Determination of stability and activity of immobilized lipase for transesterification reaction in fluorous solvent and deducing the reaction mechanism by molecular docking study

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Abstract

The objective of the present study was to immobilize lipase and use it for transesterification reaction in a fluorous solvent. Polymeric substance tylose (Tyl) was chosen as immobilization support. Tylose immobilized lipase from *Candida rugosa* (CRL) was subjected to fluorous solvents treatment and was found most stable in perfluorooctane (PFO). The immobilized Tyl-CRL was characterized for protein loading, solvent stability, surface morphology, restoration of secondary structure, and thermal stability. Immobilized CRL was further applied for the synthesis of phenethyl propionate in a fluorous solvent. All reaction parameters were optimized using the response surface method (RSM). Optimized reaction parameters were substrate ratio 1:3, immobilized lipase quantity 30 mg, and temperature 45^{o}C. Prepared biocatalyst was evaluated for recyclability, and it was found that catalytic activity decreased by 77% up to the fifth recycle run. The optimized synthesis protocol scope was checked to synthesize various flavoring esters. The molecular-level interactions of substrate and lipase were studied using docking.

1. Introduction

In the past few decades use of enzymes in nonaqueous organic media has immensely increased because it has several advantages, such as enzymes mainly working at mild reaction conditions, requiring low energy, selectivity of product is high, and usually less or no by-product formation is observed [1, 2]. Though the biocatalysis in organic solvents has many advantages, organic solvents are hazardous, pollute the atmosphere, and be harmful to human health. Moreover, using organic solvents involves a tedious down-streaming process and increment in E-factor and inhibition to enzyme activity [3–5]. So there is increased pressure on industries to reduce solvents by the US environmental protection agency (EPA). There are different strategies to minimize the use of organic solvents by replacing them with green solvents like water, ethanol, aqueous surfactant micelles and polymers, ionic liquid, fluorous solvents, and supercritical $CO_{2,}$ etc. or by eliminating them by keeping solvent-free conditions [3, 5–12]. In the coming decades, it is expected that this will stimulate total renovating or alteration of the infrastructure of numerous industrial processes presently being used since new green reaction media may reasonably substitute conventional organic solvents. Fluorous media is one green reaction media researchers are trying to explore. Fluorous solvents have the fundamental property of being effectively separable from their hydrocarbon analogs as they are immiscible in many organic solvents. Gases can be readily dissolved in fluorous solvents. They are chemically stable. They have low viscosity, which might advance the mass exchange irregularity between the active site of the enzyme and the reaction mixture. They are not harmful, do not deplete the ozone, and have fundamentally low greenhouse potential. They are non-protic, show neither solid Lewis acidity nor basicity, are latent to oxidizing conditions, and do not respond with nucleophiles or electrophiles [3, 4]. The above properties of fluorous media make them a good alternate for replacement for more conventional solvents. The main limitation of using these solvents in biocatalysis is the restricted solubility of enzymes in such solvents. To increase the recyclability and dispersion in a fluorous solvent, immobilization of the enzyme should be tried [11, 13].

More commonly, lipase is widely used for biocatalytic transformation because of its higher stability, activity, and availability [14–17]. In the present study, we used lipase from *Candida rugosa.* Four different polymers, namely tylose, chitosan, guar gum, gum acacia, and their combinations, were used to immobilize lipase from *Candida rugosa* [18]. A simple entrapment method was used for immobilization. Gum arabic is obtained from the acacia tree, and it consists of glycoproteins and polysaccharides mixture. Guar gum is also a polysaccharide obtained from guar beans. Both gum acacia and guar gum are water-soluble and used as a stabilizer in the food industry. Tylose is a cellulose derivative and is used in many foods and non-food products like toothpaste, detergents, paints, paper, etc., as a thickener and stabilizer. Chitosan is also a polysaccharide obtained from alkali treatment of chitin and has wide applications in agriculture, medicine, pharmaceuticals, etc. Immobilized CRL was used to synthesize phenethyl propionate (Fig. 1) as it has wide application in flavors, agriculture, and pharmaceutics. Phenethyl propionate has a Sweet, Rose, Fruity, Berry, and Honey like odor and is used in perfumes. It also has antifungal activity and is used as a pesticide. Phenethyl propionate is found naturally in guava, cheeses, peanut, and alcoholic beverages like brandy. In the present work, immobilized CRL were incubated into different fluorous solvents and checked for stability and activity. As per our knowledge, there is no report on the synthesis of propionate esters in fluorous solvents using immobilized CRL as a catalyst. In this work, immobilization of lipase on polymeric support was carried out. The immobilized lipase was characterized for stability and activity in a fluorous solvent. Moreover, the application of immobilized lipase for flavor synthesis was made.

