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Enhanced Activity and Stability of Lipase via Immobilization in Pectin-Based Hydrogels

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ABSTRACT

Pectin-based hydrogels are hydrophilic polymeric networks created from the cross-linking of the plant-based polysaccharide pectin. In this study, the hydrogels were synthesized using chemical modification with glycidyl methacrylate and used for the immobilization of *Aspergillus Niger* lipase (ANL). The immobilization efficiency and relative activity recovery achieved about 82% and 88.3%, respectively. Scanning electron microscopy revealed the porous three-dimensional structure of the pristine hydrogels, and the surface became rougher and the pore structure changed during the immobilization of the lipase enzymes, indicating the successful immobilization of the enzymes within the hydrogels. Fourier transform infrared spectroscopy confirmed the presence of the enzyme within the hydrogels, with the presence of the carbonyl and amide groups at 1650 and 1540 cm^{-1}, respectively. While, the functional groups of the pectin-based hydrogels remaining intact during the immobilization process. At 60°C, the immobilized lipase showed 15% of its original temperature stability compared to 5% for the free lipase. At pH 5, the immobilized enzyme was found to have 70% of its maximum pH activity, while the free enzyme was found to have 52% of its maximum pH activity. These results indicate pectin hydrogels are effective supports for lipase immobilization, improving enzyme stability for biocatalytic applications.

1 INTRODUCTION

Hydrogels are hydrophilic, water-insoluble polymer materials that are able to retain significant amounts of water within a three-dimensional network resulting from physical or chemical crosslinking. Hydrogels have been used for several different purposes due to their swelling characteristics, which are attributed to the diffusion of water molecules into the polymer network accompanied by relaxation of the polymer chains. Hydrogels have been used for food processing, drug delivery systems, tissue engineering, biosensors, water treatment, and soft electronics [1-3].

Because hydrogels are hydrophilic and have significant amounts of water content, they are biocompatible, biodegradable, and have low toxicity. These characteristics have made hydrogels suitable for enzyme immobilization matrices. Hydrogels provide enzymes with a microenvironment for stability and activity. Hydrogels made of natural polysaccharides are also suitable for enzyme immobilization matrices because they have sufficient functional groups for crosslinking and modification. Pectin-based hydrogels are thermally and mechanically stable hydrogels, especially when combined with reinforcing fibers [4-6].

Pectin is a complex biopolymer and an important structural component of the primary cell wall and the middle lamella of higher plants. It is composed mainly of a linear chain of α-(1→4)-linked D-galacturonic acid residues that are interrupted by rhamnose and have neutral sugar side chains consisting of arabinose and galactose. The carboxyl groups of the galacturonic acid are esterified with methyl and acetyl groups. These esterification patterns give rise to considerable structural and functional diversity among various pectin molecules depending on their plant origin [7-10].

Depending on the extent of esterification (DE), pectin is of two types: high-methoxyl pectin (HMP) and low-methoxyl pectin (LMP), where DE > 50% and DE < 50%, respectively. The esterification pattern of pectin is critical in determining its gelling properties. HMP requires acidic conditions and high concentrations of sucrose for gel formation, whereas LMP forms gels in the presence of divalent ions. The gel network is held together by hydrogen bonds and electrostatic forces between the

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carboxyl and hydroxyl groups. The low charge repulsion under acidic conditions favors the association of the molecules [8, 9]. Pectin is a water-loving and a water-hating molecule, so it is amphiphilic. Hence, it can be used as a natural food additive for thickening, gelling, and modifying textures. The water-loving property makes it possible for hydrogels to absorb water and body fluids as well as bioactive compounds [13, 14].

Immobilized enzymes can be obtained by considering the properties of the polymer used as a support, the immobilization technique used, and the effects on enzyme activity. The pH, temperature, enzyme-substrate binding, and stability should be taken into consideration for the immobilized enzyme. Hence, the choice of the right immobilized enzyme is very important for high enzyme activity [15, 16]. Lipases, EC 3.1.1.3, are versatile biocatalysts that mediate reactions such as esterification, transesterification, and hydrolysis, but their use in industrial applications is hindered by factors such as cost, stability, and reusability problems. Immobilization on a hydrophilic support, especially entrapment in a hydrogel, is a viable strategy to improve lipases' stability, alcohol tolerance, and long-term activity without compromising activity. There are a number of benefits associated with entrapment, including mild handling, affordability, retention of activity, and substrate accessibility, which are facilitated by the swelling ability of hydrogel matrices, thus enabling enzyme retention in its original form, making it suitable for environmental and biotechnological applications [17-19]. According to the available literature, there is no report of ANL immobilized on pectin hydrogel. So, in this study ANL immobilized and morphology characterized on pectin hydrogel. Finally, the biochemical properties of immobilized enzyme compared with free enzyme.

