295 mU/ml.

As enzyme loading capacity was 1mg of HRP/1.39 mg of composite,

Therefore, 1mg/ml of composite has nearly 0.719 mg of HRP.

As 0.719 mg of HRP in immobilized form exhibited specific activity of 295 mU/ml.

1mg equivalent of free HRP in immobilized form showed specific activity of 410.29 mU/ml. Therefore, enhancement in enzymatic activity post immobilization in MOF =

$$\frac{\text{Activity of free HRP} - \text{Activity of immobilized HRP} \times 100}{\text{Activity of free HRP}}$$

There was 46.53% increase in activity post immobilization. This increase might have occurred because of the presence of imidazole in ZIF-8 structure as reported in literature. (Massahud et al.,2023; Wang et al.,2019). To mention ZIF-8 exhibited no enzymatic activity as illustrated in graph in Fig. 5.9.

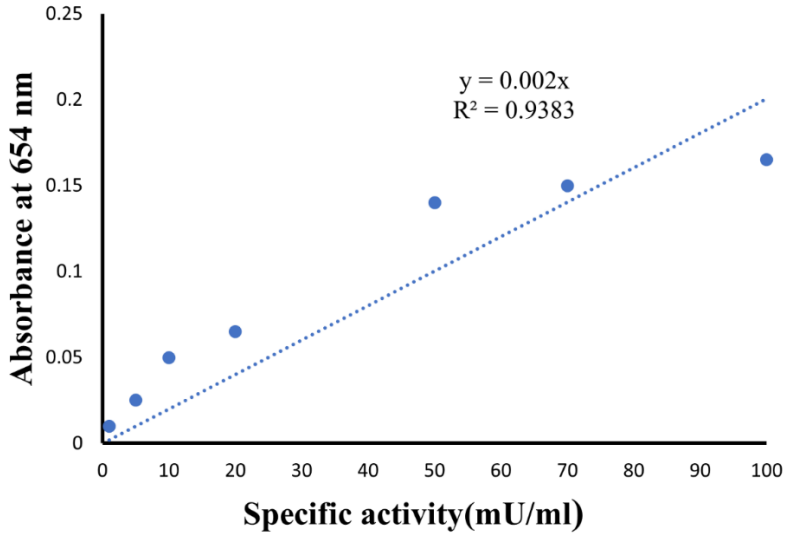


Figure 5.8 Calibration curve for specific activity of HRP standards plotted using Amplite^{R} Colorimetric Peroxidase Assay Kit

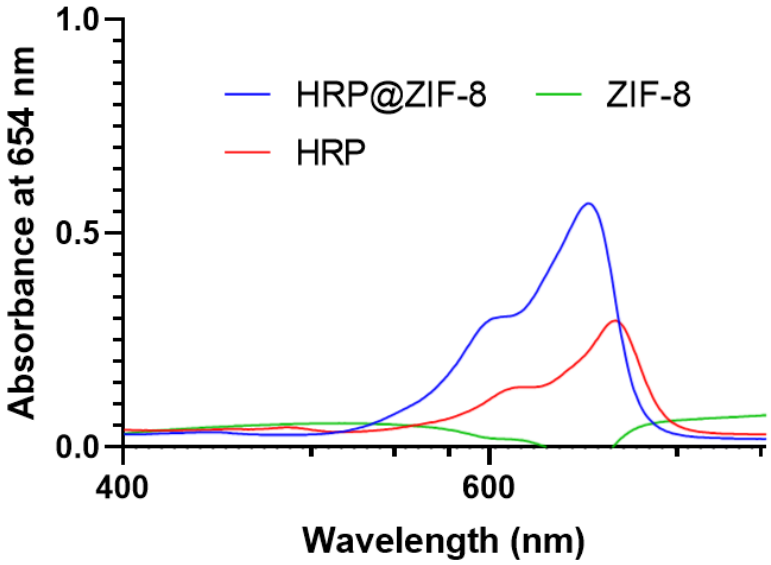


Figure 5.9 Specific activity measurements of (a) ZIF-8, (b) HRP, and (c) HRP@ZIF-8 using Amplite^{R} Blue Colorimetric Peroxidase Assay Kit

5.7 DETERMINATION OF pH AND THERMAL STABILITY OF FREE AND IMMOBILIZED HRP

The stability of HRP and HRP@ZIF-8 was determined for temperature range from 30-80˚C. For this 1mg of free and immobilized HRP was dissolved in PBS buffer at different temperature. Activity at different temperature was determined using Amplite^{R} Blue Peroxidase Activity kit. Graph representing thermal stability of HRP@ZIF-8 shown in Fig. 5.10.

