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BIOTECHNOLOGICALLY RELEVANT ENZYMES AND PROTEINS

Immobilized inclusion bodies of recombinant cold-adaptive lipase from Antarctic *Pseudomonas* sp. as catalysts

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Abstract

Immobilization of lipase has been receiving attention for a long time; this is because of the need for robust catalysts by industries. Numerous literature has reported improvements in the properties of immobilized lipase in terms of stability when exposed to extreme conditions of temperature, pH, and organic solvents commonly encountered in most industrial settings. However, some microbial lipases that have the potential to catalyze significant reactions do occur in the form of inclusion bodies when expressed in *Escherichia coli.* This research aimed to immobilize catalytically active inclusion bodies (CatIBs) of LipAMS8 lipase onto Seplite LX120 as the adsorption material. Scanning electron microscopy and Fourier infrared spectroscopy were used to ascertain the immobilization. Immobilized CatIBs have an optimum temperature of 20 °C and pH of 9.0. They exhibit high stability to broad temperatures, pH levels, and organic solvents, with excellent storage stability and reusability, retaining 50% of their residual activity after ten cycles. They demonstrated excellent activity in the transesterification of waste cooking oil with methanol, in which 2% of the immobilized CatIBs produced up to 98% biodiesel in a ratio of 1:9 at 25 °C for 7 h at 200 rpm. LipAMS8 CatIBs immobilization improved their stability and capability to produce biodiesel at lower temperatures.

Key points:

•Catalytically active inclusion bodies from recombinant AMS8 lipase occurring naturally were successfully immobilized onto seplite LX120. Morphological and structural analyses using SEM and FITR confirmed the immobilization.

•Characterization revealed the immobilized LipAMS8 CatIBs to maintain the residual activity of up to 50% at a broad temperature (10–80 °C) and pH (4–12).

•Interactions with metal ions and various organic solvents manifest their stability. Transesterification reaction with methanol using palm cooking oil to produce biodiesel at 25 °C which revealed their synthetic capacity.

Keywords Immobilization · Inclusion body · Lipase · Cold-adapted · *Pseudomonas* · Antarctic

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Introduction

Most enzymes exhibit a drawback in that it is extremely difficult to technically extract active enzymes from reaction mixtures after usage due to their high production and separation costs and relative instability (Nguyen and Kim 2017). Enzymes function as natural catalysts with excellent substrate specificity, high conversion rates, and mild pH, pressure, and temperature stability (Al-Ghanayem and Joseph 2020). This makes them more effective than traditional chemical catalysts (Rodrigues et al. 2021). Enzymes are proteins that catalyze chemical and biological reactions (Callender and Dyer 2015). They are found in many plants, animals, and microbes (de la Fuente et al. 2021). These catalysts offer several benefits,including high catalytic activity, biodegradability, selectivity, and specificity (Liu et al. 2018). Nevertheless, once the initial run is complete, enzymes cannot be retrieved and reused again, making them uneconomical (Dal Magro et al. 2020). To circumvent this and improve their efficiency, stability, and reusability, various immobilization methods have been used (Wahab et al. 2020). The interactions that bind the enzymes to the support include stable covalent bonds, reversible physical adsorption, and ionic connections (Federsel et al. 2021). The type of enzyme and carrier will determine which immobilization technique is best; however, in recent years, immobilization technology has become increasingly dependent on logical design (Santos et al. 2015). As our understanding of the principles underlying enzyme biosynthesis has advanced rapidly, the field of enzymatic productivity has been rising quickly in recent years due to advancements in genetic engineering, microbial cultivation, and improvement screening methodology (Hassan et al. 2018). The wide application of cold-active enzymes, especially, lipase, due to their ability and versatility to catalyze a huge number of reactions has resulted in their high demand, especially in the food and pharmaceutical industries (Godoy et al. 2022). The increase in available literature with detailed information on immobilizing enzymes and their possible application has promoted the interest of many researchers and industrialists (Maghraby et al. 2023).

Lipases (EC 3.1.1.3) are hydrolases that operate on carboxylic ester bonds and are classified as triacylglycerol acyl-hydrolases (Khan et al. 2017). They belong to the class of serine hydrolases and do not require cofactors (Galmozzi et al. 2014). The triacylglycerol ester hydrolase family contains many serine hydrolases, which are sometimes known as lipases (EC 3.1.1.3). As carboxylesterases, they can catalyze the hydrolysis and synthesis of long-chain triglycerides (triglycerides to fatty acids, diacylglycerol, monoacylglycerol, and glycerol). In addition to hydrolysis, they exhibit interesterification, esterification, aminolysis, and alcoholysis activities, all of which contribute to various industries (Rajendran et al. 2009). Lipases are responsible for esterification, transesterification, interesterification, acidolysis, alcoholysis, and aminolysis conversion reactions (Jiao et al. 2018). Even though enzyme immobilization progressed remarkably, it remains a study topic, as indicated by the amount of published research and review publications devoted to it (Slouka et al. 2019). Due to additional properties such as stronger stability, increased reusability, longer storage time, a larger spectrum of activities in the presence of various physical and chemical variables, and faster product recovery, enzyme immobilization has made enzymes economically useful biomolecules (Filho et al. 2019). Adsorption, trapping, and covalent binding on various supports are all strategies used to immobilize enzymes. The enzyme's thermostability is improved through immobilization, which prevents the enzyme from losing its activity. Numerous technological reasons exist for enzyme immobilization for better catalysis (Sarrouh 2012). Lipases are essential for the metabolism of fats and lipids in microbes, plants, animals, and humans (Yao et al. 2021). The function of lipases is to regulate the metabolism of lipids and lipoproteins in humans and other animals. During seed germination, plant lipases participate in the metabolism of oil stores (Kawiński et al. 2021). They supply the energy needed for the production of amino acids and glucose, two crucial substances required for embryonic growth (Mhetras et al. 2021). Lipases, rather than traditional chemical procedures, are being utilized in the modern food industry to produce a variety of items such as fruit juices, baked goods, fermented vegetables, cheese, soups, and sauces. Lipid modification is one of the most essential procedures applicable in the food industry (oils and fats) (Hasan et al. 2006). Lipases are also employed to give food a unique flavour and taste by converting fatty acids and alcohols into flavour and aroma molecules (Abdel-Hamied et al. 2017). Lipases account for over 30% of the global enzyme market and have shown promise as future enzymes. By (Pourkhanali 2023), the market value of microbial lipase is projected to grow to $590 million from its 2017 value of $400 million (Mhetras et al. 2021). Many lipases also have a movable sub-domain termed a lid, which can take one of two forms: prevents closed conformations, in which the lid closes the substrate from entering the active site, or open confirmations in which the lid opens and allows the substrate to enter the active site. The lid's opening is controlled in the presence of a micellar substrate at the catalytic site's interface (Khan et al. 2017).