2 MATERIALS AND METHODS

2.1 Materials and instruments

Pectin and glycidyl methacrylate acquired from Merck (Germany). Lipase from *Aspergillus Niger* (ANL), Albumin from bovine serum (BSA), and p-nitrophenyl palmitate (pNPP, 98%), were purchased from Merck company (Germany). All chemical reagents used in this research were of analytical grade. All water used has been purified through a water purification system (Milli-Q Integral 5, Millipore), having a resistivity of 18.2 MΩ cm. FESEM and EDX were carried out on a MIRA 3 FESEM instrument (TESCAN, Czech Republic), and FT-IR spectra were recorded in the range of 400-4000 cm^{-1} on a Tensor 27 FT-IR spectrophotometer (Bruker, Germany). Absorption spectra were recorded on a Carry 50 UV-Vis spectrophotometer (Varian, Australia).

2.2 Synthesis of Pectin Hydrogels

The pectin hydrogels were prepared in two consecutive stages, according to the methodology described previously [20, 21], with minor modifications. In the first stage, the polysaccharide modification process was performed. Specifically, 10 g of the polysaccharide was dispersed in 0.5 L of distilled water at 50 °C. The pH of the solution was adjusted to 4.0 by adding hydrochloric acid. Then, 10 mmol of glycidyl methacrylate was added to the solution. The flask containing the solution was sealed to avoid solvent loss during the process. The solution was incubated in an orbital shaker at 60 °C for 24 h. Once the polysaccharide modification process was completed, the solution was dried in a circulating-air oven at 50 °C for 24 h. The resulting films of modified polysaccharide, prepared in Petri dishes, were treated with an ethanol solution to eliminate the unreacted constituents [20, 21].

In the second stage, the hydrogel was prepared by dissolving 8.0 g of the modified polysaccharide in 40 mL of deionized water at 45 °C, under continuous magnetic stirring for approximately 30 min. Then, 0.6 mmol of potassium persulfate were added to the solution. The resulting solution was homogenized, transferred to syringes, and maintained at 50 °C for 72 h in a circulating-air oven to allow the cross-linking process of the constituents. Once the cross-linking process was completed, the hydrogel was extruded from the syringe, cut into small fragments weighing approximately 100 mg, washed with distilled water to eliminate the unreacted constituents, and dried in the circulating-air oven at 60 °C for 24 h [20, 21].

2.3 Lipase activity

The activity of lipase was measured using p-nitrophenyl palmitate (pNPP) as the substrate. The reaction mixture contained 470 µL of 40 mM sodium phosphate buffer at pH 7.0, 10 µL of 6 mM pNPP solution in isopropanol, and 30 µL of free or immobilized lipase solution with similar protein concentrations of 1 mg/mL. The reaction mixture was incubated in a water bath at 40°C for 30 minutes. The reaction was stopped by centrifuging the mixture at 3,500 rpm for 10 minutes. The absorbance of the solution was measured at 412 nm. The hydrolyzing activity of the support without the enzyme was measured under the same reaction conditions. In addition, reaction mixtures without the enzyme were prepared and contained all the components required for the reaction. These reaction mixtures were used to determine the rate of spontaneous hydrolysis of the substrate [22].

2.4 Synthesis of Immobilized Enzyme

Lipase was immobilized on pectin hydrogel by reacting 4 mg of the enzyme with 40 mg of hydrogel at 4 °C for 3 hours. The immobilized lipase was then recovered via centrifugation at 6500 rpm for 15 minutes at 4°C. The activity of the immobilized lipase was then determined as described in section 2.2. The concentration of proteins in the supernatant was determined via the Bradford method, where absorbance readings were obtained at 595 nm based on a standard curve of BSA concentrations. The interference of imidazole and other reagents was investigated via control samples [23].

$$\text{Encapsulation yield (\%)} = \frac{\text{amount of protein in pectin hydrogel}}{\text{amount of initial protein}} \times 100$$

$$\text{Activity recovery (\%)} = \frac{\text{total activity of immobilized enzyme}}{\text{initial activity of the free enzyme}} \times 100$$

2.5 Morphological Investigation of Immobilized Enzymes

Various techniques were used for the characterization of the samples, and these techniques include scanning electron microscopy (SEM), energy dispersive X-ray spectroscopy (EDX), and Fourier transform infrared spectroscopy (FT-IR).