The optimal temperature for the soluble HRP was 30° C. The enzyme activity was assessed at various temperatures from 30° to 80° C. The findings indicated that both the soluble HRP and the immobilized HRP demonstrated thermal stability within the temperature range of 30° C to 40°C. Furthermore, the soluble HRP preserved only 43% of its activity at temperature 60° C, while the immobilized HRP retained 58% at the same temperature. Overall, the results indicated that the immobilized HRP exhibits more thermal stability than the soluble HRP over the evaluated temperature range of 30°C to 80°C. The thermal stability of the immobilized

enzyme is possibly enhanced via micro-environment provided to the enzyme within the structure of MOF.

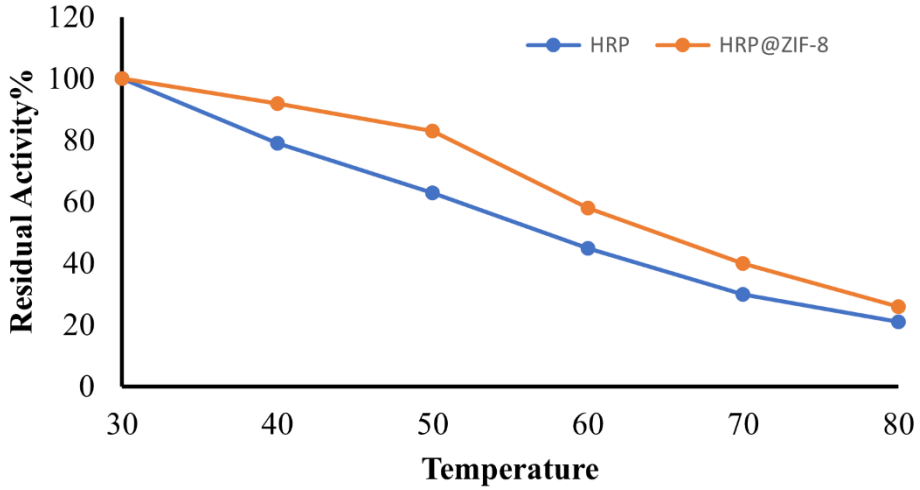


Figure 5.10 Thermal stability of HRP@ZIF-8

The effect of pH on soluble and immobilized HRP activity was tested by incubating in HRP and HRP@ZIF-8 in different buffers with pH values ranging from 4.0 to 9.0 for 10 minutes. Activity of HRP and HRP@ZIF-8 dissolved in different buffers was determined using Amplite^{R} Blue Colorimetric Peroxidase kit. Immobilized HRP had optimum working pH of 7. Optimum pH for HRP is 6.5. Similar findings were seen, with the pH shifting from 6.5 for free HRP to 7.0 for immobilized HRP which is more desirable pH for commercial and industrial application shown in Fig. 5.11. The conformational changes in the three-dimensional structure of the enzyme protein following immobilization might be a possible cause for this pH shift.