Inclusion bodies (IBs) are refractile particles that typically exist in the cytoplasm of actively generating recombinant *E. coli* cells (Ventura and Villaverde 2006), even though

secretory proteins can also produce IBs in the periplasm. IBs appear to be quite amorphous under electron microscopy (Ventura and Villaverde 2006); however, after detergent-based purification, scanning microscopy reveals that they are rod-shaped particles (Carrió et al. 2000). According to traditional proteomics, IBs' composition was mostly determined by the recombinant protein itself, which also revealed that IBs were generally homogeneous (Tang et al. 2024). Although appearing in varying amounts, the recombinant product can account for more than 90% of all embedded polypeptides (Villaverde et al. 2012). Many polypeptides make up bacterial IBs, including tiny, big, monomeric, multimeric, prokaryotic, and eukaryotic proteins. These polypeptides are not connected physically or chronologically. Because chaotic intracellular precipitates are formed, aggregation inside bacterial factories has long been thought of as an unspecific process. As a result, it has been proposed that many general characteristics, independent of the protein's composition but inherent to the specific molecular status of the protein, may favour IBs' formation (Helleckes et al. 2024). High local concentrations of the generated polypeptide are among them, as well as the brief accumulation of proteins in fully or partially unfolded conformations with decreased solubility compared to the native form (Baneyx & Mujacic 2004). The buildup of unstructured protein fragments is caused by proteolytic assault (Baneyx & Mujacic 2004) and the occurrence of improper interactions with the bacterial folding machinery. Inclusion bodies' composition and organization are significantly impacted by culture conditions (Peternel et al. 2008). A thorough review of the literature reveals that catalytically active inclusion bodies (CatIBs) of naturally occurring proteins have been described since 1989 when Worrall and Goss revealed that the enzyme β-galactosidase may be generated as CatIBs in *E. coli* (Krauss et al. 2017). Tokatlidis et al. (1991) discovered that *Clostridium thermocellum* endoglucanase D was also generated as CatIBs in 1991 (Tokatlidis et al. 1991). Human dihydrofolate reductase (Krauss et al. 2017) is another recently discovered native protein that produces CatIBs in *E. coli.* Different enzyme classes, structures, and origins of naturally occurring CatIB-producing proteins argue against this hypothesis (Heckmann and Paradisi 2020). The fact that the catalytic activity of recombinant proteins generating inclusion bodies was frequently not assessed added to the complexity of the literature search (Krauss et al. 2017). CatIBs are carrier-free enzymes that are immobilized in synthetic chemistry and biocatalysis. Because CatIBs are insoluble, they can be used as immobilizers in synthesis chemistry and biocatalysis (Santi et al. 2021). CatIB-based immobilizers have numerous advantages over conventional enzyme immobilizers. CatIBs are simple to make in pure form, especially because no chromatographic purification procedures are required (Krauss et al. 2017).

In this study, LipAMS8 CatIBs were immobilized onto adsorption support seplite (LX120). Scanning electron microscopy (SEM) and Fourier infrared spectroscopy (F-TIR) were carried out to confirm the immobilization. Characterization of the immobilized LipAMS8 CatIBs revealed their tolerance to pH, temperature, and organic solvents. Storage stability (Figure S2) and recyclability manifest high stability when compared with their free form. Transesterification with methanol using waste cooking oil revealed the potential of immobilized CatIBs to produce biodiesel at a lower temperature. The uniqueness of this research is in its ability to enhance the stability of LipAMS8 CatIBs, a crucial cold-active enzyme with greater versatility.

Materials and methods

Equipment, chemicals, and reagents

The reagents, chemicals, and equipment used in conducting this research were secured from the Enzyme and Microbial Technology Centre (EMTECH), Faculty of Biotechnology and Biomolecular Sciences, Universiti Putra Malaysia.

Isolation and solubilization of LipAMS8 CatIBs

The glycerol stock of LipAMS8 lipase was obtained from the Enzyme and Microbial Technology Centre (EMTech). It was revived using the standard procedure. The LipAMS8 lipase gene was previously cloned, and the protein sequence of LipAMS8 lipase was deposited in the NCBI GenBank database, with accession numbers HQ162821 and ADM87309 (Ganasen et al. 2016). AMS8 has been overexpressed in *E. coli* BL21(De3) harboring pET32b with the aid of the inducer isopropyl β-D-1-thiogalactopyranoside (IPTG) at a concentration of 0.3 mM for 12 h at 15 °C with agitation of 150 rpm (Salwoom et al. 2019). The culture obtained was poured into a Beckman bottle and centrifuged at 10,000 g for 10 min. The supernatant obtained was discarded. The residual cell pellet was further resuspended in a mild solubilization buffer. It has been completely solubilized, followed by sonication at 6 duty cycles at 40 °C with 30-s intervals. The sample was centrifuged again at 12,000 g for 20 min. The supernatant was discarded, and the pellet (inclusion bodies) was resuspended in 50 mM Tris–HCl (8.0) and mixed thoroughly using a rotary mixer for 4 h. It was then centrifuged at 12,000 g to obtain a clear supernatant (solubilized inclusion bodies).

Determination of protein content

The Bradford method of protein quantification was employed to measure the protein concentration (Bradford et al. 1990).

This has been evaluated with the aid of a commercial Bradford reagent obtained from Sigma. The absorbance was measured at 595 nm. Bovine serum albumin (BSA) was used as the reference protein standard.

Enzyme activity of immobilized LipAMS8 CatIBs

Immobilized enzyme activity was estimated by a simple and rapid colorimetric method that was modified from Kwon and Rhee (Kwon and Rhee 1986). Using olive oil, the reaction mixture, containing 1.0 mL of free enzyme and 10 mg of immobilized CatIBs, 2.5 mL olive oil emulsion in a ratio (1:1) of olive oil and Tris–HCl buffer (50 mM) at pH 8.0, with 0.02 mL of 20 mM $CaCl_2.2H_2O$, then incubated in a water bath shaker at 30 °C for half an hour with an agitation rate of 150 rpm. The reaction was terminated by adding 1.0 mL of 6 N HCl. The FFAs were finally extracted by the addition of 5.0 mL isooctane and mixed vigorously using a vortex mixer for 30 s. The top isooctane layer (4 mL) containing the fatty acid was carefully transferred into a test tube containing 1 mL of copper pyridine reagent and vortexed again. The reagent was prepared by adjusting the solution of 5% (w/v) copper (II) acetate-1-hydrate to pH 6.1 with pyridine. The amount of FFAs dissolved in the isooctane layer is proportional to the intensity of green color, which was used to determine the activity of immobilized CatIBs, computed at 715 nm. One unit (U) of lipase activity was defined as the rate of formation of fatty acids (µmole) per minute under standard assay conditions.

Immobilization of CatIBs

Influence of adsorption time

To optimize the adsorption time, 10 mL of LipAMS8 CatIBs in 50 mM Tris–HCl (8.0) with a protein concentration of 1 mg/mL was mixed with 1 g of seplite LX120 with agitation at 200 rpm in an incubator shaker for 1, 2, 3, and 4 h at 30 °C. The mixture was separated using filter paper. The residue was dried using a Bed fluid dryer. The protein concentration of the filtrate was determined. The enzyme activity of the immobilized enzymes was assayed using the Kwon and Rhee method (Kwon & Rhee 1986). The yield of immobilization was evaluated according to Eq. 1.

$$\text{Yield of immobilization (\%)} = \frac{(\text{CatIBs}_{\text{protein content}} - \text{Unbound CatIBs}_{\text{protein content}})}{\text{CatIBs}_{\text{protein content}}} \times 100\% \quad (1)$$

Effect of protein loading on the immobilization of LipAMS8 CatIBs

Successful immobilization can only be achieved by loading a suitable amount of enzyme, which facilitates enzyme binding, onto a given support. To determine the optimal concentration of enzyme to be loaded onto seplite LX120, various concentrations were used to optimize enzyme loading within the ranges of 0.25–1.5 mg/mL in which 10 mL of each concentration was used to immobilize onto 1 g of seplite LX120. The mixture was then incubated at 30 °C with agitation of 150 rpm for 2 h (optimized incubation time). The mixture was filtered and dried. The activity of immobilized LipAMS8 CatIBs was determined. The protein content of the unbound enzyme was computed, and the yield of immobilization was calculated using the equation previously mentioned above.

Characterization of immobilized and free LipAMS8 CatIBs

Effect of temperature on activity and stability

The temperature effect against immobilized enzyme was investigated at temperature ranges from 10 to 80 °C for half an hour at intervals of 10 °C. It was subjected to enzyme activity. The temperature effect on the immobilized enzyme stability was measured by initially incubating the immobilized enzymes at different ranges of temperatures from 10 to 80 °C up to half an hour, followed by assaying the enzyme activity at 30 °C for half an hour. An untreated enzyme was used as the control.

