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Research Article

Investigating Enzyme Activity of Immobilized *Candida rugosa* Lipase

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Candida rugosa lipase is a food-grade enzyme that is extensively utilized in the dairy processing industry for milk fat hydrolysis. The enzyme is mainly employed to modify the fatty acid chain length that results in the enhancement of flavors. The hydrolytic activities of *C. rugosa* lipase (fungal source) in its free and immobilized forms were investigated at different pH and temperature settings. The main objective of this study was to understand how different support materials (Celite-545, Sephadex G-25, and chitosan) and immobilization techniques alter lipase activity and stability. Our results indicated that hydrolytic activity increased significantly with immobilization on Celite-545. In general, immobilization resulted in considerable improvements in the stability of the enzyme with variations in pH and temperature. Immobilization on Celite-545 led to the highest catalytic efficiency. Remarkable improvements in the recovery and reusability of the immobilized lipases were noted. Comparatively, the acetone immobilization procedure resulted in higher activities than alcohol immobilization. In conclusion, the activity of *C. rugosa* lipase was enhanced most significantly when immobilized on Celite-545 using acetone as an adsorption solvent.

1. Introduction

During the last two decades, the use of lipases in the food industry has increased due to the increased attention in the products that are more natural. The major application of lipases in food industries involves biocatalysis of lipids especially fats and oils. They are mainly used for enhancing the flavor components during the production process of various products such as cheese, butter, salad dressings, sauces, and soups. The potential of lipases to work on a broad spectrum of substrates and their stability when subjected to wide ranges of pH, temperature, and organic solvents are chief reasons for their use since these properties support catalysis with lowered side reactions and reduce costs for waste treatments. Also, food flavor components synthesized with solvent-free techniques have superiority (compounds with no solvent impurities) for being safe in making delicious foods. Moreover, lipase-mediated reactions involving ester interchange can be utilized for attaining appropriate flavor esters [1].

C. rugosa lipase is a lipolytic food-grade enzyme that is synthesized using the controlled fermentation technique. This enzyme catalyzes the hydrolysis reaction of triglycerides, resulting in the production of mono- and diglycerides, glycerol, and free fatty acids. Lipase from *C. rugosa* is distinctive in its reactivity where it displays no positional specificity and results in effectual fatty acid hydrolysis from all three positions in the triglyceride. Lipase is also utilized for catalyzing esterification and acidolysis reactions (transesterification reaction) that result in the production of fatty acid esters and structured lipids, respectively. This lipase shows wide substrate specificity on the fats and oils of both vegetable and animal origins. *C. rugosa* lipase aids in the flavor development in Italian cheese, ripening

acceleration in Cheddar cheese, flavor improvement in processing blue cheese, and enhancing creamy flavor in butter oil. The enzyme principally results in the synthesis of short-chain fatty acids (C_4 and C_6) which are the prime components for tangy sharp flavor [2].

Reactions involving *C. rugosa* lipases are more environment-affable relative to some bulk chemical syntheses since the operation of lipase-catalyzed reactions nearly resembles that of natural metabolic pathways. Lower activation energies of lipase-mediated processes allow them to convert their reactants to end products by utilizing mild temperature and pH conditions, thus resulting in lower energy consumption. The enzyme is also stable in organic solvents and does not require any cofactors.

Despite its beneficial and productive uses, the employment of *C. rugosa* lipases for commercial industrial purposes faces some challenges. The harsh conditions of industrial processes usually lead to destabilization, thereby shortening its industrial lifespan. Other drawbacks include sensitivity to process controls, low solidity, and the tendency to be constrained by high concentrations of reactants [3]. Krajewska [4] stated that the strenuous recovery and reuse of an enzyme is also a challenging task since the powdered form of enzyme can form an emulsion, which results in significant loss, with their recovery from the reaction system being very low [5].

In addition, the interactions between amino acid residues in the core of a protein's sequence are not fully optimized and as such only attain the minimal prerequisites for their required functioning [6]. This suggests the need for methods that can improve the functionality of the enzyme. One such method is enzyme immobilization. Immobilization improves the stability and ease of enzyme recovery after a reaction and thus contributes to reduced production cost since the enzyme can be reused [7]. Although the technique of enzyme immobilization has become widespread, some commercial lipase enzyme products still exist in the free form—not immobilized [8, 9]. The immobilization has the potential for improving the activity, specificity, and stability of lipases as well as the ease of separating the enzyme from reaction products. In addition, the reusability of the immobilized enzyme is bound to reduce operational cost [10].

The properties of the support material matrix are predominant attributes that control the efficaciousness of the immobilization process. The matrix materials can modify the mechanism and the partitioning of the reactant and product components that are present in the reaction mixture and, because of which, they would influence the chemical properties, stability, and the activity of the enzyme. Three support materials were used in the study: Celite 545, Sephadex G-25, and chitosan.