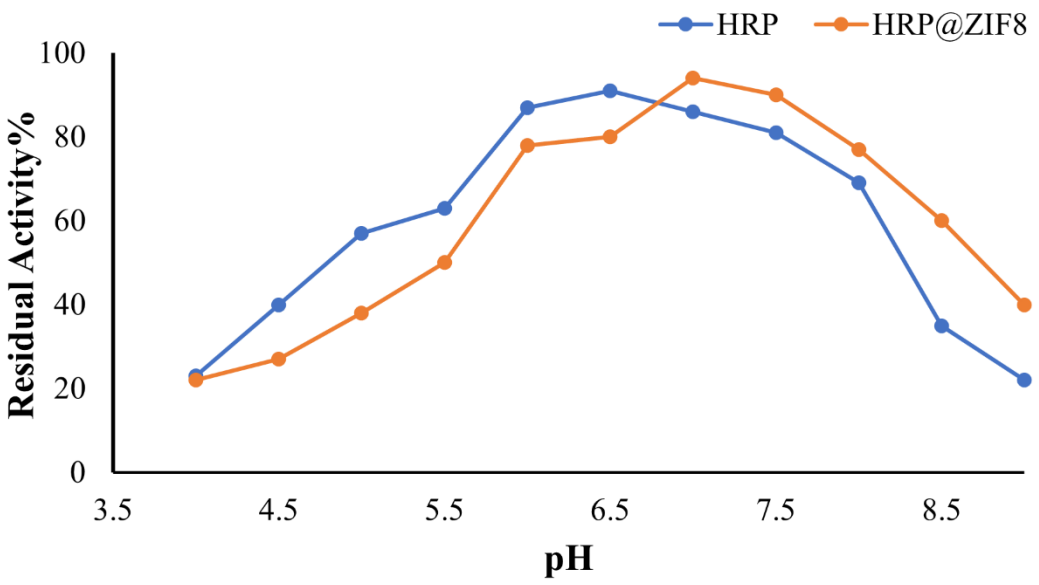


Figure 5.11 Optimum pH of HRP and HRP@ZIF-8

5.8 REUSABILITY STUDIES OF HRP@ZIF-8

Reusability of enzyme is an important factor as it reduces the overall cost of process. Given the excessive expense of soluble enzymes, reusing immobilized enzymes several times is essential. The reusability studies were performed for six cycles. Fig. 5.12 illustrates the number of times the immobilized HRP enzyme can be reused while maintaining an initial enzyme activity of 100%.

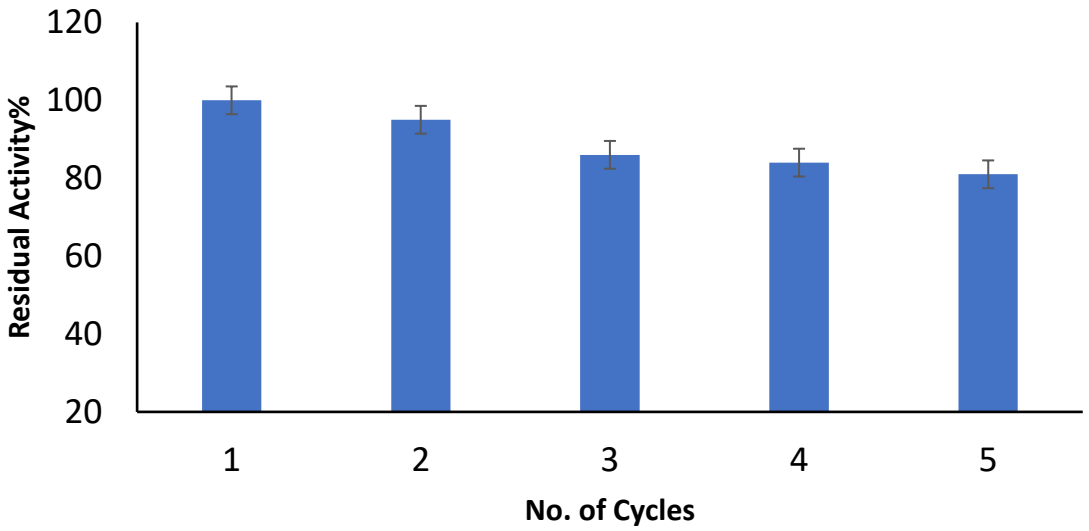


Figure 5.12 Reusability study of HRP@ZIF-8

The immobilized HRP retained around 82% of its original activity for 5 cycles. After 6 cycles the activity significantly reduced to around 30%. The decrease in activity following repeated use may be attributed to the inactivation of enzyme on repeated washing and usage. Also, the prolonged exposure to high amounts of H_2O_2 might have caused damage to the HRP and ZIF-8 structure.

