RESEARCH ARTICLE

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Properties of snail shell-immobilized lipase of *Aspergillus pseudotamarii* and its application in butyl butyrate synthesis

ABSTRACT

In this study, attempts were made to evaluate shells of egg and snail, crystal beads, and kaolin clay as suitable carriers for the immobilization of purified lipase produced by *Aspergillus pseudotamarii*. The free and immobilized lipases were characterized and applied in the synthesis of butyl butyrate. Snail shell yielded 84.17% immobilization efficiency, surpassing other carriers. Optimal activity occurred at 40℃ and 45℃, yielding 2.0U/ml and 3.0U/ml for free and immobilized lipase, respectively. Immobilized lipase demonstrated superior thermal stability (62.6% vs. 0% at 300 min). pH influenced activity, with maxima at pH 7 and 6.5 for immobilized and free lipase. Kinetic parameters: Km, Vmax—3.17mM, 0.549 µM/min (free); 0.214mM, 0.894 µM/min (immobilized). Immobilized lipase exhibited better storage stability. Butyl butyrate synthesis favored immobilized lipase (71.85% vs. 52.38% yield). Optimization studies showed that the immobilized lipase achieved the highest conversion yield at a shorter reaction time and higher temperature compared to the free lipase. The immobilized lipase also exhibited excellent reusability, maintaining a stable conversion yield over multiple cycles. Overall, the immobilized lipase on snail shell showed promising characteristics and potential for butyl butyrate synthesis.

Key words: Agro waste, immobilization, lipase, cross-linking, *Aspergillus* sp.

Introduction

Lipases, specifically triacylglycerol hydrolases (EC 3.1.1.3), are enzymes that facilitate the breakdown of various carboxyl esters, resulting in the production of fatty acids, mono and diacylglycerols, and glycerol. These enzymes have demonstrated diverse applications across numerous industries, including oleochemicals, food ingredients, pharmaceuticals, polymers, textiles, biodiesel, and detergents (Adrio & Demain, 2014; Kourist *et al.*, 2014; Barriuso *et al.*, 2016; Serri & Mooi, 2019; Bolivar *et al.*, 2022). Moreover, lipases play a pivotal role in the synthesis of natural flavor esters such as butyl butyrate, which finds extensive use in the food, cosmetic, detergent, chemical, and pharmaceutical sectors (Brault *et al.*, 2014). It is commonly added to food and beverages to enhance their sensory properties, while the cosmetic industry utilizes it to achieve specific fruity or floral scents (Sinumvayo *et al.*, 2021).

Esters are frequently derived from organic sources such as plant materials but the quantity reportedly obtained from this process is low (Salah *et al.*, 2007; López-Fernández *et al.*, 2020) for commercial purposes and the utilization of homogeneous acids as catalysts in esterification as an alternative has been suggested. Acid hydrolysis on the other hand is reported to have drawbacks such as equipment corrosion and potential hazards associated with handling corrosive acids hence, employing lipases in ester synthesis presents an appealing alternative that enhances yield while reducing energy consumption (Matte *et al.*, 2016).

However, the use of free lipases in ester synthesis is not cost-effective due to the inability to recover the enzyme after each synthesis, resulting in enzyme loss and increased demand for enzymes. To address this issue, the immobilization of lipase on solid insoluble supports has been proposed, as it allows for recycling (Bhattacharya & Pletschke, 2014; Bohara *et al.*, 2016). Moreover, this approach has been reported to enhance the activity, specificity, and selectivity of the biocatalyst (Thangaraj & Solomon, 2019). As a result of these numerous advantages associated with immobilized lipase, several researchers have explored its use in enzymatic production of sugar esters such as butyl butyrate (Groussin & Antoniotti, 2012; Lorenzoni *et al.*, 2012; Salihu *et al.*, 2014).

The most commonly employed method for enzyme immobilization involves the use of inorganic materials and

solid supports, such as silica, inorganic oxides, mineral materials (e.g., bentonite, halloysite, kaolinite, montmorillonite, and sepiolite), carbon-based materials (activated carbons, unmodified and modified charcoals), synthetic polymers, and biopolymers (chitin, chitosan, and alginate) (Daoud *et al.*, 2010; Kumar *et al.*, 2013; Saun *et al.*, 2014; Chrisnasari *et al.*, 2015; Maksym *et al.*, 2017; Kołodziejczak-Radzimska *et al.*, 2018). This approach involves immobilizing or incorporating the enzyme within a solid carrier molecule, thus creating a heterogeneous enzymatic catalytic system. Despite the advantages associated with enzymes immobilized on synthetic molecules, the cost and availability of carrier molecules, as well as the chemical modification of enzymes resulting from the multiple steps involved in enzyme attachment, have posed significant obstacles to the large-scale industrial application of immobilized enzymes.

In this study, we successfully immobilized lipase from *Aspergillus pseudotamarii* on a snail shell *(Lissachatina fulica*) and characterized both the free and immobilized lipases. Additionally, we assessed the performance of the immobilized lipase in the synthesis of butyl butyrate.

Materials and Methods

Sample collection

Shell of African Giant Snail (*Lissachatina fulica*), eggshell from hen (*Gallus gallus domesticus*), and crystal beads employed for immobilization in this study were obtained from Bodija market in Ibadan, Nigeria while the Kaolin clay was mined at Ologun eru area of Ibadan. All other chemicals used were of analytical grade and purchased from a local supplier.

Microorganism and culture condition

The lipase-producing *Aspergillus pseudotamarii* was obtained from the Culture Collection Centre of the Department of Microbiology, University of Ibadan, and sub-cultured on a tween-80 agar plate and incubated at 30℃ for a period of 48-72h in an incubator (MEMMERT, 770040).

Lipase production in submerged fermentation

Lipase production by the *Aspergillus pseudotamarii* was carried out in a 250ml Erlenmeyer flask containing 50ml of sterile basal medium, composed of the following components (in g·L-1): NaH2PO4 (12), MgSO4·7H2O (0.3), KH2PO4 (2), CaCl2 (0.25). (NH4)2SO4 was added at 1% (w/v) and olive oil was used as a carbon source at a concentration of 2% (v/v). The initial pH of the medium was adjusted to 6.0. The medium was inoculated with a 72-hour culture using a 6 mm disc and then incubated at 30ºC and 150 rpm for a duration of 96 hours. After the incubation, the mycelia were harvested by filtration using a Whatman filter paper No. 1 and the resulting filtrate was assayed for lipase activity using a titrimetric method (Schinke & Germani, 2012). Lipase activity was determined based on the liberation of 1µmol of fatty acid per minute at a temperature of 37°C and pH 7. One unit of lipase activity was defined as the amount of enzyme required to release 1µmol of fatty acid under these conditions (Lopes *et al.*, 2011; Ferraz *et al.*, 2015). Lipase activity was calculated using Equation 1:

$$Lipase\ activity\left(\frac{U}{mL}\right) = \frac{(a-b)\times N\ .\ NaOH\ \times 10}{t}$$

Equation 1

Where, *a* and *b* represent the amount of NaOH required for the sample titration using lipase and without lipase (mL), respectively, and *t* is the incubation time (30 min).

Purification of the lipase was done according to the methods of Tangtua *et al.* (2017) using cold acetone precipitation at 4°C, followed by gel chromatography as described by Shaheen *et al.* (2020).