Effect of pH on activity and stability

pH effect on the activity of the immobilized enzyme was evaluated employing several solutions within the range (4–12), 50 mM sodium acetate (pH 4–6), 50 mM potassium phosphate (pH 6–8), 50 mM Tris–HCl (pH 8–9), 50 mM glycine–NaOH (pH 9–11), and 50 mM disodium phosphate (pH 11–12). The activity was assayed using these different buffers at 30 °C for half an hour. The effect of pH on immobilized enzyme stability has been analyzed after initially incubating the enzyme at different pH values

(4–12) at 15 °C for half an hour. Immobilized enzyme activity was then determined using enzyme assay at 30 °C.

Scanning electron microscopy (SEM) of immobilized LipAMS8 CatIBs

This was conducted using JSM-1T200 In TouchScope TM (JEOL, Tokyo, Japan) to evaluate the morphology of immobilized LipAMS8 CatIBs. The morphological appearance of immobilized seplite (LX120) was compared with empty seplite (LX120). The samples were processed, coated with gold, and viewed under SEM. Various magnifications, such as × 10, × 20, × 30, × 500, × 5000, and × 10,000, were used to visualize the appearance of both the immobilized and empty support.

Fourier-transform infrared (FTIR) spectroscopy

Structural analysis of the immobilized LipAMS8 CatIBs was carried out using the FTIR spectrometer Nicolet 6700, Thermo Scientific. The analysis was conducted at the spectrum between 500 and 4000 cm^{-1} and up to 3 times scanning. The radiation source (IR) of the spectrometer came from an impaired total reflectance (ITR) crystal. Before recording the measurements, a pressure controller was carefully adjusted to improve the optimal contact between the subjected sample and the diamond plate.

Brunauer–Emmett–Teller (BET) analysis of the surface area of immobilized LipAMS8 CatIBs

The surface area of immobilized LipAMS8 CatIBs and the nature of pore size were also assessed using N_2 gas adsorption–desorption to outline the differences in the surface morphology of immobilized LipAMS8 CatIBs. This was conducted with the aid of the Micro Active TriStar II Plus version Surface Area Analyzer.

Organic solvents' effect on immobilized LipAMS8 CatIBs

The solvents' effect on immobilized enzyme strength has been investigated using distinct solvents. The immobilized enzyme has been initially incubated with 25% (v/v) organic solvents at 15 °C for up to half an hour. While initially incubated, the immobilized enzyme has been analyzed for activity at 30 °C using the oleic acid method. The immobilized enzyme without organic solvent was the control used in this experiment and was considered to have 100% activity.

Metal ion effect on immobilized LipAMS8 CatIBs

The metal interaction effect against immobilized enzyme has been analyzed by initially incubating the immobilized enzyme in a volume of 30% (v/v) of 1 mM and 5 mM of different metal ions at 15 °C for half an hour. The initially incubated immobilized CatIBs were analyzed for activity at 30 °C using the oleic acid method. Immobilized enzymes that do not contain any metal ions were served as controls and considered to have 100% activity.

Recyclability of immobilized LipAMS8 CatIBs

Recyclability of immobilized LipAMS8 CatIBs was conducted by subjecting the immobilized CatIBs to ten (10) cycles of enzyme assay. At the end of each cycle of enzyme assay, the immobilized enzyme was separated by centrifugation. 50 mM Tris–HCl (8.0) was used to wash the enzyme. It was then dried using a bed fluid dryer before the subsequent reaction. The process was repeated in all the cycles. The assay activity of the initial cycle was used as 100% which was considered the control of the experiment.

Transesterification of waste cooking oil with methanol to produce biodiesel

Waste cooking oil

Waste cooking oil was obtained from a nearby restaurant. The oil was allowed to settle and filtered before the transesterification reaction with methanol at different proportions.

Biodiesel synthesis

Waste cooking oil and methanol were used in the transesterification reaction to produce biodiesel. Free and immobilized LipAMS8 CatIBs were used as catalysts. HCl was used as a positive control. The reaction was conducted at 40 °C, 30 °C, and 25 °C for 7 h using 1:3, 1:6, and 1:9 ratios of oil to methanol, with agitation at 200 rpm. Biodiesel yield was computed according to Eq. 2.

$$\text{Biodiesel yield } (\%) = \frac{\text{weight of biodiesel produce}}{\text{weight of original oil}} \times 100 \quad (2)$$

Acid value

The acid value of the biodiesel produced (Tables S1 and S2) was evaluated using a titration method. 0.1 M sodium hydroxide was used to standardize the biodiesel. The sample was previously dissolved in ethyl alcohol and heated

in a water bath at 70 °C for 10 min. Bromophenol blue was used as an indicator, in which a persistent blue color for up to 30s indicates the reaction endpoint. The acid value was computed according to Eq. 3.

$$\text{Acid value} = \frac{V \times N \times \text{Mwt.}}{\text{Weight of biodiesel in (g)}}$$

*V*value obtained from titration.
*N*normality of sodium hydroxide.
Mwt.molecular weight of sodium hydroxide.

GC–MS analysis

The biodiesel samples were analyzed using Gas Chromatography–Mass Spectrometry GCMS-QP2010 Ultra, with an HP column Rxi-5MS (30.0 length × 0.25 ID × 0.25 mm thickness) allowing the scan mode to analyze FAME composition. The carrier gas was helium with a 0.8 mL/min flow rate. The sample was injected using split mode 1:10 at an injection temperature of 250 °C, while the GC oven temperature was maintained at 50 °C for 10 min.

Statistical analysis

All the results generated are expressed in mean ± standard error mean (SEM). Experiments carried out were in triplicate ($n = 3$). A paired *t*-test and one-way analysis of variance (ANOVA) were used between different treatments using the Microsoft Excel Analyze It 2016 version. $P < 0.05$ was significant.

Results

Effect of time and enzyme loading

The result of influence of time on the yield of immobilization was conducted using incubation time intervals 1 to 4 h. The influence of time on the yield of immobilization is presented in Fig. 1A. The effect of protein loading on LipAMS8 CatIBs immobilization was investigated using a range of concentration (0.25–1.5 mg/mL) of the enzyme (Fig. 1B). The time interval used was 1 to 4 h. The immobilization at 2 h was (86%) and the activity recorded was (340 U/g); However, at the 1–4 h, there was yield and activity less than that recorded at the 2-h incubation. Therefore, the 2-h incubation time was considered an optimum time of immobilization for LipAMS8 CatIBs. The effect of enzyme loading on immobilization yield (%) and activity (U/g) revealed that 1.5 mg/ml produced the highest yield (86%) and activity (375 U/g) at the optimum incubation time (2 h).