2.6 Biochemical Characterization of Free and Immobilized Enzymes

2.6.1 Effect of Different Temperatures on the Enzyme Activity and Stability

The catalytic activities of the free and immobilized enzymes were measured in the temperature range of 30-80 °C, with the pH maintained at 8.0. Each reaction mixture was adjusted to 1.0 mg/mL of protein concentration for the free and immobilized enzymes, with 5 µL of enzyme, 485 µL of 40 mM phosphate buffer solution (pH 7.5), and 10 µL of 5 mM substrate solution in isopropanol. Control reactions were carried out in the absence of enzyme but with all other reagents as in the enzyme-catalyzed reactions. Each reaction mixture was then subjected to the desired temperature for 30 minutes, followed by centrifugation at 3000 g for 10 minutes, with the absorbance of the supernatant measured at 410 nm [15, 16].

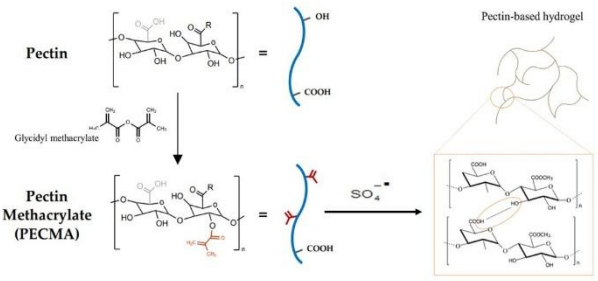
2.6.2 Effect of Various pHs on the Enzyme Activity and Stability

The enzyme activity of both free and immobilized enzymes was tested over a pH range of 4.0 to 11.0, as per the enzyme assay protocol outlined in Section 2.2. To determine the stability of the enzyme, various buffers were used to test the stability of both free and immobilized enzymes over the pH range of 4.0 to 11.0. The samples were prepared by adding equal concentrations of free or immobilized enzyme (1.0 mg/mL) to the buffers. The samples were incubated for two hours at the desired pH at room temperature. The remaining enzyme activity was measured under the optimized conditions. For each pH, control samples were also set up, which consisted of the same reaction mixture without the enzyme [18, 19].

3 RESULTS AND DISCUSSION

3.1 Immobilization of Lipase on Pectin Hydrogel

Scheme 1) shows the schematic representation of the grafting reaction mechanism of the formation of pectin hydrogel by glycidyl methacrylate [5, 24].



Scheme 1) Schematic representation of the grafting reaction mechanism of the formation of pectin hydrogel by glycidyl methacrylate [5, 24].

The immobilization efficiency was achieved 82%, along with a relative activity recovery of 88.3% for lipase. Although immobilization often results in enzyme activity loss, this value was relatively high. Enzyme deactivation can be caused by the immobilization process, the working conditions (pH, temperature, etc.), and interactions with the support, which may involve structural distortions. There are other factors that can affect enzyme deactivation, such as mass transfer, which may require special approaches to recover enzyme activity [15, 16].

3.2 Morphology Characterization

3.2.1 SEM Analysis

From the scanning electron microscopy (SEM) analysis of both the pristine pectin hydrogel and the lipase-immobilized pectin hydrogel, it can be seen that there are significant differences in the morphology of the two materials.