Lipase Immobilization

The snail shell, eggshell, crystal beads, and Kaolin clay used in this study were pretreated and activated according to the methods of Vemuri *et al.* (1998), Serri & Mooi, (2019), Sharmin *et al.* (2007) and James *et al.* (2008) respectively. To immobilize the lipase enzyme, 1g of each support matrix (snail shells, eggshells, clay, and crystal beads) was combined with 10ml of the enzyme and 10ml of a 0.75% glutaraldehyde solution (v/v). The mixture was allowed to stand undisturbed for 4 hours, followed by agitation at 150 rpm for 1 hour at a temperature of 30°C. Subsequently, the support matrices were separated by filtration using Whatman filter paper No. 1, and washed with 10ml of a 50mM potassium phosphate buffer (pH 7) to eliminate any excess glutaraldehyde and lipases. The resulting immobilized enzyme was then resuspended in a potassium phosphate buffer and stored at 4℃ until further use. The degree of immobilization was assessed by determining the immobilization efficiency. To calculate the immobilization efficiency, the filtrates and wastewater resulting from the immobilization process were collected, and the protein content that remained unabsorbed was determined using the method described by Lowry *et al.* (1951). The immobilization efficiency (IE, %) was calculated using equation 2:

$$\text{IE}(\%) = \frac{\text{Initial protein content}\left(\frac{\text{U}}{\text{mg}}\right) - \text{Final protein content}\left(\frac{\text{U}}{\text{mg}}\right)}{\text{Initial protein content}\left(\frac{\text{U}}{\text{mg}}\right)} \times 100$$

Equation 2

The support matrix with the highest immobilization efficiency after immobilization was further used in this study.

Morphological characterization of the carrier matrix

The surface of the support matrix with the highest immobilization yield was investigated before and after immobilization to check the effect of immobilization on the number of pores and pore sizes of the matrix using JEOL JSM-7600F field-emission SEM. To carry out the procedure, sample of an appropriate size that fits in the specimen chamber was mounted rigidly on a specimen holder called a specimen stub. The sample was then coated with platinum coating of electrically conducting material, and deposited on the sample by high-vacuum evaporation. The machine then places the specimen in a relatively high-pressure chamber where the working distance is short and the electron optical column is differentially pumped to keep the vacuum adequately low at the electron gun. The high-pressure region around the sample in the ESEM neutralizes charge and provides an amplification of the secondary electron signal. The amplified signal was seen on a monitor as a digital image and was then saved (Janssen, 2015).

Characterization of free and immobilized lipases

Effect of temperature and pH on the activity of free and immobilized lipase

The influence of temperature on lipase activity was examined by incubating a mixture of 1.0ml of the enzyme and 1.0ml of a 0.1 M sodium phosphate buffer (pH 7.2) containing 1% v/v olive oil at various temperatures ranging from 25°C to 70°C. To investigate the effect of pH on lipase activity, the reactions were conducted using different buffer solutions. Specifically, 1.0ml of the enzyme was combined with 1.0ml of a substrate preparation consisting of 1% olive oil, and the pH was adjusted within the range of 3.5 to 9.0 using 0.1 M sodium acetate buffer (pH 3.5-5.5), 0.1 M sodium phosphate buffer (pH 6.0-7.5), and 0.1 M tris-glycerine buffer (pH 8.0-9.0). The incubation was carried out at a temperature of 45°C for 1 hour and lipase assay was investigated as described previously.

The thermostability of the enzyme was assessed by incubating a 5ml solution of the enzyme at a temperature of 45°C for a duration of 6 hours, in the presence of olive oil at a concentration of 1% w/v. To determine the pH stability, the enzyme solution was mixed with a 0.1 M sodium phosphate buffer at the optimal pH and maintained for 6h. The residual enzyme activity was then measured at 30-minute intervals throughout the incubation period (Kareem *et al.*, 2017).

Estimation of the kinetic constant of the free and immobilized lipase

K_m and V_{max} of the free and immobilized lipase were determined by measuring enzymatic activity with various concentrations of olive oil substrate (0.5%, 1.0%, 1.5%, 2.0%, and 2.5% (v/v)) in 0.1M phosphate buffer (pH 7.0) for 1 hr. Lipase activity was then determined by titrimetric method and the kinetic constants K_m and V_{max} were evaluated by fitting the experimental data of the Michaelis-Menten model using GraphPad Prism version 9.00 for Windows, GraphPad Software, San Diego California USA, www.graphpad.com

Effect of storage on the stability of immobilized lipase

For the effect of storage on the stability of the immobilized lipase, the immobilized catalyst was stored in 0.2M potassium phosphate buffer (pH 6.2) at 4°C and the residual activity was estimated every 3 days for a period of 15 days by comparison with the first running (activity defined as 100%) using the equation 3 below:

$$Residual\ activity(\%) = \frac{(activity\ at\ t_o) \times (activity\ at\ t_s)}{(activity\ at\ t_s)}$$

Equation 3

Where activity at t_o was defined as initial activity and activity at t_s is activity after storage

Application of the free and immobilized lipases in the synthesis of butyl butyrate

Butyl butyrate was synthesized by adding 1ml/g of free and immobilized lipase to a 10ml mixture of n-butanol and butyric acid (1:1 molar ratio) to initiate esterification reaction in a stoppered conical flask and incubated in an orbital shaker (150 rpm) at 30°C for 1h. A control experiment was also set up by using a 10ml mixture of n-butanol and butyric acid (1:1 molar ratio) in a conical flask without the lipase and incubated under the same condition. After incubation, 1ml reaction sample and control were directly titrated with 0.4M of sodium hydroxide solution by adding phenolphthalein as an indicator (Serri & Mooi, 2019). The volume of sodium hydroxide solution used in the titration was substituted as in equation 4:

$$\text{Conversion yield (\%)} = \frac{\text{Vol NaOH(w/o E)} - \text{Vol NaOH(with E)}}{\text{Vol NaOH(w/o E)}} \times 100\%$$

Equation 4

Where: Vol NaOH (w/o E) = Volume of sodium hydroxide without enzyme

Vol NaOH (with E) = Volume of sodium hydroxide with the enzyme.

Optimization of butyl butyrate yield.

Optimization of the yield of butyl butyrate was done by investigating the effect of parameters such as the reaction time (1-4 h), temperature (30-60°C), the molar ratio of butyric acid: butanol (0.2-1.8), and immobilized lipase loading (0.1-4.0g) on the esterification reaction was investigated. The reusability of the immobilized enzyme was also investigated by repeated synthesis of butyl butyrate for 5 cycles. After each cycle, the enzyme was recovered, rinsed

with sterile distilled water, and reused.K_m and V_{max} of the free and immobilized lipase were determined by measuring enzymatic activity with various concentrations of olive oil substrate (0.5%, 1.0%, 1.5%, 2.0%, and 2.5% (v/v)) in 0.1M phosphate buffer (pH 7.0) for 1 hr. Lipase activity was then determined by titrimetric method and the kinetic constants K_m and V_{max} were evaluated by fitting the experimental data of the Michaelis-Menten model using GraphPad Prism version 9.00 for Windows, GraphPad Software, San Diego California USA, www.graphpad.com

Results

Purification of lipase

Lipase from *Aspergillus pseudotamarii* was purified 4.15-folds with an overall protein yield of 14.06% and a specific activity of 29.57 and the lipase preparation was immobilized on Snail shells, egg shells, crystal beads, and kaolin clay. The results as shown in Table 1 revealed that the Snail shell had the highest immobilization efficiency of 84.17% followed by eggshell, clay, and crystal beads with immobilization

efficiency of 63.89%, 45.54%, and 42.12% respectively. The snail shell with the highest immobilization efficiency was selected for further studies.

Morphological characterization of snail shell before and after lipase immobilization

Scanning electron microscopy was used to investigate the structure of the snail shell before and after lipase immobilization by glutaraldehyde linking and the results presented in Fig. 1. The images indicated numerous pores with irregular shape and roughness in the snail shell before cross-linked with lipase while snail shell cross-linked with lipase showed regular and smooth surface.