Celite 545, also known as diatomaceous earth, is a naturally occurring rocky substance. It exists in the earth as a soft siliceous rock that can be effortlessly disintegrated into fine powder. Generally, the color of the material ranges from off-white to whitish. Due to its high porosity, the density of the material is very low. Its characteristic chemical composition includes silica (80–90%), alumina (2–4%), and iron oxide (0.5–2%) along with some fossil remains of diatoms, which are a type of algae. The material is hydrophobic in nature. Sephadex is a beaded material that is utilized for gel filtration techniques. It is synthesized by crosslinking dextran (a polysaccharide) with epichlorohydrin. The degree and the extent of the crosslinking reaction can be controlled, resulting in variations in the degree of polymer gelation. Hence, five different types are available based on their sizes, with G-10 consisting of smaller molecules and G-75 of the larger ones. The research was studied on Sephadex G-25 which is relatively smaller of all types. This support material is hydrophobic and generally available in three different particle sizes: fine, medium, and coarse. Chitosan is a carbohydrate molecule made of D-glucosamine and *N*-acetyl glucosamine linked through β-1,4 linkages. It is a hydrophilic substance that is available in fine powder form. These support materials were chosen in order to understand the *C. rugosa* lipase activity when immobilized on both hydrophilic and hydrophobic support materials. The research was performed on the basis of the previous study [11], in which the LIP1 enzyme which is an isoform of *C. rugosa* lipase was studied, when immobilized on the two hydrophobic supports Celite and Sephadex.

The main objective of this study was to immobilize *C. rugosa* lipase, a commercially available enzyme, on different support materials by using physical adsorption techniques. The supports materials were Celite 545, Sephadex G-25, and chitosan. Acetone and alcohol immobilizations were utilized for physical adsorption of the lipase to the support materials [12]. The effects of pH and temperature variations on the hydrolytic activity of the immobilized enzymes were examined. The variations exhibited by immobilized lipases in the esterification and acidolysis reactions were investigated as was the reusability of the immobilized lipases.

2. Materials and Methods

2.1. Materials. *Candida rugosa* lipase, Celite 545, Sephadex G-25 (medium), chitosan (low molecular weight), glycerol, caprylic acid, and sodium hydroxide (purity > 98%) were purchased from Sigma Aldrich (St. Louis, MO). Sodium phosphate was obtained from Fisher Scientific. Extra virgin olive oil (Filippo Berio) was purchased from a local grocery store. All organic solvents (technical grade) were purchased from Sigma Aldrich (St. Louis, MO).

2.2. Immobilization Procedure

2.2.1. Ethanol Immobilization. Twelve grams of support materials was first mixed with 20 mL of ethanol for 18 hours at static condition and room temperature (20°C). Wet supports were then mixed with a 60 mL of sodium phosphate buffer (pH 7) and 3 g of the enzyme, keeping them in a shaking water bath at 30°C and 120 rpm for 12 hours. The immobilized lipase on the support was then filtered under vacuum, dried at 35°C–40°C for 72 hours, and stored at 4°C [13].

2.2.2. Acetone Immobilization. The lipase enzyme was immobilized on three different carriers (Celite 545, Sephadex G-25, and chitosan) using the acetone adsorption technique described by Wang et al. [14] and Lumor and Akoh [11].

Briefly, 3 g of the enzyme was dissolved in 60 mL of 10 mM sodium phosphate buffer (pH 6). The resulting solution was mixed with 12 g of the carrier. Then, 240 mL of cold acetone (−20°C) was added. The mixture was stirred for 30 min at room temperature (20°C) before it was filtered by suction, washed with 60 mL of cold acetone (10°C), and dried in an oven (25°C) for 72 hours. The powdered form was stored at 4°C.

2.3. Method of Analysis

2.3.1. Protein Content. The protein content was estimated using the FlashEA Nitrogen/Protein Analyzer (CE Elantech, Inc., Lakewood, NY) at USDA-ERRC, Wyndmoor, PA. After obtaining the percentage of nitrogen in the sample, a protein factor (6.25) was used to calculate the percentage of protein [15]. The samples to be analyzed were weighed in tin plates which were loaded in the analyzer along with a blank tin plate. The machine was calibrated with three different weights of aspartic acid. The samples were then heated, using a dynamic flash combustion technique which is a modified Dumas test that converts the samples to elemental gases. The percentage of nitrogen was then estimated by a detector.

2.3.2. Hydrolytic Activity. The hydrolytic activity of the lipase enzyme was assayed by preparing 200 mL of 10 mM sodium phosphate buffer of varying pH with 15% w/v of the olive oil emulsion. Gum arabic was added to the mixture at 5% w/v to act as an emulsifier. About 10 mL of the resulting emulsion was then incubated with 500 mg of each lipase at varying temperatures for 1 hour. After incubation, the products of reaction were titrated against 0.5 M NaOH with 1% phenolphthalein as an indicator. The amount of fatty acid released was estimated by calculating the difference in the volumes of titration (titer values) between samples and the blank. The time course analysis was also performed. All reactions were performed in triplicates. The activity was calculated using the following formula:

$$\text{specific hydrolytic activity} = \frac{(V * M)}{W * t}, \quad (1)$$

where V is the difference in the titer values between the blank and samples, M is the molarity, and W is the weight of lipase. The specific activity of the lipase was calculated by estimating the ratio of hydrolytic activity to the amount of protein.

