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Enzymatic Properties and Optimization of Immobilized Lipase from *Aspergillus niger* by using the ZIF-8 as a Carrier

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Abstract: ***Enzymes are biocompatible and biodegradable molecules, The esoporous hierarchical (ZIF-8) was synthesized in an aqueous solution and prepared using a modulating agent (TEA) to obtaining a hexahedral shape. The (CTAB) was added as a morphological regulating agent. The results of (PXRD) was showed that the morphological properties of ZIF-8 were modified because of the encapsulation. The ANL was encapsulated in ZIF-8, the immobilization efficiencies exceeded 91% and the activity recovery of reached 2150% under the optimized conditions. In terms of enzymatic properties, the results revealed that the temperature and pH stability of the immobilized enzyme increased compared with free lipase.***

Keywords: Enzymatic Properties; Aspergillus niger lipase (ANL); ZIF-8; Immobilization; Esterification; Biotechnology; lipases; Zeoliticimidazolate framework; Immobilization efficiency; lipase activity recovery.

1. Introduction

The *Aspergillus niger* is one of the most important microorganisms used in biotechnology. It has already been in use for decades to produce many extracellular enzymes that were considered Generally Regarded As Safe (GRAS) by the Food and Drug Administration of the United States of America (FDA)[1]. It can produce more than 30 species of enzymes such as lipase, amylase, cellulase, pectinase and glucose oxidase[2]. *A. niger*like other filamentous fungi, should be treated carefully to avoid the formation of spore dust. However, compared with other filamentous fungi, it does not stand out as a particular problem concerning allergy or mycopathology [1-2]. The intense research on *A. niger* over the past decade has resulted in a range of new processes and products [1].

In the same context,lipases (Triacylglycerol Lipase; EC 3.1.1.3) are produced by many microorganisms either alone or together with other members of the hydrolase family[3]. The fungi is widely recognized as preferable lipase sources because they generally produce extracellular enzymes, which facilitate enzyme recovery from the fermentation broth[4]. In particular, the lipase producing strains through solid state fermentation are *Penicilliumcamembertti*, *Rhizopus sp*., *Mucormeihei*, *A.niger* and *Mucorracemosus* [5]

Table I. shows some of the lipases from the fungi with their applications.

Since industrial applications of lipase require specific properties, there is still an interest in additional lipase that could be used in new applications [4-5]. Because of their wide scale applications, lipases remain a subject of intensive study [11]. Lipases research is focused particularly on structural characterization, elucidation of mechanism of action, kinetics, sequencing and cloning of lipase genes and general characterization of performance [14]. In the same line, the practical use of enzymes has been realized in various industrial and biological processes [13]. They have a wide specificity, recognizing various substrates, which permits their use as a catalyst for numerous reactions in pharmaceutical and drug production, energy, and food processes [10-13]. Different strategies have been used in order to immobilize crude or purified As per gillus spp. lipases. Furthermore, the immobilization of enzymes has proven particularly valuable and has been exploited over the last four decades to enhance activity, stability, and substrate specificity for their successful utilization in industrial processes [2-7]. Generally, several natural and synthetic supports have been assessed for their efficiency for enzyme immobilization such as; cross- linking, encapsulation, adsorbing and covalent linking onto a matrix [15].

In this approach, the zeoliticimidazolate framework (ZIF-8) is one of the most widely studied ZIFs,which is composed of Zn tetrahedral groups and 2-methyl imidazolatebridgesthe (ZIF-8) framework can be prepared via conventional methods such as solvothermal synthesis, nonsolvothermal synthesis or room temperature synthesis, and alternative methods, such as mechanochemical, electrochemical and sonochemical ways[16]. (ZIF-8) crystals are appealing candidates as heterogeneous catalysts due to their long life, easy recycling and disconnection from the stocks, high activity and selectivity toward most of the reactions [17]. (Hierarchical ZIF-8) structures fabricated with CTAB and l-

histidine as a co-template exhibit higher activity than activated carbon in the removal of toxic arsenates [16], phthalic acid, and diethyl phthalate[16-17]. This paper reports, the (ZIF-8) was selected as a carrier to improve immobilization with encapsulation and the enzyme properties and optimum conditions for the immobilization process were measured.