Assessment of LipAMS8 CatIBs immobilization

Scanning electron microscopy (SEM) of immobilized LipAMS8 CatIBs

SEM analysis was conducted by viewing the empty and immobilized CatIBs at various magnifications such as magnifications (× 10, × 20, × 500, and × 5000). The result revealed that the structure of the empty and immobilized CatIBs was distinguished based on the morphological appearance in terms of covering the cracks of the adsorption

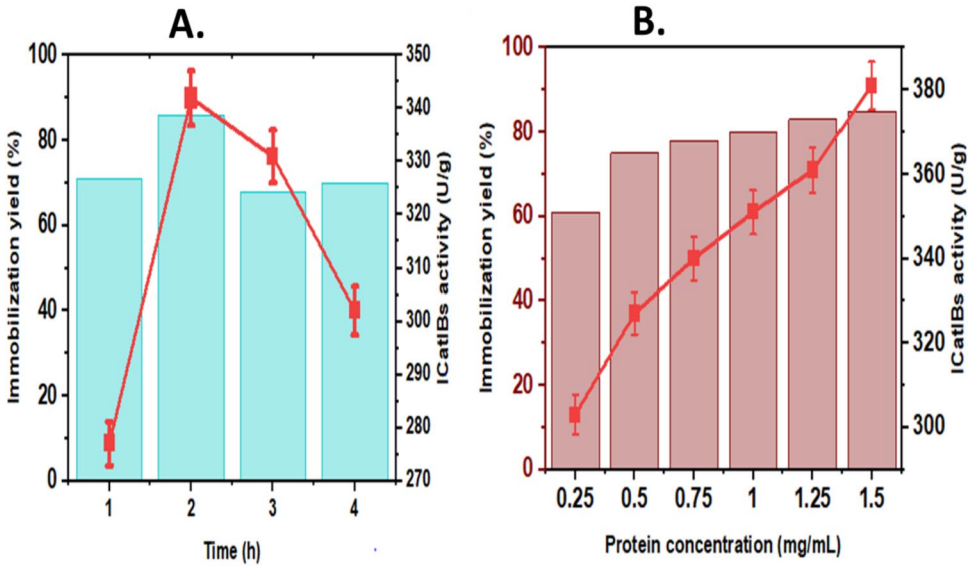

Fig. 1 Optimization adapted for effectively immobilizing LipAMS8 CatIBs onto seplite (LX120). **A** Represents the effect of incubation on immobilization yield (%) and enzyme activity of immobilized LipAMS8 CatIBs in (U/g) 10 mL of LipAMS8 CatIBs (1.0 mg/mL) was immobilized 1 g of seplite (LX120) at a different time with an interval of 1 h. **B** Shows effect of concentration of protein load on immobilization yield and the activity of the immobilized enzyme. Various protein contents (range 0.25–1.5 mg) were loaded for 2 h at 30°C with 200 rpm agitation. Experiments were carried out in triplicate

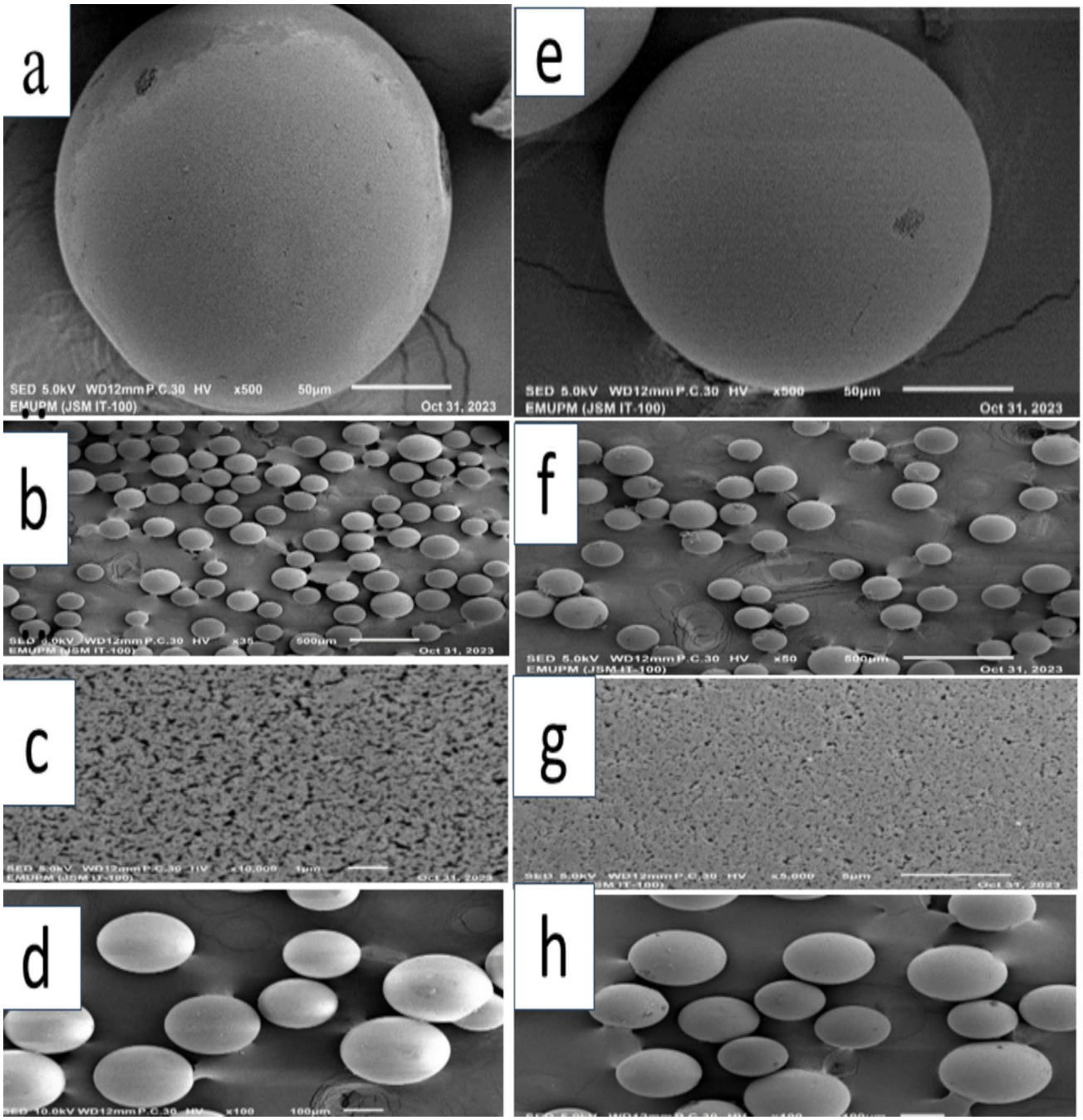

Fig. 2 Scanning electron microscopy morphology images of empty (**a–d**) empty seplite LX120 (**e–h**) immobilized LipAMS8 CatIBs viewed at various magnifications × 10, × 20, × 500, and × 5000

material (Seplite LX120) when viewed. SEM result of the empty and immobilized LipAMS8 CatIBs is presented in Fig. 2.

Structural confirmation analysis using FTIR

The FTIR analysis conducted at the spectrum between 500 and 4000 cm^{-1} included up to 3 times scanning. The results revealed the presence of a specific functional group in the immobilized sample. This is evidenced in the immobilized LipAMS8 CatIBs, which manifested the presence of some bands when compared to that of empty. FTIR analysis of empty seplite (green color) and LipAMS8 CatIBs immobilized (pink) is presented in Fig. 3.

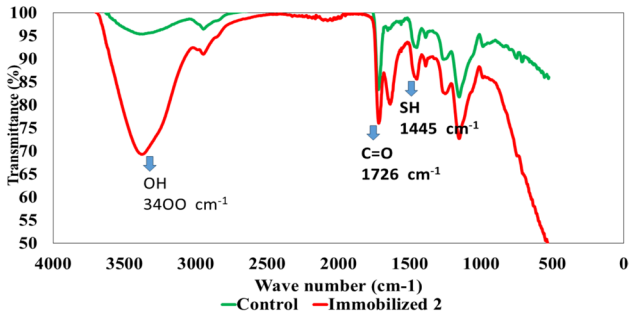

Fig. 3 Fourier infrared spectroscopy (FTIR) analysis of empty seplite LX120 (green color) and immobilized LipAMS8 CatIBs (pink color)

BET analysis of the immobilized LipAMS8 CatIBs

The surface area of the empty support and immobilized LipAMS8 CatIBs was conducted to assess the immobilization using adsorption and desorption of N_2 gas to ascertain the immobilization. The BET surface areas of the empty support and immobilized are 6.6600 m^2/g and 103.1540 m^2/g, respectively. The pore volume adsorption and desorption on empty and immobilized surfaces were 0.000135 cm^3/g, 0.018916 cm^3/g, 0.025837 cm^3/g, and 0.186442 cm^3/g, respectively. At the same time, the pore sizes are 0.8123 Å, 7.3349 Å, 155.1756 Å, and 72.2965 Å, accordingly. Isotherm plot of adsorption and desorption of empty and immobilized CatIBs is presented in Fig. 4.