2.3.3. Esterification Activity. Glycerol (1 g) and caprylic acid (1.27 g $C_8H_{16}O_2$) were chosen as reactants which were incubated with free lipase and its immobilized forms at 10% w/w. The reaction was carried out for 12 hours in screw-cap test tubes in an orbital shaking water bath at 40°C with a speed of 200 rpm. In order to pace up the reaction rate, the water produced in the reaction was absorbed by the addition of a total of 30% (w/w) of molecular sieves (4 Å in diameter, 8–12 mesh). The reaction was halted by the addition of 1 mL of methanol. After the reaction, the products from the screw-cap test tubes were titrated against 1.0 M sodium hydroxide with 1% phenolphthalein as an indicator. All reactions were performed in triplicates. Esterification activity is defined as the amount of enzyme that is consumed per minute per milligram of lipase in the reaction. The esterification activity was calculated using the following formula:

$$\text{specific esterification activity} = \frac{(V * M)}{W * t}, \quad (2)$$

where V is the difference in the titer values between the blank and samples, M is the molarity, and W is the weight of lipase.

2.3.4. Acidolysis Reaction. Structured lipids were produced in triplicates by an acidolysis reaction. One gram of olive oil and 0.3 g of caprylic acid were combined in screw-cap test tubes prior to adding lipase enzymes at 10% by weight of total reactants and placing them in an orbital shaking water bath at 45°C and 200 rpm for 12 hours. Thin-layer chromatography (Fisher Scientific, Pittsburgh, PA) separated the triacylglycerol bands. The bands corresponding to TAGs were scrapped off and modified to fatty acid methyl esters (FAMEs) using the boron trifluoride (BF_3) method: 2 mL of 0.5 M NaOH in methanol was added to the TAG bands at 90°C in screw-cap test tubes for 10 min followed by the addition of 14% BF_3 in methanol for another 10 min. FAMEs were extracted with 2 mL of hexane and then analyzed in parallel with a FAME standard (Supelco 37 component FAME Mix; Supelco, Bellefonte, PA), using the Agilent Technology 6890N Gas Chromatograph (Agilent Technologies, Inc., Wilmington, DE). The amount of caprylic acid that is incorporated after the reaction was estimated by analyzing different amounts of FAMEs which were integrated by an online computer. The area of the sample peak and the internal standard peak in the gas chromatogram were used to calculate the amount of fatty acids. The ratio of the incorporated amount of caprylic acid to the total amount of fatty acids present in the olive oil was established to determine the percentage of incorporated caprylic acid.

3. Results and Discussion

3.1. Protein Content. The percentage of protein in the lipase-immobilized support materials is an indication of the degree of immobilization achieved in our study. The percentage of protein (Table 1) found in the free form of *C. rugosa* lipase was 8.17%, which is consistent with the findings of Gitlesen et al. [16] and Ozturk [2] who reported 9.2% and 8.67% of protein, respectively. Based on our results (Table 1), the protein content of the samples was dependent on the type of the support material and the method of immobilization. Acetone-immobilized samples yielded a higher protein content than alcohol-immobilized samples, which could be due to the difference in solvent polarity. Differentiation on the basis of the support material used revealed that lipase immobilized on Celite-545 resulted in the highest amount of protein, whereas lipase immobilized on chitosan resulted in the least amount of protein, with lipase immobilized on Sephadex G-25 yielding an intermediate amount. The very low protein content of the lipase immobilized on chitosan may have been due to chitosan's hydrophilic nature. Lipase,

Table 1: Percentage of protein after lipase immobilization on different support materials.

Enzyme	Percentage of protein
Free enzyme	8.17
Celite-acetone	7.18
Sephadex-acetone	5.35
Chitosan-acetone	2.04
Celite-alcohol	6.04
Sephadex-alcohol	4.83
Chitosan-alcohol	1.41

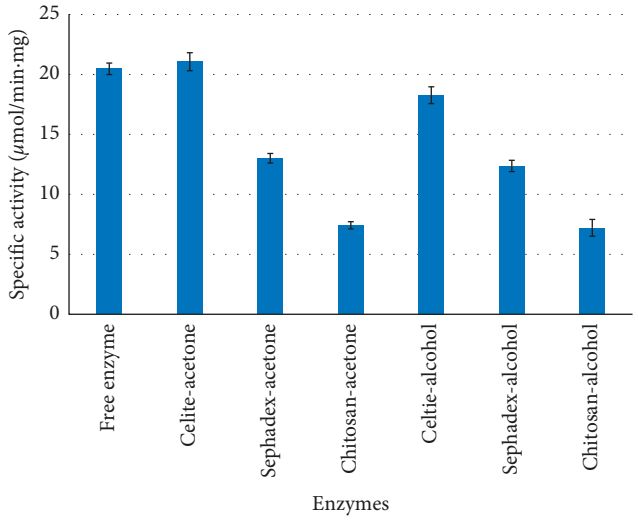

Figure 1: Graphical representation of specific hydrolytic activities of all enzymes (pH 7.0, temperature 45°C, and [S] = 1 mg/mL).

being hydrophobic in nature, binds readily to hydrophobic support materials such as Celite or Sephadex. Therefore, we concluded that the applications of acetone and Celite-545 were the most suitable for the immobilization process.