Characterization of free and immobilized lipases

Effect of temperature and thermal stability of free and immobilized lipase

The experimental findings regarding the impact of temperature on the activity and stability of free and immobilized lipase produced by *Aspergillus pseudotamarii* are presented in Fig. 2A and B. Fig. 2A demonstrates that the activity of free lipase reached its maximum level, measuring 2.0 U/ml, at a temperature of 40℃, whereas the immobilized lipase exhibited its highest activity of 3.0 U/ml at 45℃.

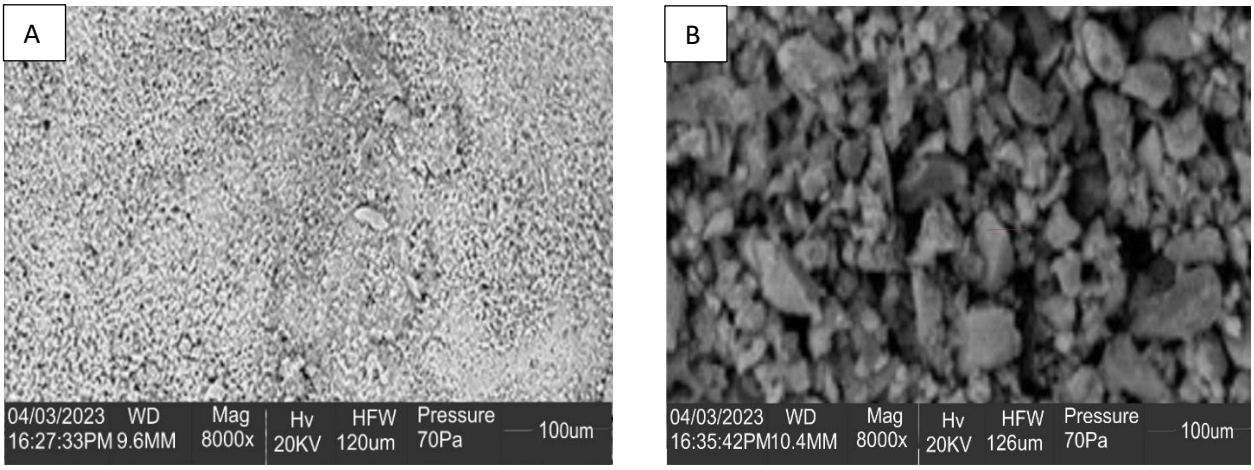

Figure 1. *Snail shell surface before and after immobilization with A. pseudotamarii lipase under scanning electron microscope at ×8,000 magnification.*

Table 1. *Immobilization efficiency of various support matrices lipase immobilization*

Support Matrix	Immobilization Efficiency (%)
	A. pseudotamarii
Eggshell	63.89
Snail shell	84.17
Clay	45.54
Crystal beads	42.12

To investigate the thermal stability of both free and immobilized lipases, incubation experiments were conducted at 40 and 45°C respectively and the residual activity was assessed at 30-min intervals over a period of 6 hours. The results revealed that the free lipase derived from *A. pseudotamarii* retained 91% of its initial activity after 30 min of incubation, which gradually decreased to 48% at 240 minutes beyond which no activity was detected (Fig. 2B). On the other hand, the snail shell-immobilized lipase exhibited a relative activity of 96.2% after 30 min of incubation. Subsequently, a gradual decline in activity was observed, with the relative activity dropping to 62.6% at 300 minutes beyond which no activity was detected (Fig. 2B).

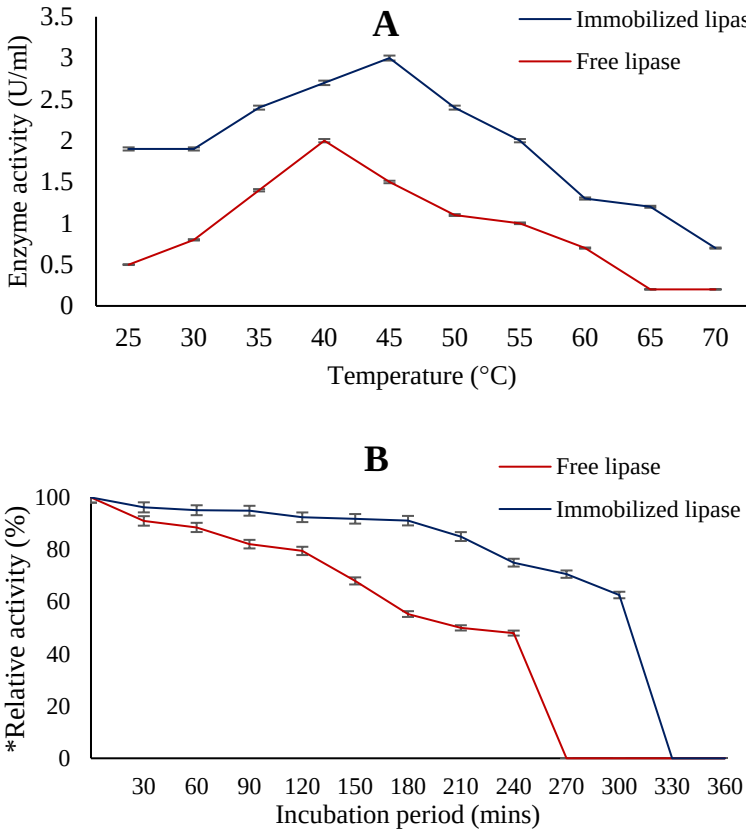

Figure 2. *Effect of temperature on activity (A) and thermal stability (B) of free and snail shell-immobilized lipase produced by Aspergillus pseudotamarii. *Relative activity of 100% corresponds to lipase activity of 2.0U/ml free lipase and 3.0U/ml for snail shell-immobilized lipase at 45 °C and 40 °C respectively.*

Effect of pH on the activity and stability of free and immobilized lipase

The effect of pH on lipase activity was investigated by carrying out the reactions in the pH range of 3.5 – 9.0 and the results presented in Fig. 3A revealed that the free lipase had the highest activity of 0.9 U/ml at pH 7 while the snail shell-immobilized lipase had the highest activity of 4.4 U/ml at pH 6.5. Similarly, the pH stability of the free and immobilized enzyme preparations was investigated at their respective optimum pH for a period of 6h, and the residual activity was determined every 30 min. The results as shown in Fig. 3B depict that free lipase showed relative activity of 88% after 30 min which further declined to 15% at 210 min. Immobilized lipase on the other had a relative activity of 99% after 30 min and a gradual decline to 75% and 72% at 60 and 90 min respectively. The lowest relative activity of 52% was recorded at 360 min (Fig. 3B).