5.9 STABILITY AGAINST LEACHING

The leaching experiments were performed by incubating HRP@ZIF-8 composite in phosphate-buffer saline (PBS) solution for various durations of 1 hour, 2 hours, 5 hours, 12 hours, and 24 hours. The pellet obtained from centrifugation was collected, and the supernatant was analyzed using BCA assay. The supernatant was examined for the presence of protein in sample at various time intervals. The enzyme was stably encapsulated within the MOF structure since negligible amount of enzyme was present in the supernatant. Therefore, the results indicate towards no significant leaching of the enzyme from MOF and stable immobilization of HRP on ZIF-8 as depicted in graph Fig. 5.13.

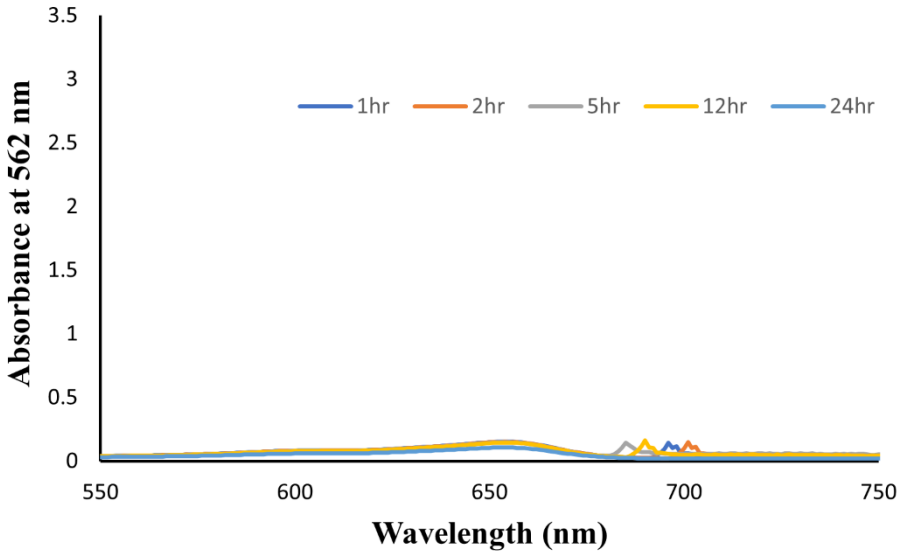


Figure 5.13 Stability of HRP@ZIF-8 against leaching

CHAPTER –6

DISCUSSION

Enzymes function as biological catalysts. They are frequently employed in various industrial applications because they increase efficiency, and have a smaller environmental effect than traditional chemical processes. Despite high efficiency and selectivity of enzymes as catalysts, enzymes often face obstacles like instability under extreme circumstances, continuous decrease in activity along with trouble in recovery and reusability. Enzyme immobilization onto the solid substrates can overcome the above-mentioned problems that limit the use of soluble enzymes in commercial applications. Moreover, immobilization extends the operational lifespan of enzymes and reduces cost and environmental impacts associated with enzyme production and disposal. For efficient of immobilization of enzyme, the substrates should be inert, biocompatible, available at low cost, highly specific and should provide resistance to enzyme from harsh environmental conditions. In this regard, metal-organic frameworks (MOFs) have been explored as efficient substrates for enzyme immobilization attribute to high surface area, adjustable pore size, and possibility of incorporating required functionality. Furthermore, immobilization onto MOFs can enhance enzyme activity by creating a favourable microenvironment, improving substrate accessibility, and using components of MOFs as cofactors/activity enhancers. Also, the enzymes can be protected from severe reaction conditions and regenerated easily for efficient reusability when present into the pores or within the structure of MOFs. Overall, the integration of enzymes into MOFs is a noteworthy breakthrough in the field of biocatalysis.

Enzyme encapsulation onto MOFs is facilitated via one of the three methods, i.e., covalent attachment onto the surface, diffusion into the pores, or *in-situ* encapsulation within the structure of MOFs. The covalent attachment of enzymes onto the surface of MOFs requires chemical agents for surface functionalization and binding. Also, the adsorption of enzyme onto the surface through weak interactions such as Van der Waal's forces and Hydrogen bonding results in high chances of leaching. On the other hand, diffusion of enzyme into the pores of MOF is limited to only a very small fraction of commercially viable enzymes that have size smaller than pore size of MOFs (approximately 2-50 nm). To overcome these limitations, *in-situ* encapsulation emerges as a great approach to immobilize enzymes onto MOFs irrespective of the size of biocatalysts, without the use of chemical agents, and under ambient conditions that do not affect the enzyme's structure and function. There is extensive research on the

increased enzymatic activity, stability (operational and storage), and reusability of enzyme *in-situ* encapsulated within the structure of MOFs.