3.2. Hydrolytic Activity. The hydrolytic activity of lipases was calculated by titrating the reaction products with 0.5 M NaOH solution to determine the amount of fatty acids released. The specific hydrolytic activities of Celite-immobilized lipase samples were significantly higher than those of other immobilized support materials or even the free enzyme (Figure 1). Lipase immobilized on Sephadex G-25 demonstrated a slightly increased specific activity compared to the free enzyme. Various authors suggested that the immobilization of lipases with appropriate support materials has proven to enhance the catalytic properties [17]. According to Patrick [17], physical adsorption on matrix materials not only improves the stability of enzymes but also promotes the catalytic efficiency by improving the interfacial specificities and substrate binding ability.

Comparing the hydrolytic activity of the different immobilization methods, acetone immobilization resulted in higher specific activities on all immobilized supports than alcohol immobilization. The specific activities of alcohol-immobilized samples did not differ significantly from the free enzyme activities. Wu et al. [18] studied the effect of different polar solvent treatments during immobilization of lipases from three microbial sources, namely, *C. rugosa*, *Mucor javanicus*, and *Rhizopus oryzae*, on support materials. They were immobilized on Amberlite support materials by physical adsorption using different polar solvents that included acetone and ethanol along with 17 other organic solvents. They also found that acetone-immobilized samples demonstrated the highest activities among all other solvents, while ethanol-immobilized samples showed significantly lower values. This could be due to the lower polarity of acetone when compared to ethanol and suggests that acetone is a better solvent for physical immobilization of support materials.

3.3. Effect of pH on Hydrolytic Activity. The surrounding microenvironment can control enzyme activity. The pH of a solution plays a pivotal role in achieving an optimum amount of fatty acids during the hydrolysis reaction because the tertiary protein structure is controlled by hydrogen bonding interactions among the R groups of amino acids. The ionization of these R groups can be altered by a small change in the pH value, disrupting the original native conformation, thereby resulting in the loss of enzymatic activity. The product inhibition becomes significant, causing a decrease in the reaction rate. Hence, every enzyme has a favorable pH range that preserves the native structural conformation and therefore its activity [19]. Generally, immobilization modifies the optimal pH of the enzyme, which may be beneficial.

In this study, the differences in hydrolytic activity of all enzymes as a function of pH were investigated. As plotted in Figures 2 and 3, *C. rugosa* lipase in its free form was less active at both lower (acidic) and higher (basic) pH values, potentially due to the structural changes caused by the variation in pH. According to Akova and Ustun [20], the cysteine amino acid residues of the enzyme can be moderately damaged due to β-elimination in alkaline solutions, whereas in the acidic solutions, the easily breakable peptide bonds that are adjacent to the aspartic acid residues are hydrolyzed. Montero et al. [21] reported a higher probability of protein aggregation in an acidic pH, specifically higher molecular weight proteins because of their hydrophobic nature. The enzyme in its free form was most active at pH 7.0, as also reported by Fadiloglu and Söylemez [22] and Ozturk [2]. For enzyme immobilization on Celite, the highest hydrolytic activity was observed at pH 7.0 for both acetone-and alcohol-immobilized enzymes, respectively. The optimal pH levels for lipases immobilized on Sephadex with acetone and alcohol were 6.5 and 6.0, respectively, and the optimal pH for lipase immobilized on chitosan were 7 and 6.5, respectively. Overall, it can be concluded that the activity of enzyme immobilized on Celite by acetone immobilization was comparatively stable at acidic and alkaline pH. This gives us an advantage of utilizing the enzyme for various reactions that may require varied pH conditions.

3.4. Effect of Temperature on Hydrolytic Activity. Temperature affects enzyme activity in two different ways. The mobility

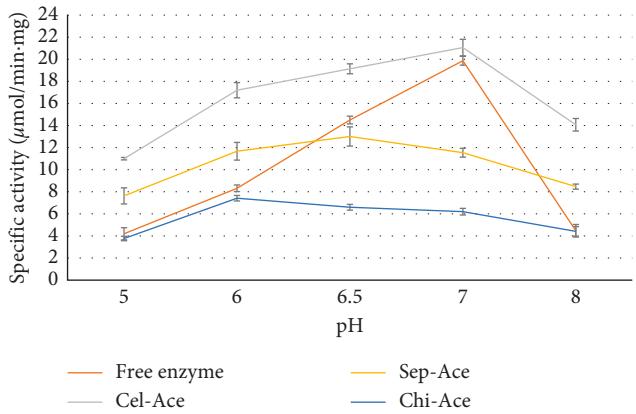

Figure 2: Specific hydrolytic activity of acetone-immobilized samples with varying pH values (temperature 45°C and [S] = 1 mg/mL).