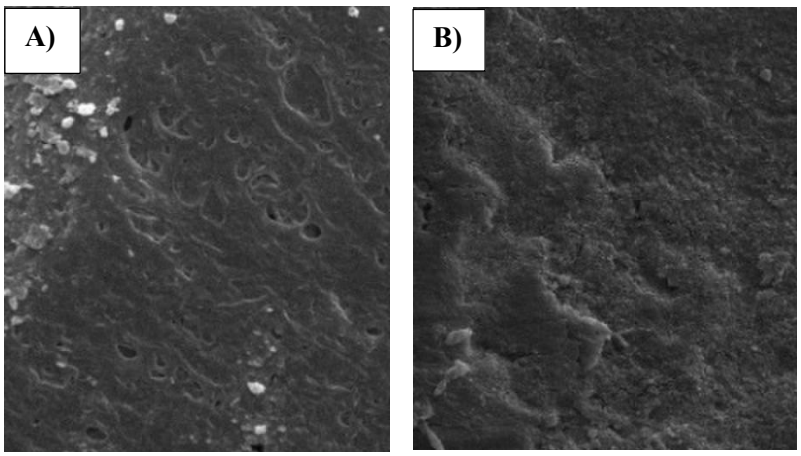


Fig. 1. A) shows the SEM image of pectin hydrogel, B) SEM image of pectin hydrogel after immobilizing the lipase

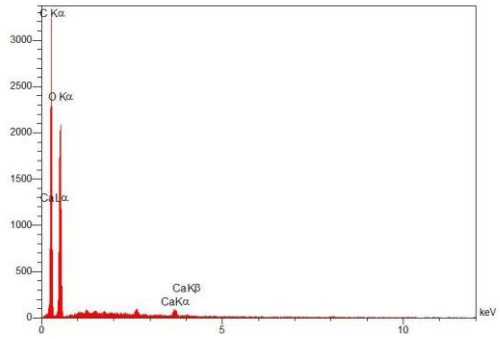
As shown in Fig. 1A, the pristine pectin hydrogel possesses its characteristic porous structure. The surface of the hydrogel is moderately rough, and the pores are homogeneously distributed in the hydrogel matrix. From

Fig. 1B, it can be seen that the morphology of the hydrogel changes significantly after immobilizing the lipase. The surface of the hydrogel becomes significantly rougher and more heterogeneous. There are also enzyme aggregates or particles present in the hydrogel structure. The pores of the hydrogel appear to be filled, which can be attributed to the successful immobilization of the enzyme using the process of physical adsorption and entrapment.

The morphological changes observed in this study, such as surface roughness and heterogeneous enzyme distribution, are consistent with established criteria for successful enzyme immobilization, as described in literature. Hasanah et al. (2019) and Cargnin et al. (2020) have reported similar transformations in pectin hydrogels after enzyme immobilization, which they attributed to enzyme-support interactions and increased enzyme stability, respectively [4, 5]. Cargnin et al. (2020) have also shown that scanning electron microscopy is an efficient method for characterizing enzyme distribution in hydrogels, where enzyme immobilization was shown to alter the hydrogel surface while retaining substrate accessibility [12]. These findings confirm that pectin hydrogel is an effective matrix for lipase immobilization, with potential applications in biodiesel production, food processing, and biosensor development. The successful interaction between the enzyme and the matrix is expected to enhance enzyme stability compared to free enzyme systems.

3.2.2 Edax Analysis

Energy Dispersive X-ray (EDX) spectroscopy was carried out to determine the elemental composition of the pectin hydrogel, which provided valuable insights into the cross-linking mechanism and chemical composition.



Elt	Line	Int	Error	K	Kr	W%	A%	ZAF	Formula	Ox%
C	Ka	473.3	7.7764	0.5493	0.3342	43.98	51.58	0.7599		0.00
O	Ka	362.2	7.7764	0.4272	0.2599	54.30	47.81	0.4786		0.00
Ca	Ka	16.5	0.6121	0.0234	0.0143	1.72	0.60	0.8294		0.00
				1.0000	0.6083	100.00	100.00			0.00

Fig. 2. Edax analysis of pectin hydrogel

The EDX spectrum (Fig. 2) indicates the presence of oxygen and calcium, as the peaks corresponding to the Kα lines of oxygen and the Lα, Kα, and Kβ lines of calcium were present in the EDX spectrum. The quantitative elemental composition (Fig. 2) showed that the composition of the hydrogel contained 43.98% carbon, 54.30% oxygen, and 1.72% calcium, along with traces of other elements. The high oxygen content (54.30%) reflects the polysaccharide nature of pectin, and the calcium content (1.72%) indicates that ionic cross-linking of pectin chains has taken place via the 'egg-box' mechanism. This hydrogel composition profile is in agreement with that reported by Beigi et al. (2023) and Aghajani et al. (2025) for pectin hydrogels, indicating that calcium cross-linking of pectin hydrogels results in hydrogels that possess high oxygen and calcium content [1, 21].