Effect of temperature on immobilized LipAMS8 CatIBs' activity and stability

The effect of temperature on immobilized LipAMS8 CatIBs activity was investigated in the range of 10–80 °C. The optimum temperature of immobilized LipAMS8 CatIBs was 20 °C. The activity of immobilized LipAMS8 CatIBs was determined by assaying the enzyme at 10–80 °C for 30 min. The immobilized LipAMS8 CatIBs retained 57.1% (200 U/g) of their activity at 60 °C. The result of the temperature effect on the activity of immobilized CatIBs is presented in Fig. 5A. The result of the effect of temperature on stability revealed that immobilized CatIBs are stable at 40, 50, and 60 °C, as shown in Fig. 5B.

Effect of pH on immobilized LipAMS8 CatIBs activity and stability

The impact of pH on the activity of immobilized enzymes was evaluated using buffers with various pH values (4–12.0). As shown in Fig. 6A. The immobilized LipAMS8 CatIBs exhibited an optimum pH of 9.0. The results obtained from the effect of pH on the stability indicate that sodium acetate (pH 6.0) has the highest activity at 65 U/mL in the free CatIBs, and Tris–HCl (pH 9.0) has the highest activity at 350 U/g in the immobilized CatIBs. The impact of pH on the stability of LipAMS8 CatIBs is presented in Fig. 6B.

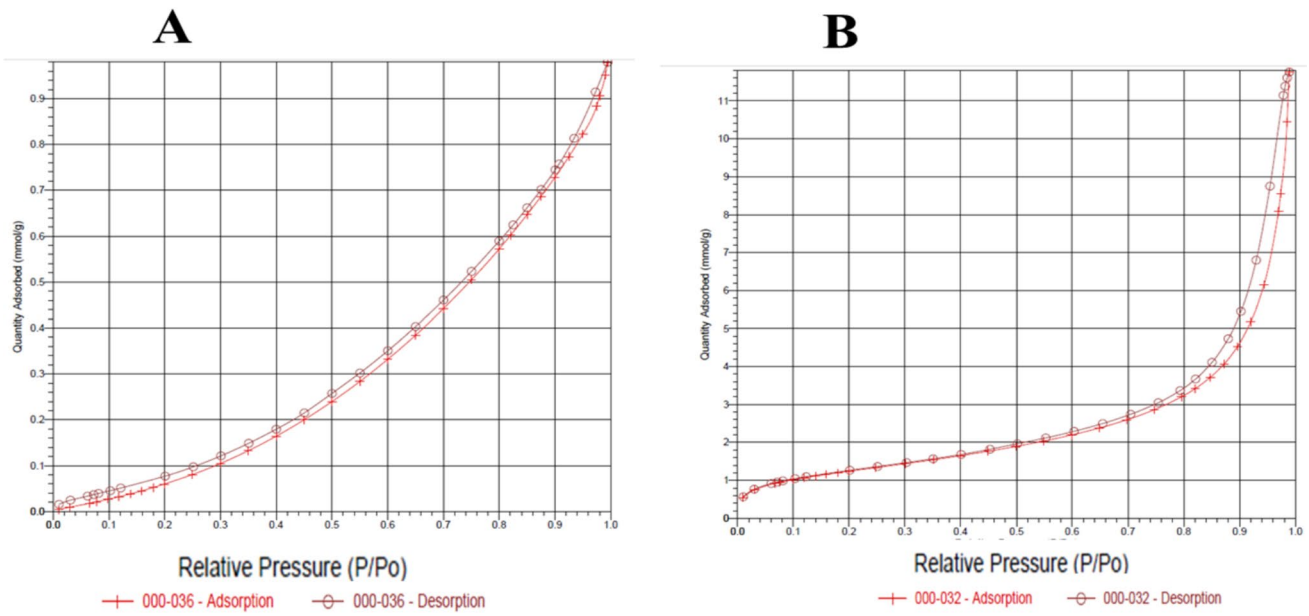

Fig. 4 Isotherm plot of nitrogen absorption and desorption of Seplite LX12O. **A** Represents empty seplite adsorption and desorption plot. **B** Shows adsorption and desorption of seplite LX120 with LipAMS8 CatIBs after immobilization

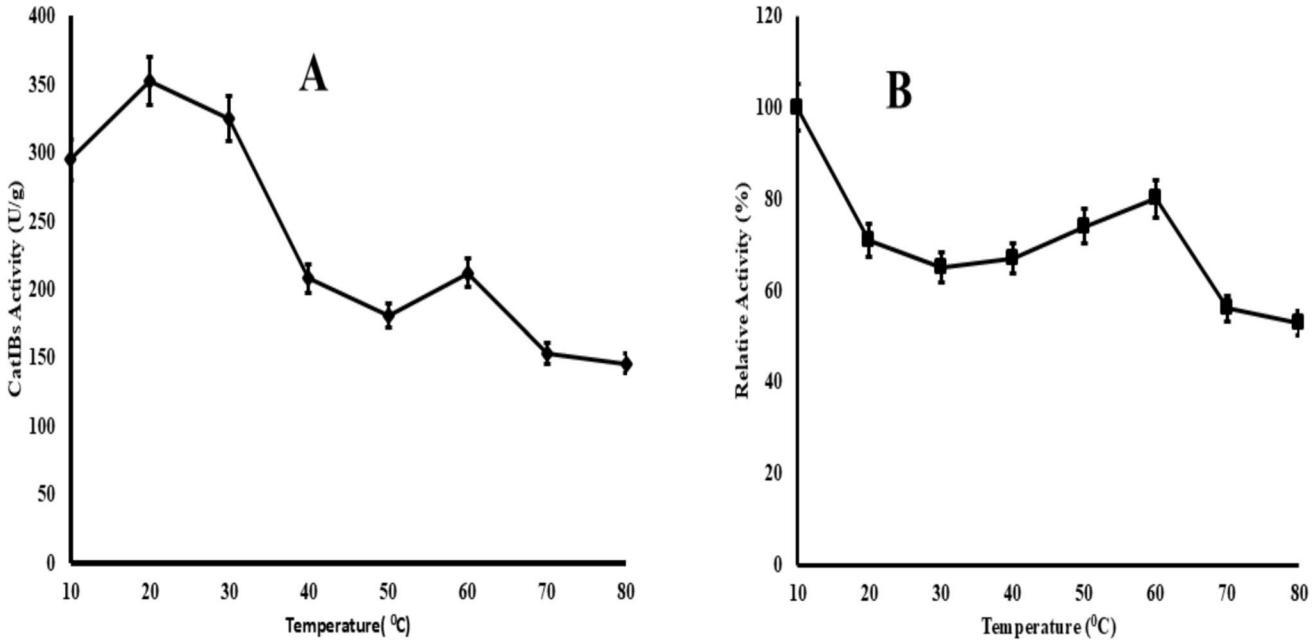

Fig. 5 Temperature effect on activity and stability of immobilized enzyme. **A** Temperature effect on activity was evaluated at the range of 10–80 °C. It was then assayed using the Kwon and Rhee method. **B** Effect of temperature on the stability of immobilized CatIBs. The immobilized LipAMS8 CatIBs were incubated at the temperature range of 10–80 °C for 15 min, followed by an assay using the Kwon and Rhee method. Parameters were measured three times ($n = 3$)