2. Materials and methods

2.1 Chemicals and enzymes

Lipase from *Candida rugosa* (CRL; activity ≥ 30,000 U/g), *Aspergillus niger* lipase *(ANL;* activity ≥ 30,000 U/g), lipase from *Mucor javanicus* (MJL; activity ≥ 10,000 U/g), chitosan (CHI; Brookfield viscosity > 200), Guar gum (GG) (Mw 9000–10 000), Gum acacia (GA), Tylose (TYL), vinyl propionate (VP) were purchased from Sigma Aldrich Pvt. Ltd., India. Fluorous solvents like perfluorohexane (PFH), perfluorooctane (PFO), and perfluoromethylcyclohexane (PFMC) were purchased from TCI. Phenethyl alcohol (PA), bovine serum albumin (BSA), and other solvents/chemicals were purchased from Hi-Media Pvt. Ltd. and Sigma Aldrich.

2.2 Immobilization of lipase

CRL was immobilized by a simple entrapment method described with slight change [19]. The 400 mg of TYL, GG, and GA were dissolved in 25 mL of deionized water at room temperature, stirring continuously at 600–800 rpm. Likewise, 400 mg of chitosan was dissolved in 25 mL 1% glacial acetic acid solution. Lipase 100 mg was dissolved in a separate 10 mL glass vial in 1–2 mL deionized water and was added to the solution of the polymer. The resulting solution was stirred slowly at 150–300 rpm for the next 30–60 min to ensure uniform lipase and polymer mixing. Then the solution was gently poured into a Teflon

Petri dish and dried at 40-42^{o}C for 24–48 h. After drying, a thin film of the polymer was obtained. The film was cut into small 2–3 mm^{2} pieces and kept in the refrigerator at 4-6^{o}C for further characterization.

2.3 Determination of protein content and lipase activity assay

Bradford method was applied to determine the amount of protein at 595 nm. Subsequent to drying, the film was withdrawn, the Petri dish was washed, and the washing sample was treated with Bradford reagent to calculate unimmobilised protein. We estimated the amount of immobilized protein by subtracting the amount of unimmobilised protein from the amount of total protein used for immobilization. [6]. Percent loading was calculated using the following formula

% loading of protein = Amount of protein entrapped in film ÷ Total protein used for loading

Through the hydrolysis of p-nitrophenyl acetate (p-NPA), the lipase activity of crude and various immobilized lipase was assessed in triplicate spectrophotometrically at 405 nm [20]. The reaction mixture in the standard assay condition contained 1 ml of n-hexane solvent and 2 mg of crude lipase (or an equivalent amount of immobilized lipase). The reaction was initiated with the addition of 1 mL of 12 mM p-NPA, which was dissolved in a 2-propanol solution. This was incubated for 10 min at 40°C. After that, 300 µL of the reaction mixture was withdrawn and combined with 700 µL of deionized water for extracting p-nitrophenol (p-NP) in the aqueous phase. Subsequently, 400 µL of pH 8.0 potassium phosphate buffer solution was added to the solution mentioned above, resulting in a pale yellow color for the extracted p-NP, which was utilized to measure the absorbance at 405 nm. Under the required standard assay conditions, lipase activity was measured in micromoles of p-NP released per minute per milligram of lipase.

2.4 Determination of stability of lipase in fluorous solvent

The effect of three fluorous solvents, PFO, PFH, and PFMC, on the activity and stability of Tyl-CRL immobilized lipase was studied. The 10 mg of immobilized lipase was placed in three solvents separately at 45^{o}C, shaking at 120 rpm for 5 h incubation. After incubation, the solvent was removed by filtration, and lipase activity was determined for immobilized Tyl-CRL as above indicated procedure. Similarly, lipase activity of 10 mg of un-incubated fresh immobilized Tyl-CRL was also selected to calculate the relative activity. Relative activity compares the activity of solvent incubated immobilized lipase and un-incubated fresh immobilized lipase.