The EDX data reported here are consistent with theoretical predictions for calcium cross-linked pectin hydrogels reported by Said et al. (2023), where calcium content ranges from 1 to 5% by weight for calcium cross-linked pectin hydrogels. The carbon-to-oxygen ratio of 0.81, as observed from EDX data, is indicative of polysaccharide hydrogels. The EDX analysis data reported here serve to corroborate the hydrogel structure and cross-linking density, indicating that the hydrogel preparation method and cross-linking agent were effective. This analysis has provided further support for the hydrogel for use as an immobilization matrix for enzymes [2].

3.2.3 FTIR analysis

Fourier Transform Infrared (FTIR) spectroscopy was employed in the characterization of the chemical structure and functional groups in the pectin hydrogel prior to and after lipase immobilization. The FTIR spectrum of pristine pectin hydrogel shows absorption bands that are characteristic of the functional groups in the polymer chain, given that pectin is a polysaccharide.

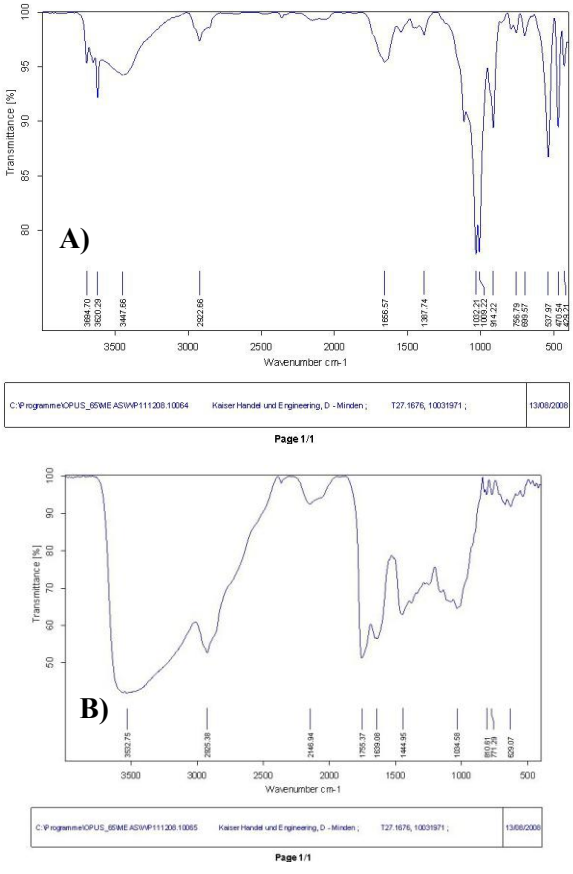


Fig. 3. A) FTIR analysis of pectin hydrogel, B) FTIR analysis of lipase immobilized in pectin hydrogel

The absorption bands in the FTIR spectrum of pristine pectin hydrogel in the region 3600-3000 cm^{-1} are due to the O-H stretching vibrations (Fig. 3A). The absorption bands at 2920 cm^{-1} and 2850 cm^{-1} are due to C-H stretching. The absorption bands in the region 1735 cm^{-1}, 1615 cm^{-1}, 1420 cm^{-1}, 1150 cm^{-1}, and 1080 cm^{-1} are characteristic absorption bands in the FTIR spectrum of a pectin hydrogel.

Significant changes are observed in the FTIR spectrum of lipase-immobilized pectin hydrogel (Fig. 3B), including the presence of bands due to amide groups within the 1700-1500 cm^{-1} region, specifically at 1650 cm^{-1} and 1540 cm^{-1}, assigned to amide I and amide II bands, respectively, thus confirming lipase immobilization. Slight changes and variations in bands within the hydroxyl and carboxylate regions may be attributed to interactions between lipase and pectin hydrogel. These changes are similar to those observed by Miao et al. (2021) for pectinase immobilization, and das Neves et al. (2024) for lipase immobilization onto pectin hydrogels [11, 25]. The presence of bands due to amide groups confirms interaction between the enzyme and hydrogel, and the presence of bands characteristic of pectin hydrogel indicates that its structure remains intact after immobilization.