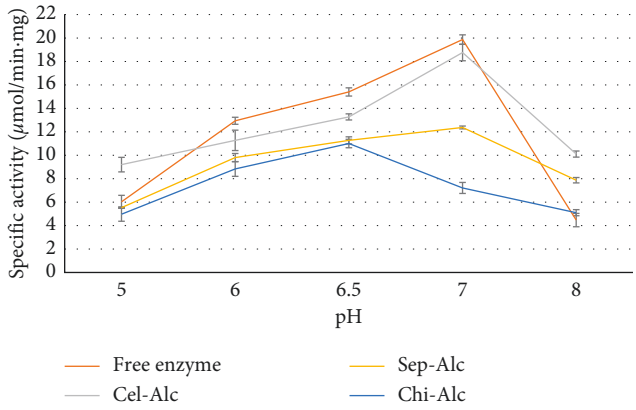

Figure 3: Specific hydrolytic activity of alcohol-immobilized samples with varying pH values (temperature 45°C and [S] = 1 mg/mL).

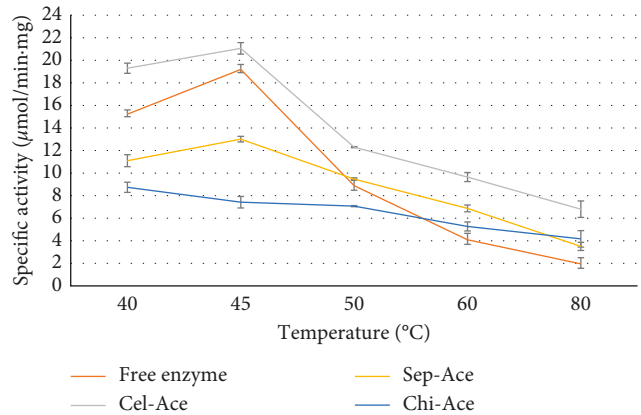

Figure 4: Specific hydrolytic activity of acetone-immobilized samples with varying temperatures (pH 7.0 and [S] = 1 mg/mL).

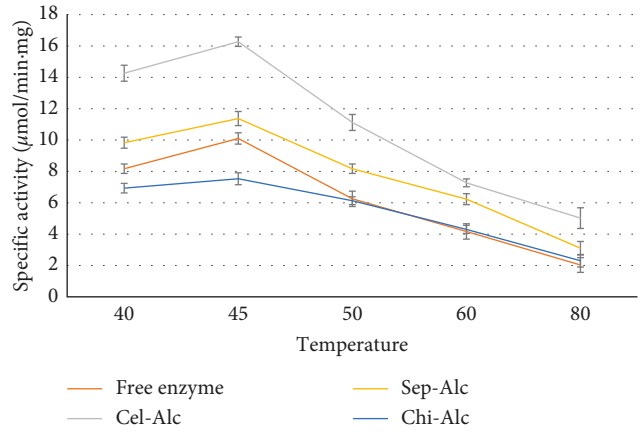

Figure 5: Specific hydrolytic activity of alcohol-immobilized samples with varying temperatures (pH 7.0 and [S] = 1 mg/mL).

of the substrate reactants increases when raising the temperature, but this substantial effect on the catalytic rate is limited as temperature-dependent enzyme denaturation becomes more significant. At an enzyme-specific temperature, an even small increase in the temperature can lead to so much denaturation that the loss of enzyme activity results in decreased product formations.

In this study, the specific hydrolytic activity of all enzymes was determined at different temperatures ranging from 40°C to 80°C. Except for lipase immobilized on chitosan using acetone adsorption, all lipases demonstrated the highest activity at 45°C (Figures 4 and 5), suggesting that immobilized enzymes were significantly more active at higher temperatures than the free enzyme. Unimmobilized *C. rugosa* lipase lost 80% of its activity at 80°C. Similar to unimmobilized C. rugosa lipase, around 72% of the specific hydrolytic activity was lost by both Sephadex-immobilized lipases at 80°C. The immobilization on Sephadex conferred less thermal stability to the lipase than immobilization on chitosan, which showed a 53% reduction in enzymatic activity at 80°C, and on Celite with approximately 60% reduction. Although immobilization on chitosan showed little activity, it conferred significant thermal stability to the lipase.

3.5. Timeline and Kinetics of Hydrolytic Activity. The rate at which an enzyme catalyzes a reaction is crucial to its progress. Every enzyme has its own rate phenomena in the conversion of substrates into products. Enzyme kinetics explains the catalytic behavior of enzymes. In this study, the effect of support materials on the rate of enzyme catalysis was measured through a time-course study of the hydrolysis reaction.

The hydrolytic activities of free and immobilized lipase enzymes were analyzed over an extended period of time until 16 hours to determine the progress of catalysis. As presented in Figures 6 and 7, the amount of fatty acids produced by all the samples increased with the reaction time. At a constant temperature and pH, changing the substrate concentration resulted in varying amounts of fatty acids. Two different substrate concentrations were chosen to explain enzyme kinetics, using the Michaelis–Menten equation. With two values for initial velocities and substrate concentrations, $V_{\max}$ and K_m values of the reactions could be established (Figure 8).