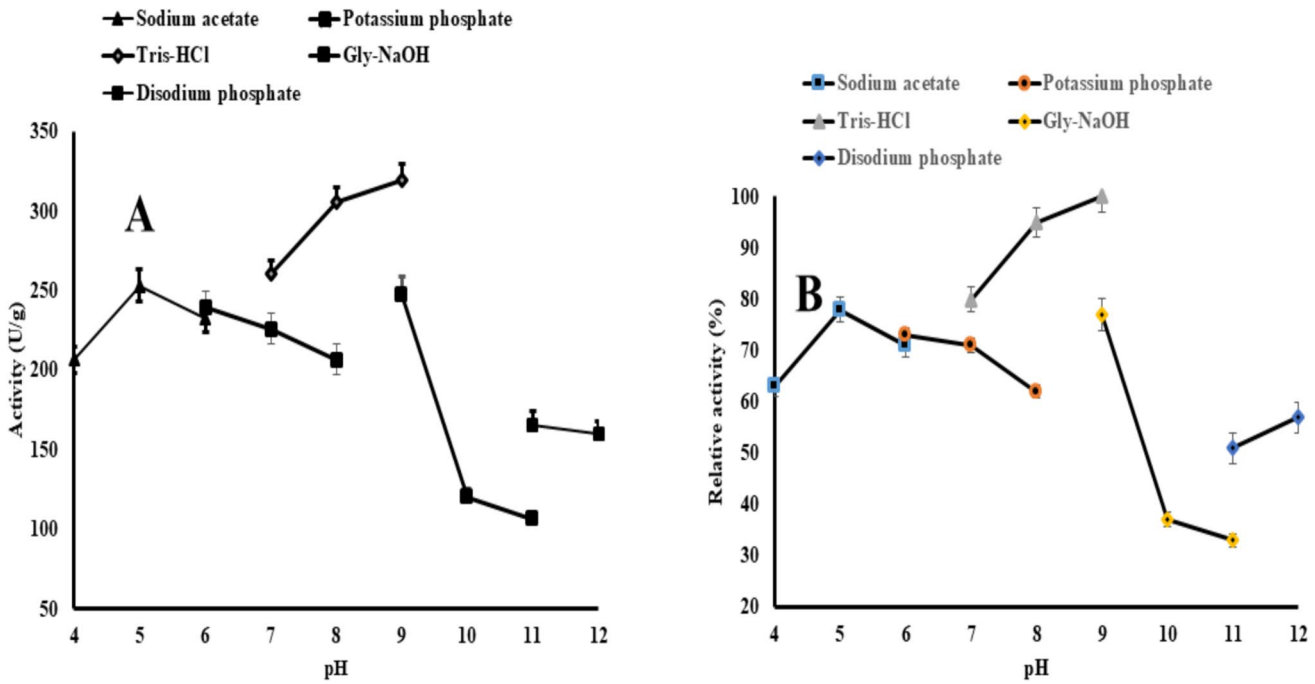

Fig. 6 pH Effect against immobilized enzyme. **A** Effect of pH on activity of immobilized LipAMS8 CatIBs. It was conducted by emulsifying an equal volume of buffers pH (4–12) with olive oil and then analyzed at 30 °C using the Kwon and Rhee method. Parameters measurements were conducted three times ($n = 3$). **B** Effect of pH on stability was evaluated by preincubating the immobilized enzyme in the same volume of buffers pH (4–12) at 15 °C for up to 30 min followed by enzyme assay using the Kwon and Rhee method. Parameters were measured three times ($n = 3$)

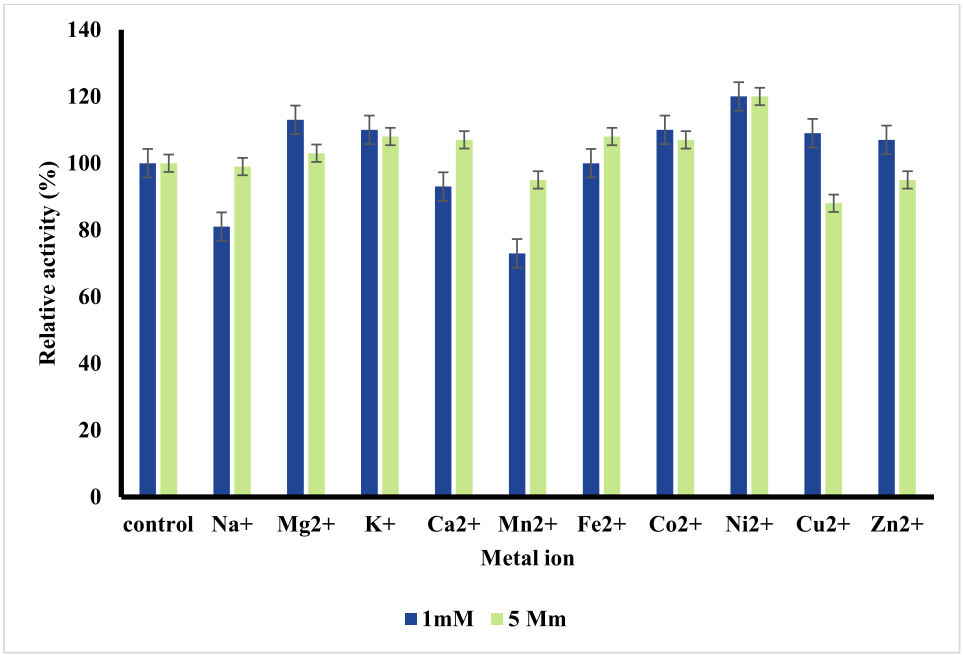

Fig. 7 Effect of metal ions on the stability of the immobilized enzyme. This was conducted by preincubating the immobilized enzyme in a volume of 30% (v/v) of 1 mM and 5 mM of various metallic ions at 15 °C for half an hour. followed by enzyme assay using the Kwon and Rhee method. All measurements were carried out in triplicate

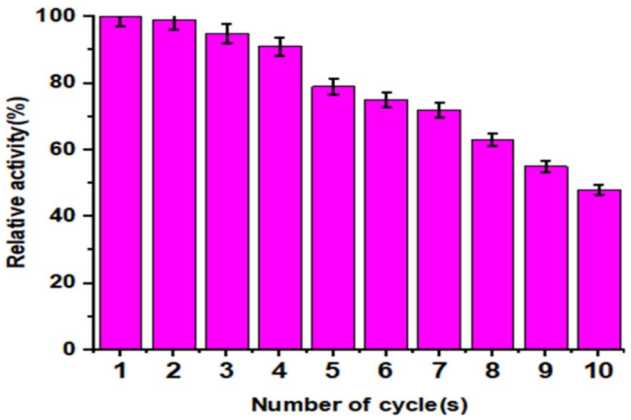

Fig. 8 Reusability of immobilized enzyme. Immobilized LipAMS8 CatIBs recyclability was conducted by subjecting the enzyme to ten (10) cycles of enzyme assay. It was centrifuged and washed with 50 mM Tris–HCl (8.0) at the end of each cycle. All measurements conducted were triplicate($n=3$)

Table 1 FAME composition of biodiesel produced from waste cooking oil, using immobilized LipAMS8 CatIBs as a catalyst

Retention time (min)	Methyl ester	Structure	Molecular weight (g/mol)	Weight (%)
49.8	Hexadecanoic acid	C16:0	256.43	31.56
55.3	octadecadienoic acid	C18:2	280.447	6.39
55.5	Octadecenoic acid	C18:2	282.46	22.83
56.3	stearate acid	C18:0	284.48	2.9
61.46	Hexadecanoic acid	C16:0	256.43	2.2
66.49	Octadecanoic acid	C18:0	284.48	2.31
67.3	Hexadecanoic acid	C16:0	256.43	10.8
72.1	Octadecenoic acid	C18:2	282.46	9.92

Metal ion interaction against immobilized CatIBs

The metal ion interaction of the immobilized enzyme was conducted after initially incubating the immobilized enzyme alongside 30% (v/v) 1 mM and 5 mM concentrations of different metal ions at 15 °C for half an hour. The result indicates LipAMS8 CatIBs exhibit reasonable activity in Fe2 +, Co2 +, and Ni2 + ions as well as in Ca2 +. The effect of metal ions is presented in Fig. 7.