3.3 Biochemical characterization of immobilized lipase

3.3.1 Enzyme activity and stability at different temperatures

The temperature activity profile of the immobilized lipase on pectin hydrogel was compared to that of the free enzyme.

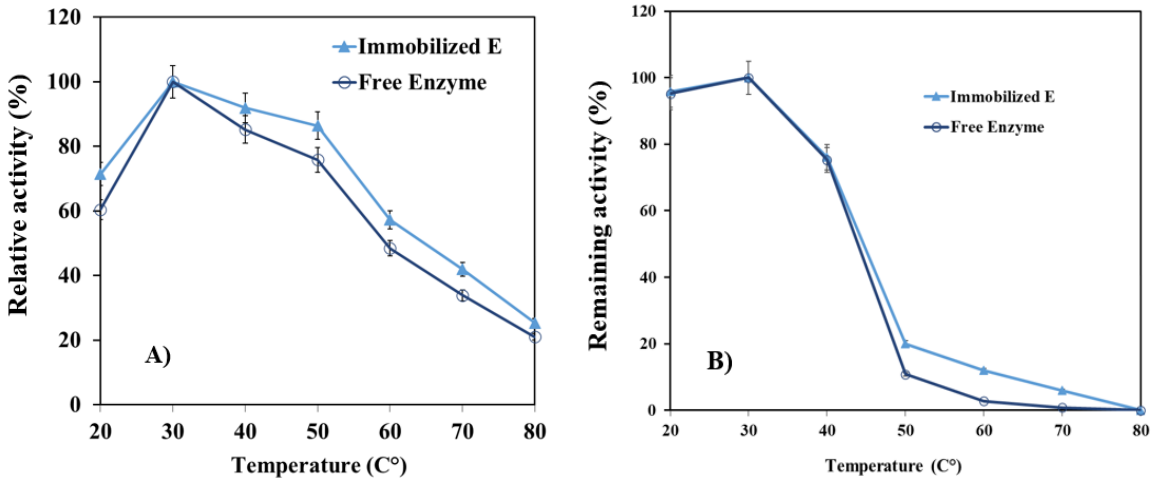


Fig. 4. A) Temperature activity analysis free and immobilized lipases; enzyme activities have been investigated at different temperatures based on section 2.6.1. B) Temperature stability analysis free and immobilized lipases; enzymes have been incubated at different temperatures for 2 h and then the remaining activities have been measured based on section 2.6.1

Both enzymes had their optimal activity at a temperature of 30°C, though the immobilized lipase had 100% relative activity compared to 98% for the free enzyme at the same temperature (Fig. 4A). At 40°C, the immobilized lipase retained about 92% of its maximum activity, while the free enzyme showed a slight loss, retaining about 85% of its original activity. However, the difference between the immobilized and free enzyme activities increased at higher temperatures. When the temperature was raised to 50°C, the immobilized enzyme retained 88% of its original activity, while the free enzyme retained 75%. When the temperature was raised to 70°C, the immobilized enzyme retained 44% of its original activity, while the free enzyme retained 35%.

The temperature stability profile of the immobilized and free enzymes on pectin hydrogel was investigated based on the enzymatic activity remaining after enzyme incubation at different temperatures (Fig. 4B). The optimal temperature for the stability of both free and immobilized lipases was around 30°C. However, notable differences in thermal stability were obtained at higher temperatures. Furthermore, the thermostability of the enzyme was slightly enhanced after immobilization. For instance, at 60°C, the immobilized lipase showed 15% of its original stability compared to 5% for the free lipase.

These results reinforce the concept of thermal stabilization of enzymes when immobilized in pectin hydrogel matrices, as supported by literature. The same thermal stability results were also reported by Abdel-Mageed et al. (2022) and Muanruksa et al. (2020), which showed improved thermal stabilities of immobilized lipase in pectin-alginate hydrogels [26, 27]. The underlying principle of this improved thermal stability is based on the enzyme microenvironment provided by the pectin hydrogel matrix, which helps protect the enzyme from thermal denaturation, thus enhancing enzyme rigidity against thermal unfolding.

3.3.2 Enzyme Activity and Stability at Different pHs

The pH activity profile of the immobilized lipase on pectin hydrogel was investigated by measuring residual enzyme activities in the pH range of 3.0-11.0 (Fig. 5A).