The Michaelis–Menten equation determines the rate of reaction:

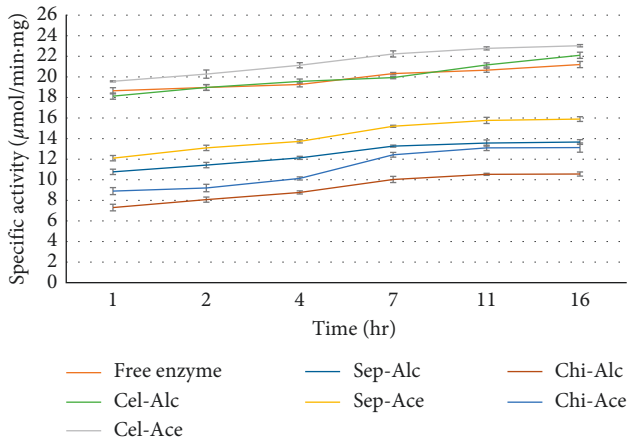

Figure 6: Timeline of hydrolysis reaction at [S] = 1 mg/mL (pH 7.0 and temperature 45°C).

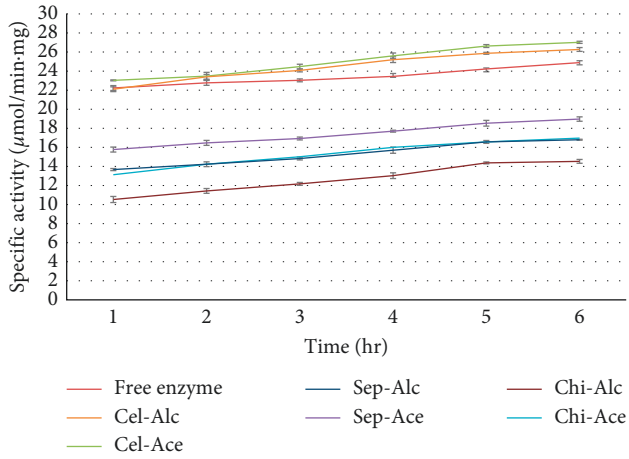

Figure 7: Timeline of hydrolysis reaction at [S] = 2 mg/mL (pH 7.0 and temperature 45°C).

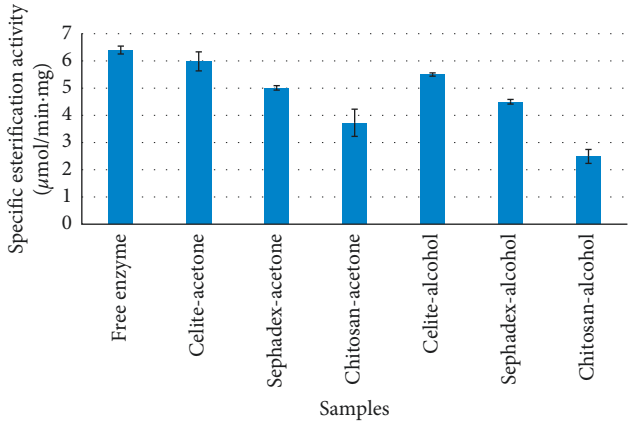

Figure 8: Specific esterification activities of all enzymes (pH 7.0, temperature 40°C, and time 12 hours).

$$V = \frac{V_{\max} * [S]}{K_m + [S]}, \quad (3)$$

Table 2: Variation in the Michaelis–Menten constants.

Enzyme	$V_{\max}$ (μM/min)	K_m (μM)	$V_{\max}/K_m$ ($\min^{-1}$)
Free enzyme	0.41	0.97	0.43a
Celite-acetone	0.50	0.87	0.56b
Sephadex-acetone	0.42	0.92	0.43a
Chitosan-acetone	0.25	1.19	0.21
Celite-alcohol	0.46	0.89	0.52c
Sephadex-alcohol	0.41	1.10	0.37
Chitosan-alcohol	0.23	1.25	0.19

where V = rate of the reaction, $[S]$ = substrate concentration, $V_{\max}$ = maximum rate that can be achieved, and K_m = substrate concentration at half of $V_{\max}$. K_m can also be defined as the substrate concentration at which half of the enzyme's active sites are utilized by the substrate. The higher the K_m value, the higher the amount of substrate required to saturate the enzyme and therefore the lower the affinity of that enzyme for the substrate. The $V_{\max}$ value indicates the maximum reaction speed.

The initial hydrolytic velocity of the samples at different substrate concentrations was estimated from the slope of the line. The two constants, $V_{\max}$ and K_m, of the Michaelis–Menten equation were determined for all enzymes by solving the two substituted linear equations with the two unknowns. The calculated $V_{\max}$ and K_m values of these samples are reported in Table 2.

The ratio of the Michaelis–Menten constants allowed the calculation of the catalytic efficiency of the enzymes. The catalytic efficiency of lipase immobilized on Celite supports was significantly higher than that of other enzymes, suggesting that the maximum rate of reaction can be achieved with a lower substrate concentration. Free lipase and Sephadex-immobilized lipase demonstrated similar catalytic efficiencies. Hence, immobilizing *C. rugosa* lipase on Sephadex could add to the cost of reaction if other factors such as pH and residual recovery are not considered. Chitosan-immobilized lipase, being less capable of binding to the support, resulted in lower catalytic efficiency and may not be suitable for immobilization of *C. rugosa* lipase with a physical adsorption technique.