Immobilized LipAMS8 CatIBs recyclability

Immobilized LIPAMS8 CatIBs' reusability was investigated by subjecting them to ten cycles of an enzyme assay. The result of the recyclability of immobilized LipAMS8 CatIBs for ten cycles of enzyme assay indicated that it can be maintained at more than 50% after ten cycles of enzyme assay. The result of immobilized CatIBs' reusability is presented in Fig. 8.

Table 2 FAME composition of biodiesel produced from waste cooking oil, using HCl (control) as a catalyst

Retention time (min)	Methyl ester	Structure	Molecular weight (g/mol)	Weight (%)
9.2	Hexadecanoic acid	C16:0	256.43	2.81
49.8	Octadecadienoic acid	C18:2	280.447	10.41
55.5	Hexadecanoic acid	C16:0	256.43	10.74
61.4	Hexadecanoic acid	C16:0	256.43	7.16
66.4	Hexadecanoic acid	C16:0	256.43	6.92
67.3	Octadecanoic acid	C18:0	284.48	25.72
72.1	Octadecanoic acid	C18:0	284.48	14.82

GC–MS analysis

The individual peaks chromatogram and the retention time were verified against a FAME standard mixture, and individual FAMEs were identified with the use of the MS database. The FAME composition was generated from the free and immobilized LipAMS8-generated biodiesel, and the FAMEs are composed of methyl lineolate, methyl palmitate, methyl stearate, and methyl laurate. The result revealed a composition of about 88.91% of biodiesel-catalyzed transesterification compared with control 72.4% as shown in Tables 1 and 2.

Discussion

Immobilization of LipAMS8 CatIBs

Seplite LX120 is a nonionic, hydrophobic, cross-linked polymeric adsorbent. Its adsorption function comes from its macromolecular pore structure, high surface area, designed pore size distribution, and excellent osmotic and thermal stability. The pore size of an adsorption material plays an important role in determining its loading capacity (Santos et al. 2015). It was used as the adsorption material for immobilizing LipAMS8 CatIBs. Strategic optimization for time and the concentration of protein loading was conducted to ensure effective immobilization with high yield and activity. 10 mg/g of seplite LX120 was used to study the effect of time on the immobilization yield (%) as well as the activity (U/g) of the immobilized LipAMS8 CatIBs. The influence of time on the yield of immobilization is presented in Fig. 1A, which displays the yield of immobilization (%) and immobilized enzyme activity (U/g). The time interval used was 1 to 4 h. The immobilization at 2 h was (86%) and the activity recorded was (340 U/g). However, at 1, 3, and 4 h, there was yield and activity less than that recorded at the 2-h incubation. Therefore, the 2-h incubation time was considered an optimum time of immobilization for the LipAMS8 CatIBs. The immobilization of LipAMS8 lipase using chitosan as an adsorption material recorded the highest yield of 72.6% (Musa et al. 2018).. The effect of enzyme loading on immobilization yield (%) and activity (U/g) revealed that 1.5 mg/ml produced the highest yield (86%) and activity (375 U/g) at the optimum incubation time (2 h). However, reports have revealed that up to 30 mg of lipase can be loaded and incubated for a longer period (12 h) (Jacob and Suthindhiran 2020). A study of the effect of enzyme loading and immobilization conditions on *Pseudomonas fluorescene* lipase revealed that both small and higher enzyme loading have significant effects on the immobilized enzyme properties (Arana-Peña et al. 2020). Therefore, in this study, 1.5 mg/ml of enzyme produces the highest immobilization yield and activity.

Morphology analysis of immobilized LipAMS8 CatIBs with SEM

SEM images depicted the morphology of an empty support and immobilized CatIBs structure viewed at various magnifications. It can be deduced from the images seen that the nature of the immobilized LipAMS8 is strikingly different from that of the empty seplite LX120 in terms of smoothness and darker appearance when compared with the empty. This change observed in the immobilized seplite LX120 is an indication of successful immobilization. It has been reported that a change in the appearance of immobilized enzymes can serve as proof of successful immobilization (Aghabeigi et al. 2023). Considering the SEM result from the morphological point of view, LipAMS8 CatIBs indicate a clear change in the immobilized seplite in terms of compactness and covering of the crack structure of the seplite when compared with the empty. This result is in line with that reported in the morphological analysis of immobilized lipase from *Penicillium* sp. (Pourkhanali et al. 2022). The result obtained in the SEM analysis of Immobilized LipAMS8 CatIBs demonstrated successful immobilization of the enzyme onto seplite LX120. SEM analysis of empty and immobilized LipAMS8 CatIBs is presented in Fig. 2.

Confirmational analysis using FTIR

It can be seen clearly, as presented in Fig. 3, that the vibration of infrared has triggered some changes in the spectrum of immobilized LipAMS8 CatIBs compared to the empty. The characteristic band of the sample appeared at

3400, 1726, and 1445 cm^{-1}. The sharp and broad appearance of the band at 3400 cm^{-1} because of the overlap of the bands of the NH_2 and OH groups present. The band of around 1726 cm^{-1} indicated the presence of C = N and 1445 cm^{-1} due to various groups such as C–O, CH_2, SH, and amide (Aghabeigi et al. 2023). The C = O stretching at 1726 $cm^{-1}$and the SH group around 1445 cm^{-1} in the immobilized CatIBs sample indicate LipAMS8 CatIBs, upon exposure to infrared radiation, have produced characteristic bands that revealed the presence of an amide and sulfhydryl group that might be from the active site of the enzyme. The presence of bands in the immobilized LipAMS8 CatIBs is proof of the covering of the enzyme onto the surface of the adsorption material seplite LX120. Therefore, the result revealed successful immobilization.

BET analysis of the immobilized LipAMS8 CatIBs

The result obtained is in line with Gilani et al. (2016), who reported an increase in the BET analysis of the surface area of lipase immobilized on chitosan (Gilani et al. 2016). The pore volume adsorption and desorption on empty and immobilized surfaces were 0.000135 cm^3/g, 0.018916 cm^3/g, 0.025837 cm^3/g, and 0.186442 cm^3/g, respectively. At the same time, the pore sizes are 0.8123 Å, 7.3349 Å, 155.1756 Å, and 72.2965 Å, accordingly. This revealed the successful immobilization of LipAMS8 CatIBs onto seplite LX120, as evidenced by the report of Bae et al. (2010) for evaluating the BET method for determining surface areas (Bae et al. 2010). The isotherm plots of the BET adsorption and desorption are presented in Fig. 4.

Effect of temperature on immobilized CatIBs

The activity of immobilized LipAMS8 CatIBs was determined by assaying the enzyme at 10–80 °C for 30 min. The immobilized LipAMS8 CatIBs retained 57.1% (200 U/g) of their activity at 60 °C. The effect of temperature on stability, as shown in Fig. 5A, was evaluated by initial incubation of immobilized LipAMS8 CatIBs at 10–80 °C for 30 min., followed by an enzyme assay. The immobilized stability was observed at 40, 50, and 60 °C. There is an increase in temperature stability compared with free LipAMS8 CatIBs (Figure S5). This improved stability may result from the protection provided by the support to the enzyme, which drastically reduces inactivation at elevated temperatures (de Andrade Silva et al. 2022). The immobilized LipAMS8 CatIBs exhibit efficient stability over a range of temperatures. This is crucial because for an enzyme to be successfully used in an industrial process, it must attain a certain level of stability. The effect of temperature on immobilized LipAMS8 CatIBs stability is presented in Fig. 5B.