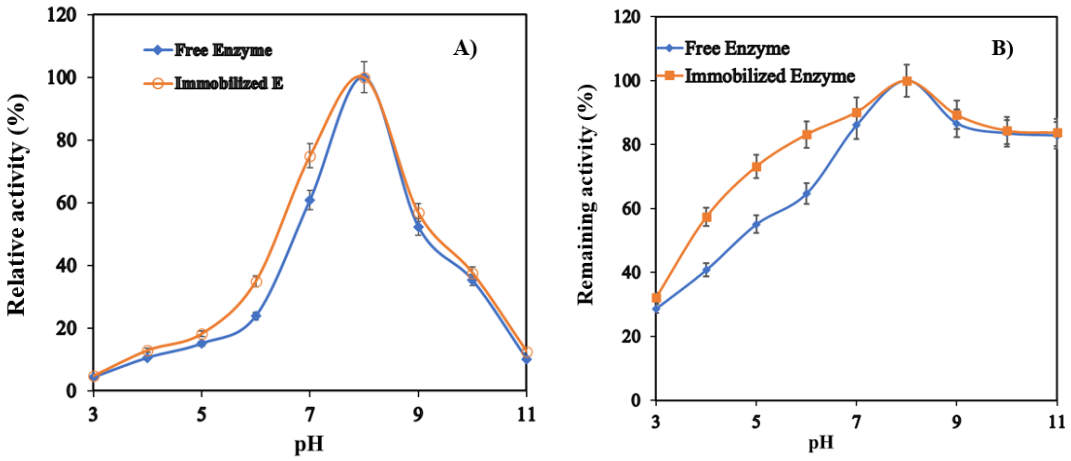


Fig. 5. A) pH activity analysis free and immobilized lipases; enzyme activities have been investigated at different pHs based on section 2.6.2. B) pH stability analysis free and immobilized lipases; enzymes have been incubated at different pHs for 2 h and then the remaining activities have been measured based on section 2.6.2

Both immobilized and free enzymes have similar bell-shaped profiles with maximum enzyme activities at pH 8.0, suggesting that the native pH optimum of the enzyme was not altered after immobilization. In the acidic pH range of 6.0-7.0, the immobilized lipase was found to have higher residual enzyme activities (about 10 %) than the free enzyme. This observation is concordant with the results obtained by Li et al. (2008), where they observed that the optimal pH of the immobilized pectinase on an agar-gel support remained unchanged [28].

The pH stability profile of the immobilized lipase in the hydrogel matrix was characterized through the assessment of the residual enzymatic stability across a wide range of pHs (3.0 to 11.0) (Fig. 5B). Results showed that the highest pH stability of immobilized and free lipases observed around pH 8.0. In the acidic region, ranging from pH 3 to 5, the immobilized enzyme was observed to be more stable than the free enzyme, retaining around 70% of the maximum activity at pH 5, compared to the free enzyme stability which retained only around 52%. This shows that the enzyme has retained its native pH optimum and improved pH tolerance upon immobilization. However, in the alkaline region, ranging from pH 9 to 11, there isn,t significant difference between free and immobilized enzyme stability.

These results clearly show that the pH stability achieved by the immobilization within the pectin hydrogel, is in agreement with the results of Adetunji (2023) and Mazzocato (2024). They observed the pH stability of the immobilized enzyme within the range of 8.0-9.0 [29, 30]. The pH stability is due to the microenvironment created by the pectin hydrogel, which protects the enzymes from pH denaturation by limiting the conformational changes and aggregation of the enzymes. Thus, the pH stability observed in the present study confirms the potential of the pectin hydrogel as a support material for the immobilization of lipase.

4 CONCLUSION

Finally, the current investigation aims to highlight the potential of pectin hydrogels as a biofriendly immobilization tool for lipase enzymes. The results of this study confirm that the unique structural and chemical characteristics of the pectin hydrogel, including the presence of numerous functional groups and the potential for the development of three-dimensional structures, provide the ideal conditions for the immobilized enzyme to exhibit maximum stability. The hydrogel matrix, which confers greater stability to the enzyme against denaturation under conditions of temperature and pH, helps to overcome the inherent disadvantages of free lipase, thus extending the potential for its application in a wide range of biotechnological processes.

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