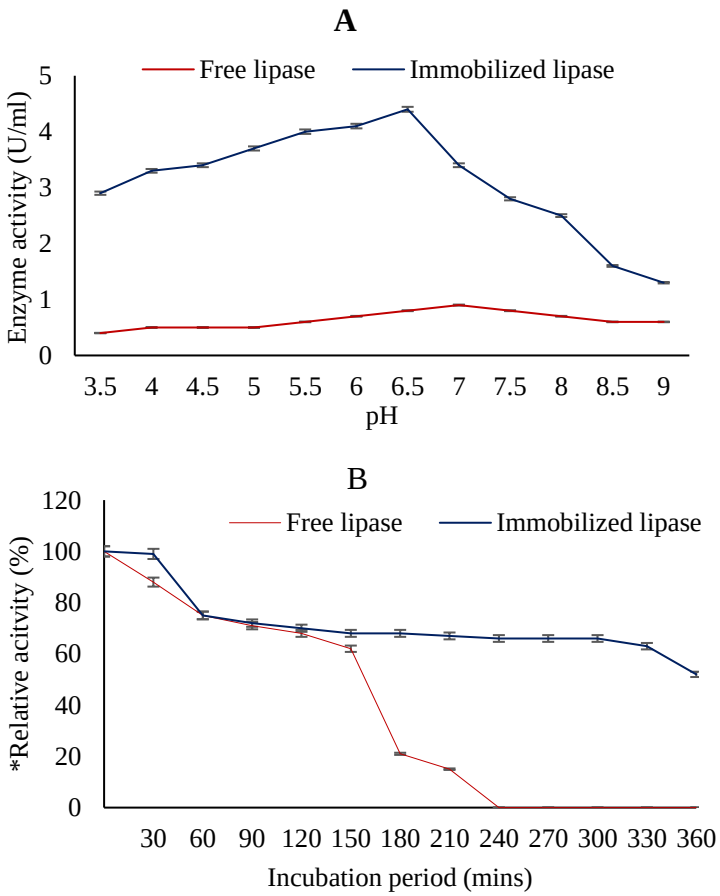

Figure 3. *Effect of pH on the activity (A) and stability (B) of free and snail shell-immobilized lipase produced by Aspergillus pseudotamrii. *Relative activity of 100% corresponds to lipase activity of 0.9U/ml for free lipase and 4.4U/ml for snail shell-immobilized lipase at 45°C and 40°C respectively*

Effect of substrate concentration on the activity of free and immobilized lipase

To access the affinity of the free and immobilized lipase preparations, the Lineweaver-Burk plot was employed, and the results are presented in Fig. 4. The data displayed adherence to the Michealis-Menton kinetics, yielding K_m and V_{max} values of 3.170 mM and 0.549 µM/min for free lipase and *Km* and *Vmax* values of 0.214 mM and 0.894 µM/min for snail shell-immobilized lipase.

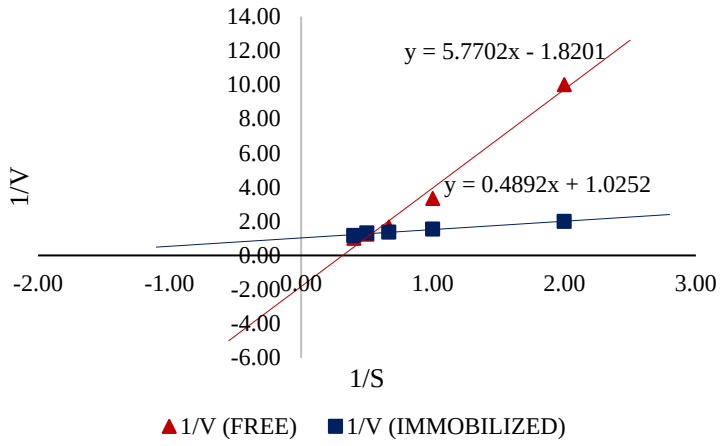

Figure 4. *Lineweaver-Burk plot for the hydrolysis of olive oil by free and immobilized lipases produced by Aspergillus pseudotamarii.*

Effect of storage on the activity of the free and immobilized lipases

The investigation of stability under storage for free and immobilized lipases involved monitoring the residual activity at intervals of 3 days over a period of 15 days and the findings illustrated in Fig. 6. A slight decline in residual activity was observed for the lipase immobilized on snail shell. Specifically, the activity decreased from 95% to 92% after 3 days, followed by a further decline to 87% by day 15 of storage. In contrast, the free lipase exhibited a decline in activity as well. After the 3rd and 6th days, the residual activity decreased to 90% and 85%, respectively. This downward trend continued, reaching 65% by day 15 as presented in Fig. 5.

Application of free and snail shell-immobilized lipase preparation in the synthesis of butyl butyrate

The free and immobilized lipase biocatalysts were applied in the synthesis of butyl butyrate from butyric acid and n-butanol. The results revealed that free lipase had a butyl butyrate yield of 52.38% and snail shell-immobilized lipase had a butyl butyrate yield of 71.85% after one hour of reaction time. From these results, the process of butyl butyrate synthesis by the free and immobilized lipase was optimized taking into consideration the effect of different reaction times, temperature, different ratios of butyric acid and n-butanol, and different enzyme concentrations.

Effect of different reaction time on esterification yield

For the effect of different reaction times on butyl butyrate yield by the free and immobilized lipase preparations synthesis was allowed to proceed for four different reaction times and the result presented in Fig. 6. The results showed that the free lipase had the highest conversion yield of 62.24% obtained at 3 h while the snail shell-immobilized lipase the highest conversion yield of 71.85% was observed at 1 h.

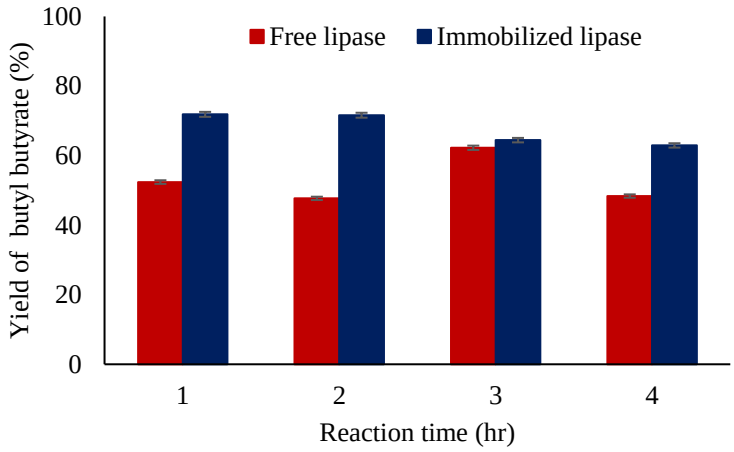

Figure 6. *Effect of different reaction times of butyl butyrate yield by the free and snail shell-immobilized lipase from Aspergillus pseudotamarii*

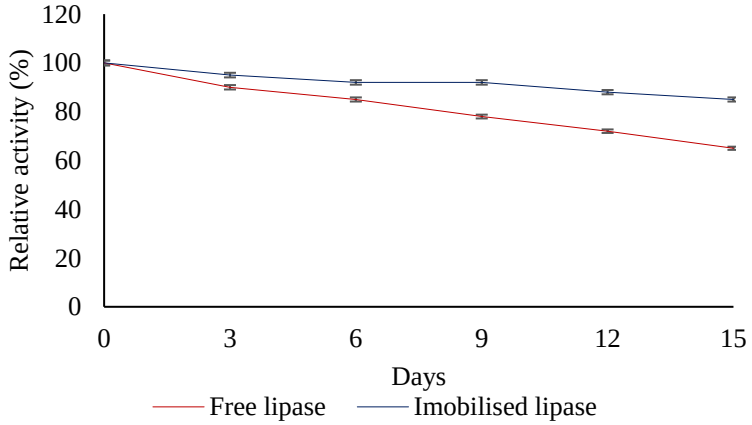

Figure 5. *Effect of storage on the activity of free and snail shell-immobilized lipases produced by Aspergillus pseudotamarii*

Effect of temperature of butyl butyrate yield

The optimal temperature for synthesis of butyl butyrate was investigated by conducting experiments with the reaction mixtures containing free and immobilized lipases, within the temperature range of 30°C to 60°C. As shown in Fig. 7, the free lipase exhibited a relatively consistent yield as the temperature increased, reaching its highest conversion yield of 57.24% at 40°C. On the other hand, the

immobilized lipase achieved its highest conversion yield of 74.6% at 45℃, and a gradual decrease in yield was observed beyond this temperature with the lowest yield of 60.36% at 60℃ (Fig. 7).