Effect of pH of immobilized LipAMS8 CatIBs activity and stability

pH value affects the formation of hydroxy radicals and the surface and interface potential properties of a catalyst. It is a significant factor in the effective functioning of enzymes. Immobilized lipase possesses high activity at alkaline pH, as reported in an earlier study on soluble AMS8 lipase (Al Angari et al. 2023). It has been reported that many lipases have an optimum pH within the range of 8.0–10.0. In this study, both free and immobilized LipAMS8 CatIBs exhibited some degree of stability in both acidic and alkaline media. LipAMS8 CatIBs maintained significant activity at different pH levels. The ability of enzymes to withstand different pH conditions is not universal (Pourkhanali et al.2022). The findings of this study highlight the ability of LipAMS8 CatIBs in both free (Figure S6) and immobilized form to withstand a broad range of pH values, which is a crucial attribute for any given enzyme to be exploitable in various industrial applications. The impact of pH on the stability of LipAMS8 CatIBs is presented in Fig. 6.

Effect of organic solvents on immobilized LipAMS8 CatIBs activity

This study investigated the level of solvent tolerance of immobilized enzymes compared to that of free CatIBs. A study conducted using wild enzymes revealed the influence of toluene (Musa et al. 2018). These non-polar and polar dissolving agents exert different influences on the activity of an enzyme, especially their log *P*-value. The use of hydrophobic organic solvents, such as heptane, hexane, and toluene, preserved the catalytic activity without distorting the nature of the enzyme (Liaquat and Apenten 2000). This differs from utilizing hydrophilic organic solvent, which tends to distort the enzyme structure and, as such, predispose it to inactivation (Latip et al. 2018). The stability of the immobilized enzyme has been observed to increase in toluene, 1-propanol, benzene, and methanol in a decreasing manner, although low stability was observed in n-hexanol, n-heptane, and DMSO increasingly. This organic solvent's presence may affect the activity of an enzyme. Immobilized enzymes retained up to 50% of their activity, which correlates with various reports that revealed lipase's ability to function in organic solvents (Sharma and Kanwar 2014). The result of organic solvent effects is presented in Figure S3.

Metal ion interaction against immobilized CatIBs

The results indicated less stability in calcium ions to remaining ions by an almost 15% decrease, contrary to that reported for the soluble LipAMS8 lipase, in which calcium ions influence its activity (Ali et al. 2020). An

increment in enzyme catalytic capacity by triglycerol hydrolases was shown when Ca2 + is present. The structure and activity of enzymes are significantly influenced by metal cations, especially Ca2 + . It has been observed that many lipases are stimulated by calcium (Cherif et al. 2011). At the same time, it manifested a certain underlying distinctness between CatIBs and the soluble enzyme. Similarly, the effect of iron revealed that an increase in its concentration from 1 to 5 mM led to inactivation and loss of almost 90% of activity in both soluble enzymes and free CatIBs. Whereas that of immobilized CatIBs revealed an increase in activity from 95 to 110% in 1 mM and 5 mM, respectively. The metal interaction results in immobilized enzyme stability, as shown in Fig. 7. The outcome showcased the strong stability of the immobilized enzyme in most of the metal ions in the 1 to 5 mM concentration, which differs from free LipAMS8 CatIBs that become inactive at 5 mM iron concentration. The result obtained from iron is contrary to the wild type, which was drastically dormant at a concentration reaching 5 mM of copper and zinc (Massadeh & Sabra 2011). Numerous reports revealed that Fe2 + has an inhibitory effect on most lipases (Borkar et al. 2009). The effect of metal ions is presented in Fig. 8.

Immobilized LipAMS8 CatIBs recyclability

Reusability is a prerequisite for an enzyme to be widely utilized in an industrial setting (Kumari et al. 2024). The result of recyclability of immobilized LipAMS8 CatIBs for ten cycles of enzyme assay, indicated that it can be maintained at more than 50% activity after ten cycles of enzyme assay. This proved its reusability, which can be of industrial importance. The ease of separation and reusability of an enzyme are excellent properties that determine the viability of enzyme application in industries (de Andrade Silva et al. 2022). The result also revealed that, initially, the immobilized LipAMS8 CatIBs decreased at the beginning of the reaction and later maintained a steady state, as reported in the characterization of *Penicillium* lipase (Pourkhanali et al.2022). The result obtained shows a little bit higher than that obtained in another study (Aghabeigi et al. 2023). Narwal et al. (2014) reported in a comparative study that immobilized enzyme retained more than 50% of its residual activity after 6 cycles, which is lower compared to this current study in which immobilized LipAMS8 CatIBs maintained 52% of their residual activity after 10 cycles (Narwal et al. 2014). The improvement witnessed in the reusability of immobilized LipAMS8 CatIBs might be protection offered by the seplite to prevent enzyme linkages and denaturation. The result of recyclability is presented in Fig. 8.

Biodiesel yield

The biodiesel yields at 40 °C, 30 °C, and 25 °C are presented in Figure S4. The yield obtained at 40 °C revealed that the yield was lower at a ratio of 1:3, even though there was more yield in free and immobilized lipase when compared with the positive control. It also reveals an increase in yield at ratios of 1:6 and 1:9, which is in line with many studies (Sarno and Iuliano 2019). It was observed that the yield at 30 °C exhibited an almost a similar pattern to that of 40 °C with a higher yield associated with an increase in the methanol ratio. However, the yield of 1:3 was higher at 30 °C compared with that at 40 °C. This might result from a temperature alteration, since the enzyme is psychrophilic, and the positive control works optimally at higher temperatures (Mandari and Devarai 2022). The yield of biodiesel observed at 25 °C also indicated that the value obtained in free and immobilized CatIBs at a ratio of 1:3 was higher than that of the positive control. It also exhibited the highest yield compared to the control, with almost 98% yield. This might be due to the gradual increment of methanol, which contributes to preventing the inactivation of the enzyme. The result of the transesterification reaction of waste cooking oil using methanol, which is catalyzed by free and immobilized CatIBs, indicates that both are good candidates to produce biodiesel at lower temperatures, even though immobilized CatIBs produce higher yield than free CatIBs.

GC–MS analysis

The FAME composition was generated from the free and immobilized LipAMS8-generated biodiesel, and the FAMEs are composed of methyl lineolate, methyl palmitate, methyl stearate, and methyl laurate. GC–MS analysis was carried out to estimate the amount of fatty acid methyl ester in the biodiesel synthesized from cooking oil. Individual FAMEs were identified based on their retention time as appeared in the chromatogram (Parandi et al. 2022). The peaks were identified using the FAME internal standard and MS database (Figures S1A and S1B). The FAMEs composition obtained from the transesterification reaction catalyzed by immobilized LipAMS8 CatIBs revealed the methyl ester profile to consist of mainly octadecanoic as the major methyl ester, followed by hexadecenoic and stearic acid. The result is presented in Table 1 for biodiesel composition analysis, indicating a composition of about 88.91% of biodiesel-catalyzed transesterification by immobilized CatIBs compared with that of free LipAMS8 CatIBs 85.91% (Table S3) and that of control 72.4% in Table 2. It has been observed that the FAMEs of methyl ester content catalyzed by immobilized LipAMS8 CatIBs showcased hexadecanoic acid and octadecanoic acid as the main methyl esters. This finding coincides with that reported by Poudel et al. (2017).

Therefore, the free and immobilized CatIBs have the potential to catalyze the transesterification reaction effectively at lower temperatures at 25 °C for 7 h.

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Data availability The data presented in this study are available on request.

Declarations

Ethical approval The research does not involve humans or animals.

Consent to participate The research does not use human subjects.

Consent for publication The manuscript does not contain any images that are from other authors.

Competing interests The authors declare no competing interests.

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