Table I: Some of the lipases from the fungi with their applications

Lipases	Application	References
Penicilliumroquefortii	Dairy industry	[6]
Aspergillus oryzae	Detergents	[7]
*Acinetobacter*sp.	Waste management	[8]
Aspergillus niger	Food industry	[9]
Thermomyceslanuginosus	Breaded products and derivatives	[10]
Rhizopusmiehei	Cosmetics and perfumes	[11]
Rhizopusjavanicus	Bioenergy	[12]
Candida rugosa	Pulp and paper industry	[13]

2. Material and Method

2.1 Materials

Aspergillus niger lipase (ANL) was purchased from Sigma. Coomassie Brilliant Blue G250 and bovine serum albumin (BSA) was purchased from Sigma. Other analytical-grade reagents, including $Zn(NO_3)_2{\cdot}6H_2O$, K_2HPO_4 and KH_2PO_4, lauric acid, acetone, 1-dodecanol, ethanol, isooctane, phenolphthalein, 2-Methylimidazole (M-IM), CTAB and sodium hydroxide (NaOH), were purchased from Sinopharm Chemical Reagent Co., Ltd. (Shanghai, China).

2.2 Methods

2.2.1 Identification of Protein Content

Coomassie brilliant blue was prepared to define the protein content[18]. The standard curve with different (BSA) concentrations was prepared (0 as a blank control), and the concentrations started from (100, 200, 400, 600, 800, and 1000 µL). The final volume of each sample was 1000 µL. Afterward, 5 mL of Coomassie blue solution (G250) was added to each concentration and optical density was determined at 595 nm for all of the samples. A standard curve was drawn see Fig. 1.

2.2.2 Preparation of Substrate For Esterification

For esterification, lauric acid and 1-dodecanol as substrates were mixed at a molar ratio of 1:1 in 1 L of isooctane.

2.2.3 Stop Solution

Ethanol–acetone (1:1, v/v) was mixed to obtain a stop solution and terminate the esterification reaction.

2.2.4 Synthesis of Mesoporous Hierarchical ZIF-8

Hierarchical ZIF-8 was prepared in accordance with a previously described method [17] with slight modifications. In brief, 1.48 g of $Zn(NO_3)_2{\cdot}6H_2O$, 1 g of CTAB, and 1.688 g of His were dissolved in 100 mL of H_2O (DI) and stirred until they were completely dissolved. Subsequently, 100 mL of H_2O was added to a mixture containing 3.24 g of 2-methylimidazole and 4 g of triethylamine (TEA) and stirred until they were completely dissolved. A solution of M-IM and TEA was mixed slowly with a solution of $Zn(NO_3)_2{\cdot}6H_2O$, CTAB, and His at 300 rpm and kept at room temperature (28 ± 2 °C) for 14 h. The obtained white precipitates were collected through centrifugation and then washed with 50% water–ethanol solution (v/v) at 60 °C for 4 h twice to remove the unreacted CTAB. Finally, the precipitate was washed with a buffer to extract ethanol, and the sample was dried at 100 °C under vacuum for 10 h. PXRD analyses were carried out using potassium and copper radiation (40 kV and 40 mA) to examine the crystalloid structure of the samples.