Effect of different ratios of butyric acid and butanol on the yield of butyl butyrate

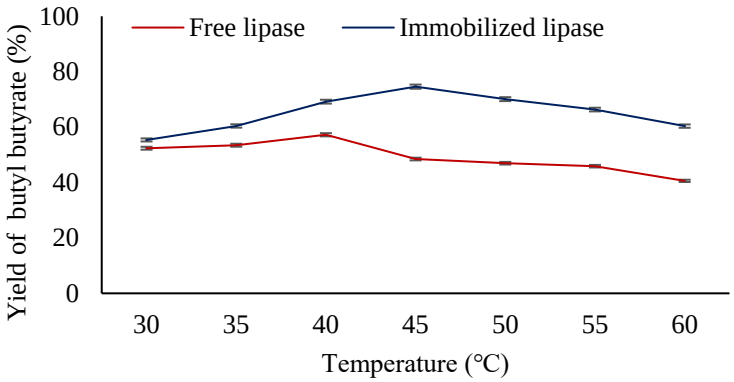

Figure 7. *Effect of different temperatures of butyl butyrate synthesis by free and snail shell-immobilized lipase of Aspergillus pseudotamarii*

The impact of different ratios of butyric acid to butanol on the yield of butyl butyrate was examined across a range of 0.2 to 1.6 and the findings are depicted in Fig. 8. Highest butyl butyrate yield of 54.93% was recorded at butyric acid ratio 0.8 to 1 for the free lipase while highest conversion yield of 77.81% at a molar ratio 1.2 to 1.

Effect of different enzyme concentrations on butyl butyrate yield

The impact of increasing the lipase loading, ranging from

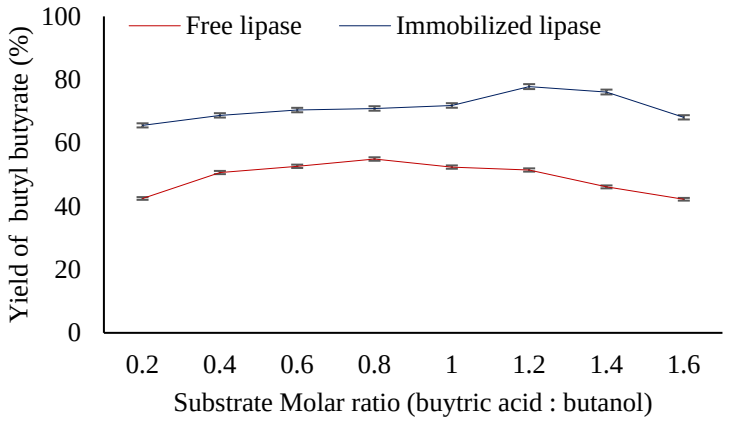

Figure 8. *Effect of different molar ratios of butyric acid and butanol on butyl butyrate synthesis using free and snail shell-immobilized lipase from Aspergillus pseudotamarii*

0.1 ml/g to 2.0 ml/g, on the conversion yield of butyl butyrate by the free and immobilized lipase preparations was investigated and the results are presented in Fig. 9. With the free lipase, highest butyl butyrate yield of 58.1% was obtained at an enzyme loading of 1.5ml while highest yield of 76.74% was obtained at an enzyme loading of 1.0 ml/g.

The optimal conditions determined from the single parameter study were employed in the final synthesis of butyl butyrate. In the case of free lipase, the synthesis utilized 1.5 ml of the enzyme at 40°C, with a molar ratio of 0.8 to 1 (butyric acid:n-butanol) and a 3-hour incubation period while for the immobilized lipase, 1.0g of the immobilized snail shells was loaded at 45°C, with a molar ratio of 1.2 (butyric acid:n-butanol) and a 1-hour incubation period. The maximum yield achieved for butyl butyrate was 78.22% and 88.49% for the free and immobilized lipases respectively.

Reusability of the immobilized lipase

The investigation into the reusability of the immobilized lipase involved multiple cycles of butyl butyrate synthesis, and the outcomes are illustrated in Fig. 10. The results showed that snail shell-immobilized lipase from *A. pseudotamarii* exhibited a relatively stable conversion yield throughout the cycles investigated. Following the initial cycle, a conversion yield of 83.49% was recorded, gradually decreasing to 81.9% after the third cycle and eventually reaching 80.00% after the fifth cycle.

Discussion

Owing to the numerous advantages of immobilized lipases, lipases from various sources have been immobilized on diverse carrier molecules, as documented in previous studies (Sadh *et al.*, 2018; Girelli *et al.*, 2020; Bolivar *et al.*, 2022). Nevertheless, the cost and inaccessibility (among other factors) of these commercial support matrices have prompted investigations into alternative carrier molecules that are more cost-effective and readily available (Sadh *et al.*, 2018; Girelli *et al.*, 2020). This present study focused on examining the viability of cost-effective and easily accessible materials such as eggshells, snail shell, clay, and crystal beads as carrier molecules for lipase immobilization via covalent bonding through cross-linking with glutaraldehyde. The remarkably high immobilization efficiency of between 42.12 to 84.17% observed in this study by these materials can be comparable to those documented for synthetic carriers in literature (Kumar *et al.*,

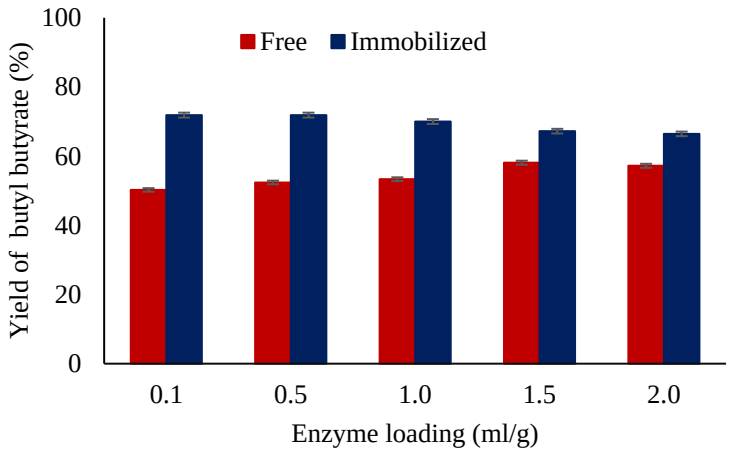

Figure 9. *Effect of different lipase loading on butyl butyrate yield using free and snail shell-immobilized lipases from Aspergillus pseudotamarii*

immobilization process, which can influence the enzyme's activity and specificity (Nadar & Rathod, 2017).

The measure of hydrogen ion concentration in a solution (pH) has been reported as an important factor influencing enzyme activity hence, its investigation. The result of the effect of pH on lipase activity showed that there was enhanced activity and stability by the immobilized lipase for all the pH regimes investigated. Furthermore, a shift towards the acidic range in the optimum pH for the snail shell immobilized lipase was observed. A similar observation has been previously reported by Karimpil *et al.* (2011). The author reported an enhanced activity when lipase from *Thermomyces lanuginosus* was immobilized

2013; Krishna *et al.*, 2021). The highest immobilization efficiency with the snail shell in this study could be a result of its typical porous structure, which provides a larger surface area for enzyme immobilization as shown by the scanning electron micrograph of the snail shell before immobilization. Furthermore, snail shells are known for their robustness and resistance to mechanical stress which can help maintain the integrity of the immobilized enzyme during the reaction, ensuring a higher yield porous structure porous nature may additionally facilitate substrate accessibility, allowing for efficient enzyme-substrate interactions (Aina *et al.*, 2018; Laskar *et al.*, 2018; Nhung *et al.*, 2023).