3.6. Esterification Activity. Percentage esterification was estimated as the ratio of moles of caprylic acid consumed in the reaction to the number of moles of initial caprylic acid. The reaction between 1 g of caprylic acid and 1.27 g of glycerol in the presence of *C. rugosa* lipase was carried out at 40°C for 12 hours at pH 7. *C. rugosa* lipases in both free and immobilized forms were moderately successful in achieving appreciable ester conversion rates. Average percentages ranging from 28 to 72% esterification were achieved during 12 hours of this experiment (Table 3).

Although appreciable amounts of esters were formed, the immobilization procedure was not able to significantly improve specific activity. The specific esterification activity of free lipase and Celite-immobilized lipase did not differ significantly. Lipase immobilized on chitosan and Sephadex resulted in lower specific activities. Chitosan-immobilized lipase, being hydrophilic, has less lipase-binding capacity,

Table 3: Percentage ester conversions.

Enzyme	% ester conversions
Free enzyme	70.72
Celite-acetone	68.95
Sephadex-acetone	62.95
Chitosan-acetone	35.45
Celite-alcohol	66.25
Sephadex-alcohol	59.54
Chitosan-alcohol	28.86

resulting in lower esterification activity. The significantly lower specific esterification activities of Sephadex-immobilized lipases are not well understood and should be further investigated.

Lumor and Akoh [11] reported that *C. rugosa* lipase had no significant esterification activity at 60°C for 1 hour. Since the enzymes in this study showed considerable ester formation at 45°C for 12 hours, further optimization of reaction conditions might obtain even higher ester yields.

3.7. Acidolysis Reaction. Acidolysis reaction, one of the reactions catalyzed by lipase enzymes, can result in the development of novel structured lipids used to incorporate a desirable fatty acid into an oil/fat to improve its nutritional value. The goal here was to study how lipase immobilization affects this reaction, determining the degree of incorporation of caprylic acid into olive oil. Caprylic acid is not present in olive oil but is found in coconut oil, corn oil, and palm kernel oil and in trace amounts in grape seed oil. The acidolysis reaction of 0.3 g of caprylic acid and 1 g of olive oil was carried out at pH 7.0, at 45°C, and at 200 rpm for 12 hours.

The reaction did not yield significant results with about 0.03%–0.16% of the fatty acid per unit protein incorporated into olive oil samples (Table 4). Srivastava et al. [23] successfully incorporated oleic acid and palmitic acid into milk fat by a transesterification reaction in the presence of *C. rugosa* lipase. The reason for lower incorporation of caprylic acid by *C. rugosa* lipases could be due to fatty acid selectivity.

Although the enzyme incorporation of caprylic acid was low, acetone-immobilized Celite-545 was capable of incorporating a slightly higher percentage, while immobilization on the two other support materials resulted in lesser incorporation of caprylic acid when compared to the free enzyme. *C. rugosa* lipases seem more efficient in catalyzing the incorporation of long-chain unsaturated fatty acids such as oleic acid than medium-chain saturated fatty acids such as caprylic acid. Further study using long-chain unsaturated fatty acids might help us estimate the effect of immobilization.

3.8. Residual Activity. Although the use of enzymes in industrial production improves the quality of the reaction products, the expense of enzymes compared to chemical catalysts usually makes the enzymatic process unaffordable. By immobilizing enzymes, they can be reused and thereby minimize production cost. In this study, all the enzymes—both free and immobilized—were reused up to six times to determine residual activity. The enzyme in the

Table 4: Percentage of caprylic acid incorporated into olive oil samples.

Enzymes	% of caprylic acid incorporated	% of caprylic acid incorporated per mg of protein
Free enzyme	0.54 ± 0.04	0.15 ± 0.01^{a}
Celite-acetone	0.517 ± 0.09	0.16 ± 0.03^{b}
Sephadex-acetone	0.17 ± 0.02	0.087 ± 0.01^{c}
Chitosan-acetone	0.05 ± 0.01	0.053 ± 0.00^{d}
Celite-alcohol	0.35 ± 0.22	0.13 ± 0.11^{a}
Sephadex-alcohol	0.138 ± 0.01	0.08 ± 0.00^{c}
Chitosan-alcohol	0.025 ± 0.00	0.039 ± 0.00^{e}

Note. Different letters indicate statistically significant differences between the groups.

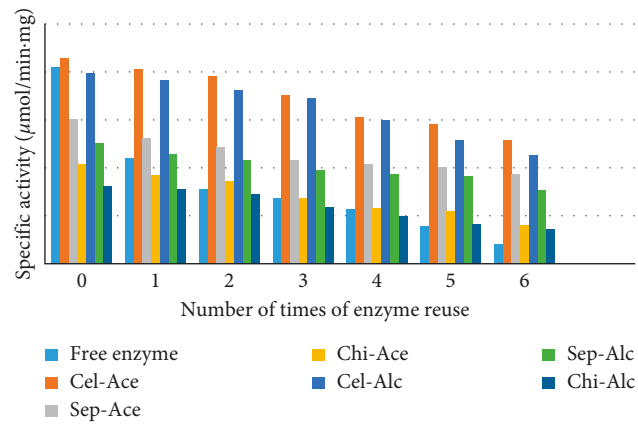

Figure 9: Residual enzyme specific activity (pH 7.0, temperature 45°C, and [S] = 1 mg/mL).