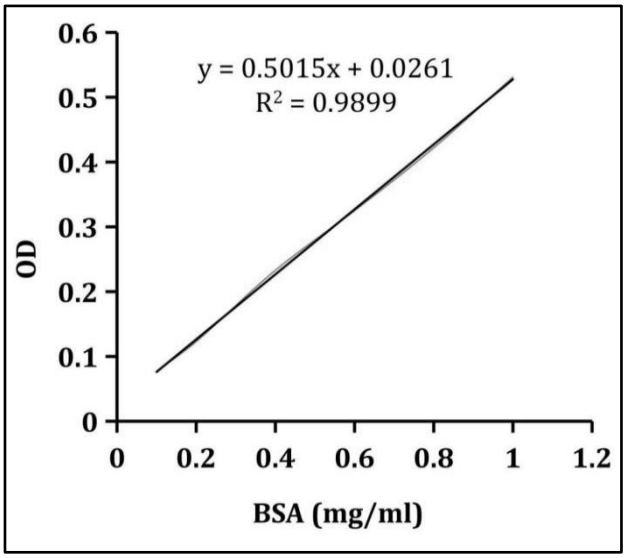

Figure 1: Standard curve of BSA in Bradford protein assay

2.2.5 ANL Lipase Encapsulation in ZIF-8

ZIF-8-encapsulated lipase was prepared in accordance with a modified method[19]. In brief, 0.148 g of 0.5 M $Zn(NO_3)_2$ was dissolved in 1 mL of DI water and dripped through a syringe into a mixture of a certain amount of lipase and 2-methylimidazole (0.656 g, 0.8 M, and 10 mL) with stirring at 200 rpm and at (25 °C to 50 °C) for (10–60 min). The formed nanoparticles were collected by centrifuging at 8000 rpm for 6 min and washed twice with DI water to remove the excess unbound lipase. (ANL@ZIF-8) was vacuum dried and stored at 4°C until further use see Fig.2.

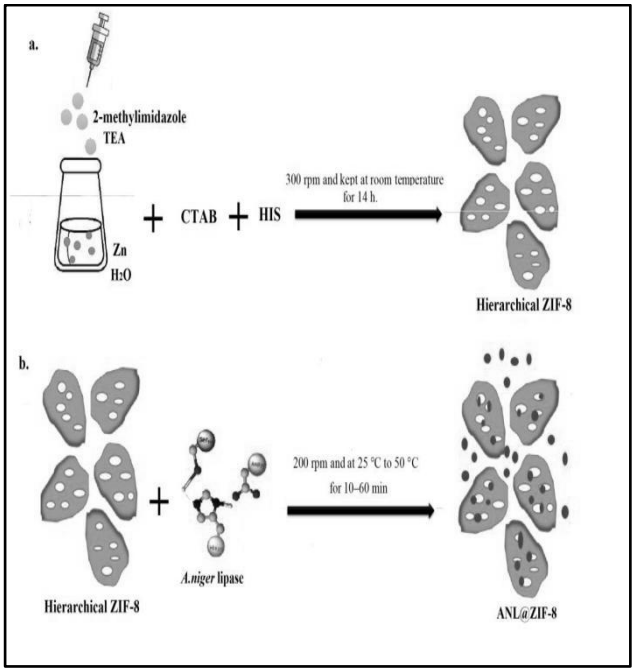

Figure 2: Schematic model to illustrate procedure of this paper study.

2.2.5.1 Measurement of the Protein Content And Activity of The Immobilized Enzymes

The remaining protein content in the supernatant was measured to determine the amount of immobilized enzyme through the Bradford protein assay with BSA as a standard protein[18].

2.2.5.2 Lipase Activity Assay on Esterification Reaction

The activity of (ANL@ZIF-8 and free ANL) was examined with esterification in accordance with previously described methods[20]. A specific amount of free and immobilized enzymes was added to 10 mL of a mixture containing 1-dodecanol (0.2 M) and lauric acid (0.2 M) in isooctane with the addition of 0.01 mL of water. The reactions were performed at 40°C for 30 min with continuous stirring at 200 rpm. Afterward, the reactions were stopped by adding 1 mL of the samples to 5 mL of the stop solution consisting of acetone–ethanol 1:1 (v/v). NaOH (0.05 M) was used in a titration to determine the residual acid in the sample. Phenolphthalein solution (0.05%, w/v) was used as a pH indicator. One unit of enzyme activity (U) was expressed as the quantity of lipase required to liberate 1 μmollauric acid per 1 min under the assay conditions. Specific activity (U/g protein), activity recovery (%), and immobilization efficiency (%) were determined using following equations:

$$\text{Immobilization efficiency}\,(\%)=\frac{\text{immobilized protein}}{\text{total loading protein}}\times 100\% \quad (1)$$

$$\text{Specific activity}\,(\text{U g}^{-1}\text{protein})=\frac{\text{initial activity}}{\text{protein content of immobilized lipase}}\times 100\% \quad (2)$$

$$\text{Activity recovery}\,(\%)=\frac{\text{specific activity of immobilized lipase}}{\text{specific activity of adding free lipase}}\times 100\% \quad (3)$$

2.2.5.3 Optimization of ANL@ZIF-8

The parameters of immobilization should be investigated and optimized to conserve the initial activity of enzymes and to obtain a high immobilization efficiency. In the immobilization procedure using 100 mg support, the effects of enzyme loading (4 mg of protein /g of support to 7 mg of protein /g of support), period immobilization (10 to 60 min), pH (4 to 8)and immobilization temperature (30 °C to 55 °C) on specific activity, immobilization efficiency, and lipase activity recovery were examined.