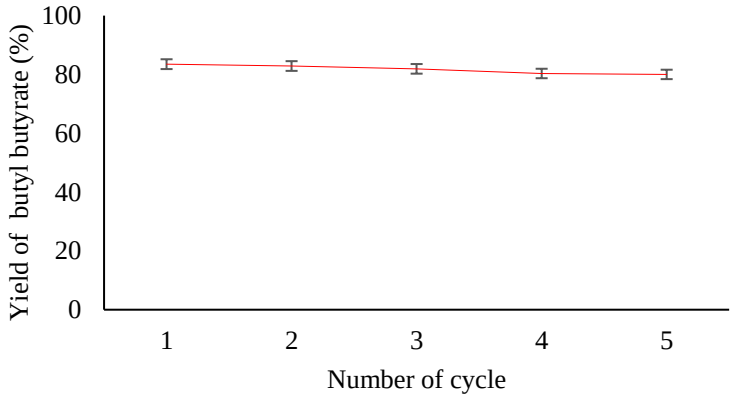

Figure 10. *Reusability of snail shell-immobilized lipase from Aspergillus pseudotamarii in the synthesis of butyl butyrate*

SEM analysis of snail shell after immobilization revealed topological changes. Similar topological changes have been noticed after the successful attachment of enzyme on functionalized nanoparticles, an epoxy group-containing polymer and aluminum oxide pellets (Li *et al.*, 2010; Liu *et al.*, 2012; Khoshnevisan *et al.*, 2019; Xu *et al.*, 2023)

The impact of temperature on the activity and stability of the free and immobilized lipase was investigated across various temperature ranges, considering its crucial role in the activity and stability of biocatalysts (Asmat *et al.*, 2017). A shift in optimum activity from 40°C to 45°C was observed for the immobilized lipase. Furthermore, enhanced stability under temperature was observed for the immobilized enzyme as compared to the free lipase. This enhanced stability and activity for immobilized lipase has been previously reported by Sufficiency *et al.* (2022). This enhancement in activity can be attributed to conformational changes induced by the on synthetic carrier molecules. The shift towards the acidic range in the optimum pH for the snail shell immobilized lipase indicates that the immobilization process might have altered the enzyme's microenvironment. Enhanced activity and stability by the immobilized lipase could be a result of an altered microenvironment, and changes in the ionization state of the active site of the lipase arising from the snail shell immobilization protocols which might have resulted in the distribution of negative charges in the support system (Costa-Silva *et al.*, 2015).

In this study, a reduction in *Km* and an increase in *Vmax* was observed when lipase from *A. pseudotamarii* was immobilized on a snail shell as compared with the free lipase. Similar changes in the kinetic parameters of lipase from various sources after immobilization have been previously reported (Mikawlrawng, 2016). This reduction in the *Km* and increase in *Vmax* after immobilization could suggest a higher affinity of immobilized enzymes towards their substrate

owing to conformational changes near the active site of the enzyme owing to the proximity between the enzyme and support which can result in substrate affinity or allosteric properties and subunit dissociation (Dal Magro *et al.*, 2021).

In this study, storage stability results showed an increase in stability during storage for immobilized lipases compared with the free ones. A similar observation was earlier reported by Asmat *et al.* (2017) who documented enhanced stability under storage conditions when evaluating the stability of immobilized lipase preparations from *Aspergillus niger*. Generally, immobilized lipases are more stable than free lipases due to the reduction in conformational changes and unfolding of the enzyme molecule. When enzymes are free in solution, they are subject to conformational changes and unfolding, which can result in the loss of activity and stability. However, when lipases are immobilized, they are confined to a specific space and are less likely to undergo these changes (Thangaraj & Solomon, 2019). Furthermore, the stability observed in the immobilized lipases could be due to the protection provided by the snail shell, which shields the enzyme from environmental factors such as pH, temperature, and organic solvents, all of which can affect enzyme stability (Liu *et al.*, 2018).

Application of the free and immobilized lipase in the synthesis of butyl butyrate showed that the snail shell immobilized lipase had a higher yield compared to the free lipase preparation. A similar observation was reported by (Serri & Mooi, 2019). The higher yield observed from the immobilized lipase could be a result of the fact that the immobilization provides a stable and confined microenvironment for the lipase, allowing for better retention of its activity. This stability ensures that the lipase remains active throughout the reaction, leading to a higher conversion rate and yield (Choi *et al.*, 2015). Furthermore, snail shell carriers (as seen under the electron microscope) possess unique properties, such as high surface area, porous structure, and functional groups, which can facilitate enzyme immobilization and provide an ideal environment for lipase activity. These properties may enhance the accessibility of substrates to the immobilized lipase, thereby increasing the efficiency of the catalytic reaction and leading to higher product yields.

In the synthesis of butyl butyrate, reaction time is of great significance hence the investigation of the effect of time on the yield of butyl butyrate in this study. For the immobilized lipase highest butyl butyrate yield was achieved at 1h of reaction time, while for the free lipase, the highest yield was achieved at 3h of reaction time. This short reaction time is among the shortest reaction times reported for the synthesis of esters using immobilized lipase (Ozyilmaz & Gezer, 2010). The short reaction time observed with the snail shell immobilized lipase in this study means that a higher number of reaction cycles can be completed within a given timeframe, and this can be particularly beneficial in industrial settings where rapid production and high yields are desirable as it will allow for an increased number of reaction cycles to be completed within a given timeframe, thereby enhancing process throughput. Additionally, shorter reaction times contribute to cost savings by reducing energy consumption and optimizing resource utilization. The diminished energy requirements for heating and cooling contribute to overall process efficiency, resulting in reduced operational costs (Friedrich *et al.*, 2013).

The optimum temperature of 40°C and 45°C by the free and snail shell for the highest butyl butyrate yield in this among the temperature ranges reported in literature (Champagne *et al.*, 2016). Beyond these temperatures, a decrease in yield was observed probably due to enzyme denaturation. Optimum butyl butyrate yield at these temperatures (by the free and immobilized lipase) could be because enzymes' active sites are optimally activated at these temperatures thereby allowing for efficient substrate binding and conversion. Additionally, these temperatures may promote the proper conformational changes in the enzyme structure, enhancing its stability and facilitating the desired esterification reaction. The higher temperature of 45°C for the snail shell immobilized lipase compared to the free lipase's optimum temperature of 40°C suggests that the immobilization process might have influenced the enzyme's thermal stability and broadened its optimal temperature range for butyl butyrate synthesis (Spinelli *et al.*, 2014).

In this study, the results of the substrate molar ratio showed that the molar ratio of the substrate is a significant factor in butyl butyrate conversion. Theoretically, until the active sites of the enzyme become saturated, the reaction increases dramatically when the substrate concentration increases. The decrease in conversion yield beyond a certain substrate molar ratio could be a result of excess water produced from the esterification reaction which facilitated hydrolysis reaction where the butyl butyrate produced is reversely converted to the substrate (Elias *et al.*, 2019).

The immobilized lipases exhibited excellent reusability and were able to maintain over 86% of its initial activity at 8 cycles of repeated use. The reduction in activity observed could be a result of gradual structural changes or damage to the enzyme. These changes may include conformational alterations and loss of active sites which can impede the enzyme's catalytic efficiency (Nguyen & Kim, 2017). Additionally, repeated washing of the immobilized lipases after the esterification reaction has been reported to cause the immobilized lipase to be washed off thus lowering its activity (Silva *et al.*, 2013). The high activity exhibited by the snail shell-immobilized enzyme after repeated use provides confidence in its long-term performance, stability, and

economic viability. This will allow for sustained enzymatic activity, consistent results, and potential cost savings, making it advantageous for various biotechnological and industrial applications.