In the present thesis, HRP was immobilized via *in-situ* encapsulation in ZIF-8. HRP is commercially important enzyme which has its use in research, diagnostics, waste water treatment and probe-based assays. ZIF-8 is the extensively used MOF for immobilization of enzymes because of its large surface area, adjustable pore size, easy synthesis can be synthesized under mild conditions.

After immobilization, a 46.53% increase in the activity of HRP enzyme was observed which may be attributed to the presence of imidazole in ZIF-8 as imidazole is reported to enhance the HRP activity. In cases exploiting other combinations of MOFs and enzymes, the metal ion component of MOF may also act as cofactor for immobilized enzyme, thereby resulting in enhanced activity of enzymes. The encapsulation of HRP enzyme within the structure of MOF resulted in increased operational and storage stability in terms of temperature, pH, and shelf life. Moreover, *in-situ* encapsulation stably incorporated the enzyme onto MOF as indicated by non-significant leaching of enzyme from substrate post incubation in buffer for varying period of time. Also, the HRP immobilization onto MOF facilitated easy regeneration and extensive reusability of the biocatalytic platform which can further reduce the enzyme wastage and operational costs making the processes commercially viable. To summarize, *in-situ* immobilization increases the enzymatic activity, stability, and reusability which is crucial for industrial and commercially viable biocatalytic processes.

Although explored, there is need to explore aspects related to the immobilization of enzymes onto MOFs as there are some loop holes which need to be addressed in future research. First and foremost, MOFs, which require high pressure and temperature for their synthesis, must be synthesized at room temperature without any loss in enzymatic activity. It is necessary to investigate and study MOFs whose metallic components function as co-factors for the enzyme in order to potentially increase the catalytic activity of the enzyme. The heat durability of mounted enzymes is crucial for industrial applications that need high temperatures to improve the reaction rate. Future research must focus on investigating the potential of porphyrins, biopolymers, and amino acids as naturally occurring organic linkers to extend the shelf life and reusability of enzymes. Chemical-free MOF production is needed to make sure the enzyme doesn't degrade.

Enzyme-MOF composite can be used in medicine industry for targeted delivery of therapeutic products. Enzymes encapsulated in MOFs can be used to degrade pollutants in environment.

Future studies should focus on simplifying MOF production and investigating novel MOF compositions that improve enzyme efficiency. Furthermore, examining the relationships between distinct enzyme classes and a range of MOF frameworks may offer a more profound understanding of the processes leading to increased stability and activity.

Overall, enzyme immobilization onto MOFs is a potentially effective way facilitating the improved functioning of enzymes, providing significant advantages in terms of activity, stability, and reusability and lay a solid framework for developing enzyme-based technologies in the future and their use in many industrial processes.

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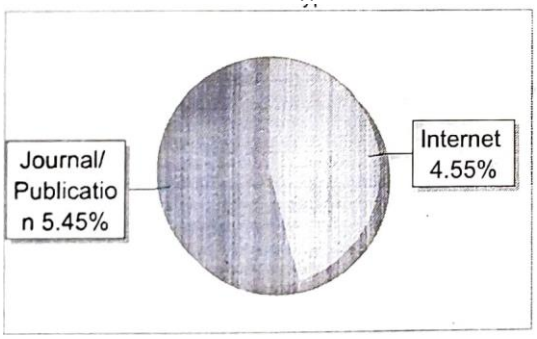

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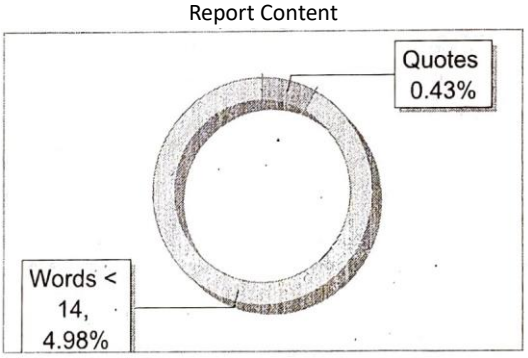

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