2.2.5.4 pH Stability and Temperature Stability of ANL@ZIF-8

The immobilized and free enzymes were added to various pH buffers for 1 h, and their enzyme activities were measured. Residual activity was the percentage of the treated enzyme activity relative to the untreated enzyme activity. In the same context, the (ANL@ZIF-8) and free enzymes were processed at various temperatures for 1 h, and their enzyme activities were determined. Residual activity was the percentage of the treated enzyme activity relative to the untreated enzyme activity.

2.2.5.5 Calculation of Stability of ANL@ZIF-8

The relative activity of (free ANL and ANL@ZIF-8) was calculated with the following equation:

$$\text{Relative activity}\,(\%)=\frac{\text{residue activity}}{\text{origin activity}}\times 100\% \quad (4)$$

3. Result

3.1 PXRD Analysis

PXRD analysis demonstrated the differences in the peak intensities of (ZIF-8 and ANL@ZIF-8) see Fig.3. The main peaks denoted the increase in intensity for ZIF-8 at 2θ = 10.98°, 15.11°, 17.04°, 18.01°, 21.76°, 27.78°, and 29.05°, which corresponded to 1830, 1842, 2800, 3708, 1387, 1506, and 2280 plates of the crystal face structure, respectively. By contrast, the (ANL@ZIF-8) intensity decreased due to the diminished distance between the atomic layers in the crystalline material and the decrease in(ANL@ZIF-8) crystallinity after immobilization[21]. This result showed that themorphological properties of (ZIF-8) were modified because of the ANL encapsulation.

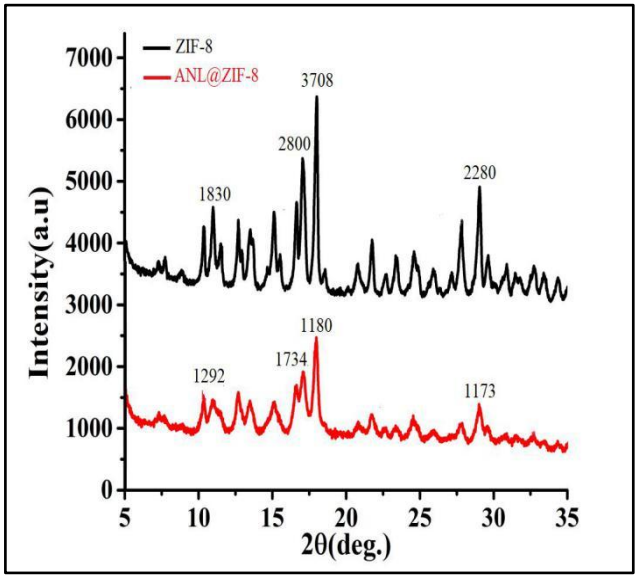

Figure 3: Powder X Ray diffraction (PXRD) patterns of ZIF-8 and ANL@ZIF-8

3.2 Effects of Immobilization Parameters

3.2.1 Effect of The Amount of Enzymes

In (ANL@ZIF-8), the amounts of lipase were from (4 mg of protein /g of support to 7 mg of protein /g of support). The maximum activity recovery was 2000% when ANL loading was (6.5 mg of protein /g of support), whereas the immobilized efficiency consistently decreased when loading increased see Fig. 4.

3.2.2 Effect of Time

The activity recovery increased as encapsulation time increased and reached the highest value of 2100%and the immobilization efficiencies was 90% at 30 min. This value then decreased when the encapsulation time exceeded 30 min. The time did not significantly impact the immobilization efficiency see Fig. 5.