Table 5: Percentage loss of enzymatic activity after six successive uses.

Enzyme	% loss of activity
Free enzyme	99.30
Celite-acetone	38.21
Sephadex-acetone	61.07
Chitosan-acetone	40.05
Celite-alcohol	38.54
Sephadex-alcohol	55.35
Chitosan-alcohol	43.13

product mixture was filtered at room temperature and washed in two steps with the 10 mM sodium phosphate buffer solution followed by washing with *n*-hexane twice. Retaining and recovery of free enzyme from the mixture was not accurate due to its miscible nature. Hydrolytic activities of the enzymes were determined, and residual activity was calculated.

Free enzymes retained significantly less enzymatic activity than the immobilized enzymes (Figure 9) due to their inability to recover the powdery form of the free enzyme adequately after each reaction cycle. The free enzyme lost 99.3% of its activity after six successive cycles (Table 5), while losses in the enzymatic activities of the immobilized enzymes after 6 cycles ranged from 38.2% to 61.1%. Lipase

immobilized on Celite-545 and chitosan exhibited comparatively lower activity loss. Celite-immobilized lipase demonstrated intermediate residual activity. Sephadex-immobilized lipase lost around 55–60% of its activity. Although lipase immobilized on chitosan exhibited intermediate specific activities, residual performance in residual activity was the lowest. Chen et al. [24] reported similar results when they examined storage stability and reusability of immobilized enzymes and found that the immobilized enzymes showed higher stability after 10 cycles of reuse than the free enzyme. To determine the storage stability, the authors stored all enzymes in a phosphate buffer for 30 days and reported that free enzymes retained only about 30% of their activity, whereas the immobilized enzymes retained all of theirs. In another study, Alagoz et al. [25] immobilized *Candida* methylica formate dehydrogenase on glyoxyl agarose-, glyoxyl silica-, and aldehyde-functionalized Immobeads. After 5 cycles of reuse, the researcher reported that the free enzyme lost around 88% of activity, while the immobilized enzymes lost only 50–60%.

4. Conclusion

The current research examined the activity of *C. rugosa* lipase when immobilized using acetone or alcohol on three different support materials (Celite-545, Sephadex G-25, and chitosan). Of the two solvents, acetone was more effective in the immobilization process due to its lower polarity. The hydrolytic activities of the enzymes, both free and immobilized, under different pH and temperature conditions allowed to optimize the reaction conditions and to analyze the degree of stability provided by the support materials. *C. rugosa* lipase immobilized on the Celite-545 support material exhibited significantly greater activity than all the other immobilized enzymes in terms of hydrolytic reaction, fatty acid incorporation, and stability at varying pH and temperatures along with good recovery and reuse qualities. Sephadex G-25 did not perform well as expected. The specific hydrolytic activities of immobilized Sephadex G-25 were comparable to those of the free enzyme, suggesting that its use as a support material provides no economic benefit. Moreover, it did not exhibit either significant ester formation or thermal stability. Immobilized chitosan displayed very low activity values due to its less porosity. However, the support material conferred good stability on the enzyme concerning temperature variations. In conclusion, the activity of *C. rugosa* lipase was enhanced most significantly when immobilized on Celite-545 using acetone as an adsorption solvent. Per our study, the acetone-immobilized *C. rugosa* lipase can be utilized in the oil/fat industry for hydrolysis reactions to achieve an improved quality and quantity of hydrolysis products and reduced resource wastage and manufacturing costs.

Additional Points

Practical Application. C. rugosa lipase is used for producing glycerol and fatty acids which help in enhancing the flavor in food substrates such as milk, butter, cheese, ice, and cream. Even though the enzyme can be synthesized commercially, the higher cost is the prime factor to achieve successful application [26]. Immobilization improves the stability and ease of enzyme recovery after a reaction and thus contributes to reductions in production cost since the enzyme can be recycled. Although the technique of enzyme immobilization has become widespread, some commercial enzyme products still exist in the free form—not immobilized [9]. The immobilization of these enzymes, especially lipases, would be beneficial to the field of lipid chemistry. This has the potential for improving the activity, specificity, and stability of the enzyme as well as the ease of separating the enzyme from reaction products. In addition, the reusability of the immobilized enzyme is bound to reduce operational cost [10].

Conflicts of Interest

The authors have no conflicts of interest to declare.

Authors' Contributions

Mrs. Bhagya Sri Kaja was the principal researcher, conducted the research, and drafted the manuscript. Dr. Stephen Lumor directed and assisted in the laboratory experiments and provided training to the principal researcher. Dr. Samuel Besong assisted with the results and overall application of the manuscript. Dr. Bettina Taylor provided suggestions to improve the project planning and manuscript editing and revision. Dr. Gulnihal Ozbay provided suggestions regarding the experimental approach and assisted with research write-ups, dissemination, and preparation of the manuscript.

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