Conclusion

Conclusively, this present study focused on evaluating the viability of eggshell, snail shell, clay, and crystal beads, which are cost-effective and easily accessible materials, and carrier molecules for lipase immobilization. Amongst them, the snail shell exhibited the highest immobilization efficacy probably due to its porous nature and mechanical strength. The immobilized lipases demonstrated enhanced activity and stability under temperature and pH variations compared to the free lipase, which can be attributed to the altered microenvironment and changes in the ionization state of the active site caused by the immobilization process. While the kinetic parameters of the immobilized lipases showed a reduction in *Km* and *Vmax*, indicating a higher affinity towards the substrate, the immobilized lipases exhibited increased stability during storage and higher yields in the synthesis of butyl butyrate compared to the free lipase. The snail shell immobilized lipase, in particular, showed a short reaction time, allowing for increased reaction cycles and cost savings in industrial applications. Moreover, the immobilized lipases demonstrated excellent reusability and maintained a high level of activity after multiple cycles. Overall, the findings highlight the potential of using snail shell as a carrier for lipase immobilization, providing a stable microenvironment, improved catalytic efficiency, and economic viability for various biotechnological and industrial applications.

Declarations

Ethics approval and consent to participate

Not applicable

Consent for publication

Not applicable.

Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author upon reasonable request.

Competing interests

The authors declare that they have no competing interests

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Authors' contributions

EOG designed the work. OTO collected the data and also prepared the manuscript together with EOG. EOG analyzed some of the data. All authors read and approved the final manuscript.

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Abbreviations

K_m Michaelis-Menten constant

V_{max} Maximum velocity

References

Adrio JL, Demain AL. 2014. Microbial enzymes: tools for biotechnological processes. Biomolecules, 4(1): 117-139.

Aina OE, Agbaji EB, Nwokem NC. 2018. Biodegradation of Naphthalene using *Pseudomonas putida* and *Bacillus subtilis* Immobilized on Snail Shell. J. Environ. Manage., 22(7): 1065-1070.

Asmat S, Husain Q, Azam A. 2017. Lipase immobilization on facile synthesized polyaniline-coated silver-functionalized graphene oxide nanocomposites as novel biocatalysts: stability and activity insights. RSC Adv., 7(9): 5019-5029.

Barriuso J, Vaquero ME, Prieto A, Martínez MJ. 2016. Structural traits and catalytic versatility of the lipases from the *Candida rugosa*-like family: A review. Biotechnol. Adv., 34(5): 874-885.

Bhattacharya A, Pletschke BI. 2014. Magnetic cross-linked enzyme aggregates (CLEAs): a novel concept towards carrier free immobilization of lignocellulolytic enzymes. Enzyme Microb. Technol., 61: 17-27.

Bohara RA, Thorat ND, Pawar SH. 2016. Immobilization of cellulase on functionalized cobalt ferrite nanoparticles. Korean J Chem Eng., 33: 216-222.

Bolivar JM, Woodley JM, Fernandez-Lafuente R. 2022. Is enzyme immobilization a mature discipline? Some critical considerations to capitalize on the benefits of immobilization. Chem. Soc. Rev., 51: 6251–6290.

Brault G, Shareck F, Hurtubise Y, Lépine F, Doucet N. 2014. Short-chain flavor ester synthesis in organic media by an E. coli whole-cell biocatalyst expressing a newly characterized heterologous lipase. PLoS One., 9(3): .e91872.

Champagne E, Strandman S, Zhu XX. 2016. Recent developments and optimization of lipase-catalyzed lactone formation and ring-opening polymerization. Macromol. Rapid Commun., 37(24): 1986-2004.

Choi JM, Han SS, Kim HS. 2015. Industrial applications of enzyme biocatalysis: Current status and future aspects. Biotechnol. Adv., 33(7): 1443-1454.

Chrisnasari R, Wuisan ZG, Budhyantoro A, Widi RK. 2015. Glucose oxidase immobilization on TMAH-modified bentonite. Indones. J. Chem., 15(1): 22-28.

Costa-Silva TA, Souza CRF, Said S, Oliveira WP. 2015. Drying of enzyme immobilized on eco-friendly supports. Afr. J. Biotechnol., 14(44): 3019-3026.

Dal Magro L, de Moura KS, Backes BE, de Menezes EW, Benvenutti EV, Nicolodi S, Klein MP, Fernandez-Lafuente R, Rodrigues RC. 2019. Immobilization of pectinase on chitosan-magnetic particles: Influence of particle preparation protocol on enzyme properties for fruit juice clarification. Biotechnol. Rep., 24: e00373.

Daoud FBO, Kaddour S, Sadoun T. 2010. Adsorption of cellulase *Aspergillus niger* on a commercial activated carbon: kinetics and equilibrium studies. Colloids Surf. B., 75(1): 93-99.

Elias N, Wahab RA, Chandren S, Razak FIA, Jamalis J. 2019. Effect of operative variables and kinetic study of butyl butyrate synthesis by *Candida rugosa* lipase activated by chitosan-reinforced nanocellulose derived from raw oil palm leaves. Enzyme Microb. Technol., 130: 109367.

Ferraz LIR, Possebom G, Alvez EV, Cansian RL, Paroul N, de Oliveira D, Treichel H. 2015. Application of home-made lipase in the production of geranyl propionate by esterification of geraniol and propionic acid in solvent-free system. Biocatal. Agric. Biotechnol., 4(1):44-48.

Friedrich JL, Pena FP, Garcia-Galan C, Fernandez-Lafuente R, Ayub MA, Rodrigues RC. 2013. Effect of immobilization protocol on optimal conditions of ethyl butyrate synthesis catalyzed by lipase B from *Candida antarctica*. J. Chem. Technol. Biotechnol., 88(6): 1089-1095.

Girelli AM, Astolfi ML, Scuto FR 2020. Agro-industrial wastes as potential carriers for enzyme immobilization: A review. Chemosphere, 244: 125368.

Groussin AL, Antoniotti S. 2012. Valuable chemicals by the enzymatic modification of molecules of natural origin: Terpenoids, steroids, phenolics and related compounds. Bioresour. Technol., 115: 237-243.

James OO, Mesubi MA, Adekola FA, Odebunmi EO, Adekeye JID. 2008. Beneficiation and characterization of a bentonite from north-eastern Nigeria. J. North Carolina Acad. Sci., 124(4): 154-158.

Janssen GJ. 2015. Information on the FESEM (Field-emission Scanning Electron Microscope). Radboud University Nijmegen. 1-5.

Kareem SO, Adebayo OS, Balogun SA, Adeogun AI, Akinde SB. 2017. Purification and Characterization of Lipase from *Aspergillus flavus* PW2961 using Magnetic Nanoparticles. Niger. J. Biotechnol., 32: 77-82.

Karimpil JJ, Melo JS, D'Souza SF. 2011. Hen egg white as a feeder protein for lipase immobilization. J. Mol. Catal. B Enzym., 71(3-4): 113-118.

Khoshnevisan K, Poorakbar E, Baharifar H, Barkhi M. 2019. Recent advances of cellulase immobilization onto magnetic nanoparticles: an update review. Magnetochemistry, 5(2): 36.

Kołodziejczak-Radzimska A, Zdarta J, Jesionowski T. 2018. Physicochemical and catalytic properties of acylase I from *Aspergillus melleus* immobilized on amino-and carbonyl-grafted stöber silica. Biotechnol. Prog., 34(3): 767-777.