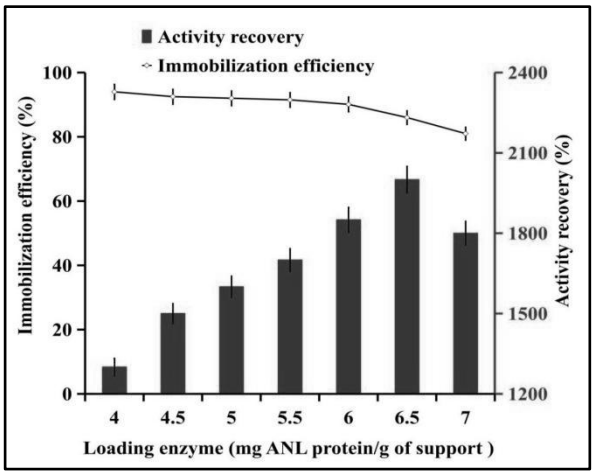

Figure 4: The effect of enzyme loading on immobilizationANL@ZIF-8

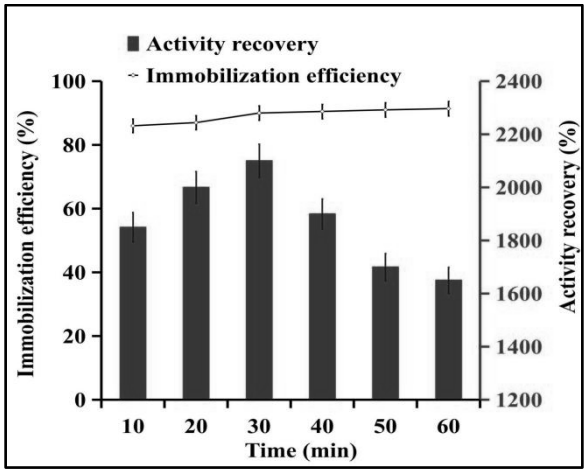

Figure 5: The effect of reaction time on immobilization ANL@ZIF-8

3.2.3 Effect of Reaction Temperature

The immobilization efficiency increased gradually. Simultaneously, the activity recovery increased and reached a maximum value of 2155% and the immobilization efficiencies was 91% at 45 °C. Thereafter, the activity recovery declined because of the thermal inactivation of lipase see Fig. 6.

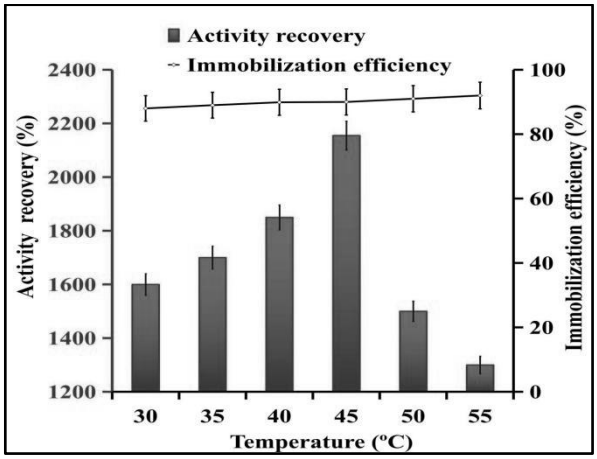

Figure 6: The effect of reaction temperature on immobilization ANL@ZIF-8.

3.2.4 Effect of pH on Immobilization

pH is an essential factor. The activity recovery improved gradually to reach the highest value of 2150% and the immobilization efficiencies was 91% at pH 6. At the same time, an alkaline medium is helpful for ANL immobilization on ZIF-8 see Fig. 7.

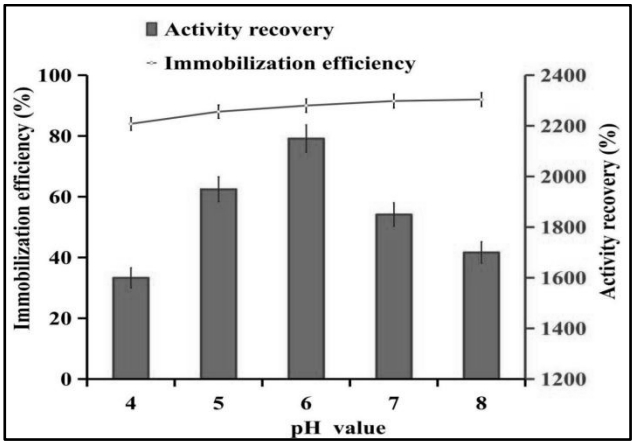

Figure 7: The effect of pH value on immobilization ANL@ZIF-8

3.3 pH Stability and Temperature Stability

At pH 4, the residual activities of the free enzyme and the immobilized enzyme were 55% and 70.2%, respectively. By that point,(ANL@ZIF-8) was more stable than the corresponding free enzymes at pH 8 see Fig. 8. And also, the immobilized enzymes was more stable to temperature changes than the free enzymes. The free enzyme began to be strongly inactivated from 45 °C. When the temperature increased the remaining activity was 62.2%. By contrast, immobilized ANL was slowly inactivated at 55 °C see Fig. 9.

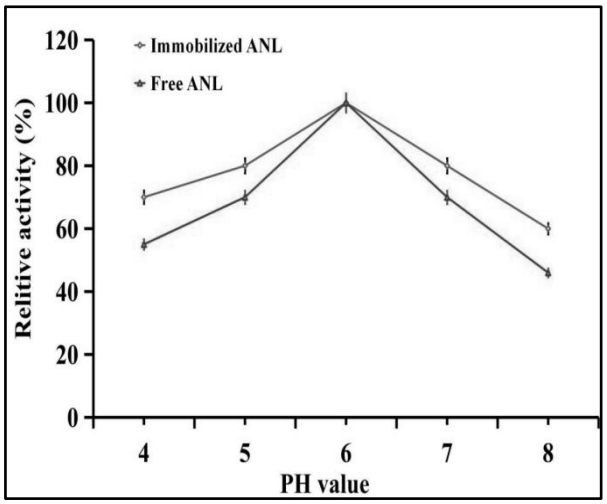

Figure 8: The effect of various pH values on the immobilized enzyme activity.

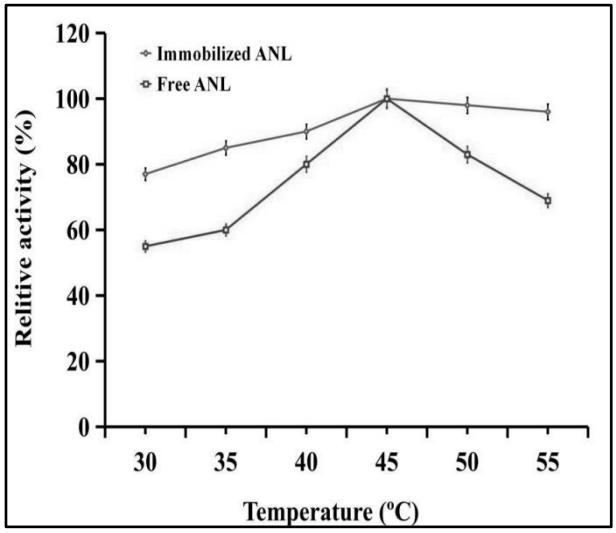

Figure 9: The effect of temperature on the immobilized enzyme activity

3. Discussion

ZIF-8 was used as a carrier for the immobilization of ANL via encapsulation. The morphological characteristics of ZIF-8 changed to after ANL was encapsulated, and these changes referred to the enzyme that overlap with the components of ZIF-8 during formation by the affinity of protein toward imidazole, which is defined as the basic building block of ZIF-8.In this procedure, ANL regulates the crystal size, morphological characteristics and crystallinity as it encapsulates itself within a porous crystal and concomitantly creates new cavities that tightly surround ANL and form bonding interactions with proteins [21-22]. Another study, constructed hierarchical ZIF-8 with 5–20 nm pore sizes via utilizing (CTAB) as a morphology-regulating agent and histidine (His) as an aiding factor and used it as a kind of porous absorbent to remove arsenate [16].