Kourist R, Hollmann F, Nguyen GS. 2014. Lipases as sustainable biocatalysts for the sustainable industrial production of fine chemicals and cosmetics. JSM biotechnol. biomed. eng., 2:1029.

Krishna J, Perumal AS, Khan I, Chelliah R, Wei S, Swamidoss CMA, Oh DH, Bharathiraja B. 2021. Synthesis of nanomaterials for biofuel and bioenergy applications. In Nanomaterials. Academic Press, 97-165.

Kumar A, Sharma V, Sharma P, Kanwar SS. 2013. Effective immobilisation of lipase to enhance esterification potential and reusability. Chem. Pap., 67: 696-702.

Laskar IB, Rajkumari K, Gupta R, Chatterjee S, Paul B, Rokhum SL. 2018. Waste snail shell derived heterogeneous catalyst for biodiesel production by the transesterification of soybean oil. RSC Adv., 8(36): 20131-20142.

Li M, Su E, You P, Gong X, Sun M, Xu D, Wei D. 2010. Purification and in situ immobilization of papain with aqueous two-phase system. PLoS One, 5(12): e15168.

Liu DM, Chen, J, Shi YP. 2018. Advances on methods and easy separated support materials for enzymes immobilization. TrAC, Trends Anal. Chem., 102: 332-342.

Liu H, Liu J, Tan B, Zhou F, Qin Y, Yang R. 2012. Covalent immobilization of Kluyveromyces fragilis β-galactosidase on magnetic nanosized epoxy support for synthesis of galacto-oligosaccharide. Bioprocess Biosyst Eng., 35: 1287-1295.

Lopes DB, Fraga LP, Fleuri LF, Macedo GA. 2011. Lipase and esterase: to what extent can this classification be applied accurately? Food Sci. Technol., 31: 603-613.

López-Fernández J, Benaiges MD, Valero F. 2020. *Rhizopus oryzae* lipase, a promising industrial enzyme: Biochemical characteristics, production and biocatalytic applications. Catalysts, 10(11): 1277.

Lorenzoni AS, Graebin NG, Martins AB, Fernandez-Lafuente R, Záchia Ayub MA, Rodrigues RC. 2012. Optimization of pineapple flavour synthesis by esterification catalysed by immobilized lipase from *Rhizomucor miehei*. Flavour Fragr. J., 27(2): 196-200.

Lowry OH, Rosebrough NJ, Farr AL, Randall RJ. 1951. Protein measurement with the folin phenol reagent. J. Biol. Chem., 193: 265-275.

Maksym P, Tarnacka M, Dzienia A, Matuszek K, Chrobok A, Kaminski K, Paluch M. 2017. Enhanced polymerization rate and conductivity of ionic liquid-based epoxy resin. Macromolecules, 50(8): 3262-3272.

Matte CR, Bordinhão C, Poppe JK, Rodrigues RC, Hertz PF, Ayub MA. 2016. Synthesis of butyl butyrate in batch and continuous enzymatic reactors using *Thermomyces lanuginosus* lipase immobilized in Immobead. J. Mol. Catal. B Enzym., 127: 67-75.

Mikawlrawng K. 2016. *Aspergillus* in biomedical research. In New and Future Developments in Microbial Biotechnology and Bioengineering. Elsevier, 229-242.

Nadar SS, Rathod VK. 2017. Ultrasound assisted intensification of enzyme activity and its properties: a mini-review. World J. Microbiol. Biotechnol., 33: 1-12.

Nguyen HH, Kim M. 2017. An overview of techniques in enzyme immobilization. Appl. Sci. Converg. Technol., 26(6): 157-163.

Nhung NTH, Long VD, Fujita T. 2023. A Critical Review of Snail Shell Material Modification for Applications in Wastewater Treatment. Materials, 16(3): 1095.

Ozyilmaz G, Gezer E. 2010. Production of aroma esters by immobilized Candida rugosa and porcine pancreatic lipase into calcium alginate gel. J. Mol. Catal. B Enzym., 64(3-4): 140-145.

Sadh PK, Duhan S, Duhan JS. 2018. Agro-industrial wastes and their utilization using solid state fermentation: a review. Bioresour Bioprocess., 5(1): 1-15.

Salah RB, Ghamghui H, Miled N, Mejdoub H, Gargouri Y. 2007. Production of butyl acetate ester by lipase from novel strain of *Rhizopus oryzae*. J. Biosci. Bioeng., 103(4): 368-372.

Salihu A, Alam MZ, AbdulKarim MI, Salleh HM. 2014. Esterification for butyl butyrate formation using *Candida cylindracea* lipase produced from palm oil mill effluent supplemented medium. Arab. J. Chem., 7(6): 1159-1165.

Saun NK, Narwal SK, Dogra P, Chauhan GS, Gupta R. 2014. Comparative study of free and immobilized lipase from *Bacillus aerius* and its application in synthesis of ethyl ferulate. J. Oleo Sci., 63(9): 911-919.

Schinke C, Germani JC. 2012. Screening Brazilian *Macrophomina phaseolina* isolates for alkaline lipases and other extracellular hydrolases. International Microbiology. Barcelona. 15: 1-7.

Serri NA, Mooi LS. 2019. High reusability of lipase immobilized on eggshells for butyl butyrate synthesis under optimum condition. ASM Sci. J., 12: 10-21.

Shaheen M, Ullah I, Rafiq M, Maqsood-Ur-Rehman M, Shah AA, Hasan F. 2020. Purification and characterization of lipase from psychrophilic bacteria *Pseudomonas mandelii* htb2 from Batura glacier, Pakistan. Appl. Ecol. Environ. Res., 18(3): 4103-4114.

Sharmin F, Rakshit SK, Jayasuriya HW. 2007. Enzyme immobilization on glass surface for the development of

phosphate detection biosensors. Agric. Eng. Int.: CIGR J., 9:6-19.

Silva AL, Nascimento RG, Arakaki LN, Arakaki T, Espínola JG, Fonseca MG. 2013. Organofunctionalized silica gel as a support for lipase. J. Non-Cryst. Solids., 376: 139-144.

Sinumvayo JP, Li Y, Zhang Y. 2021. Microbial production of butyl butyrate: from single strain to cognate consortium. Bioresour. Bioprocess., 8(1): 1-11.

Spinelli D, Coppi S, Basosi R, Pogni R. 2014. Biosynthesis of ethyl butyrate with immobilized Candida rugosa lipase onto modified Eupergit® C. Biocatalysis, 1(1): 1-12.

Sufficiency E, Qamar SA, Ferreira LFR, Franco M, Iqbal HM, Bilal M. 2022. Emerging biotechnological strategies for food waste management: A green leap towards achieving high-value products and environmental abatement. Energy Nexus, 6: 100077.

Tangtua J, Techapun C, Pratanaphon R, Kuntiya A, Sanguanchaipaiwong V, Chaiyaso T, Hanmoungjai P, Seesuriyachan P, Leksawasdi N, Leksawasdi N. 2017. Partial purification and comparison of precipitation techniques of pyruvate decarboxylase enzyme. Chiang Mai J. Sci., 44: 184-192.

Thangaraj B, Solomon PR. 2019. Immobilization of lipases–A review. Part I: Enzyme immobilization. ChemBioEng Rev., 6(5): 157-166.

Xu Y, Wang H, Lin Q, Miao Q, Liu M, Ni H, Zhang L, Lyu M, Wang S. 2023. Immobilization of Dextranase Obtained from the Marine *Cellulosimicrobium* sp. Y1 on Nanoparticles: Nano-TiO_2 Improving Hydrolysate Properties and Enhancing Reuse. Nanomater., 13(6): 1065..