The optimal enzyme loading, immobilization time, temperature, and pH were the chosen immobilization conditions for optimization. The maximum activity recoveries of (ANL@ZIF-8) was 2000% at (6.5 mg of protein /g of support) of lipase loading. This recovery decreased when lipase loading exceeded these concentrations[23]. A high concentration of enzyme loading could lead to the formation of an adsorption multilayer on a hydrophobic structure, thereby decreasing the porous diameter and limiting the diffused growth[14-19]. Although a high lipase loading likely results in a high reaction rate in terms of reaction kinetics, the corresponding increase in viscosity at a high lipase loading can decrease the mixing efficiency of a reaction mixture containing ANL lipase and ZIF-8[24]. The kinetic advantage of a high lipase concentration was reduced by a poor mass transfer efficiency when lipase loading was too high, resulting in low lipase activity.

In immobilization time, the maximum activity recoveries of (ANL@ZIF-8) was reached at 30 min. When the immobilization time exceeded 30 min, these values decreased. The watery phase negatively affected the enzyme activity as the immobilization period was prolonged. This result was consistent with that of Su *et al.*[25], who reported

enzyme absorption occurring for a long period through immobilization, which might have caused instability in an aqueous phase. Therefore, immobilization should be completed within a short period.

In terms of immobilization temperature, the highest activity recoveries of (ANL@ZIF-8) was obtained at 45 °C . Thereafter, activity recovery declined because low temperature retains enzyme properties, including conformation and stability, and inhibits protein denaturation in an aqueous buffer. This observation was consistent with that of Li *et al.*[24], who demonstrated that the enzymatic reaction rate increases as temperature increases to a certain level; high temperature subsequently causes protein denaturation and thus decreases the reaction rate.

The pH is an important factor that affects (ANL@ZIF-8) immobilization. For example, an alkaline environment is beneficial for the Schiff base reaction between lipase and (ZIF-8). Thus, the immobilization efficiency increased slightly at high pH. An acidic buffer was likely unfavorable to enzyme activity, whereas basic conditions positively affected enzyme activity [19]. The highest enzyme activity was obtained at pH 6. This phenomenon occurred probably because the difference in pH of a reaction mixture affects the reactivity of a functional group, such as COOH, of an enzyme, thereby reducing the adsorption of lipase in ZIF-8 pores[19-23]. After immobilizing (ANL) in (ZIF-8) and obtaining a high activity recovery, we should further investigate enzyme properties, such as pH stability and temperature stability[21]. Given that the stability of an enzyme is decisive to its practical applications, the enzymatic properties of ANL was examined in this study.

After an immobilization was completed, the residual activity remained high but decreased under the influence of various pH values. The dissociation effects of an active centre on proteins differ, and their specific spatial conformations are altered, resulting in a reduction in catalytic activities [16-19]. At above 55 °C, the activity of the free enzyme rapidly decreased. By contrast, the activity of the immobilized enzymes remained relatively stable until 55 °C and then gradually decreased. The improvement in the temperature stability of the immobilized enzyme is due to the rigidity of the carrier ZIF-8 that enhances the rigid structure of lipase and prevents the enzyme from melting the chain or configuration at high temperatures, thereby delaying the decrease in enzyme activities [23].

4. Conclusion

The PXRD patterns exhibited the same results about the successful immobilization of lipase through differences in the peak intensities of ZIF-8 and immobilized lipases. The *A. niger* lipase was successfully immobilized in (ZIF-8) through encapsulation. The activity recovery of (ANL@ZIF-8) was as high under the optimized conditions. These conditions resulted increase in the activity recovery of the enzyme compared with that of the free form.

5. Abbreviations and acronyms

ANL	*Aspergillus niger* lipase
ANL@ZIF-8	ANL was encapsulated in ZIF-8
BSA	Bradford protein assay
CTAB	Cetyltrimethylammonium bromide
H_2O	Water
HIS	L-histidine
K_2HPO_4	Dipotassium phosphate
KH_2PO_4	Potassium dihydrogen phosphate
M-IM	2-Methylimidazole
NaOH	Sodium hydroxide
TEA	Triethylamine
ZIF-8	Zeoliticimidazolate framework
$Zn(NO_3)_2{\cdot}6H_2O$	Zinc nitrate hexahydrate

Acknowledgments

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