2.5 Determination of kinetic parameters

The reaction kinetics was carried out to determine the maximum reaction velocity (V_{max}) and Michaelis-Menten constant (K_m) of both crude and immobilized Tyl-CRL. Hydrolytic lipase assay was carried out at a concentration of p-NPA ranging from 2–26 mM for both immobilized Tyl-CRL and crude CRL. The procedure for this assay was the same as described in the above section 2.3 lipase activity assay.

2.6 Instrumentation

Surface characterization of the control tylose and immobilized tylose: lipase polymer film was done by scanning electron microscopy (SEM) analysis (Field emission ionization, Tescan MIRA 3 model Quanta 200). The polymer films were placed on a carbon stub and coated with gold using a sputter coater. 5–15 kV voltage was applied to take images in a low vacuum. Thermal gravimetric analysis (TGA) (PerkinElmer STA 6000 analyzer) was carried out to evaluate the thermal stability of polymer films. 8–10 mg of sample was taken into the ceramic crucible and heated from 30 to 600°C with a 10°C/min increase in temperature in an inert atmosphere with 100 mL/min flow. FTIR analysis (PerkinElmer, Spectrum Two) was carried out to check whether the native structure of lipase was conserved or not in the process of immobilization.

2.7 Synthesis of propionate esters

The reaction was carried out in 8 ml glass vial having an internal diameter of 1.5 cm and a glass stopper. In all experiments, known quantities of substrate phenethyl alcohol and vinyl propionate were taken into a glass vial and diluted up to 2 ml with solvent. The reaction mixture was kept in an incubator shaker at the required temperature and stirred for 5–10 min at 120 rpm. Later on, the immobilized lipase was added to initiate the reaction and kept at required conditions. The effect of speed of agitation on reaction progress was studied from 40 to 140 rpm. It was noticed that the rate remained the same after 120 rpm. As no mass transfer barrier was found at 120 rpm, all experiments were conducted at 120 rpm.

Progress of the reaction was checked by Gas Chromatography (GC)(YL Instrument, 6500GC System) provided with a capillary column (MXT-1; Lenth15 m, Diameter 0.53 mm, Thickness 1 μm) and flame ionizing detector. The oven temperature was maintained at 80^{O}C initially for 2 min and then raised to 200°C at a rate of 20°C/min for 15 min. The injector and detector temperature was kept at 250°C. The product was identified and confirmed by analysis of gas chromatography-mass spectroscopy (Shimadzu GCMS-QP2010 Ultra, RTX-17 column). The column oven temperature was maintained at 60°C for the first 3 min then increased to 250°C with the rate of 10°C/min for 20 mins

2.8 Experiments designing

Response surface methodology is widely used for process optimization because of its advantages over the conventional one parameter at a time optimization method. RSM allows checking the effect of more than one factor on one or more than one response simultaneously. In RSM, one can get a model equation that shows the correlation between different reaction parameters and reaction outcomes. The model developed using RSM can be very useful in reactor designing also. In the present study, the RSM was applied to optimize the synthesis of phenethyl propionate in PFO and to evaluate the result of different process variables and their interactions. A three-factor three-level central composite design (CCD) method was utilized. The independent variables are the reaction parameters like the ratio of phenethyl alcohol (mmol) to vinyl propionate (mmol), immobilized lipase amount (mg), reaction temperature (◦C), and the dependant variable is the percent yield. The coded values for the three independent variables were given in Table 1, while the three-factor-three-levels central composite design consisted of 20 experiments, as shown in Table 1. Table 1 also has the corresponding yield after 3 h of

reaction. The experiments were performed in random order. All experiments were performed in triplicates, and the average was taken. The percent yield response was then analyzed by the tools provided by Design Expert Software (Version 12)

Table 1
Central composite design and the corresponding response.

No.	Temperature (°C)	Substrate ratio (Vinyl propionate:Phenethyl alcohol	Catalyst loading (mg)	% Yield	%Yield (Quadratic model)
1.	25 (-1)	3 (+ 1)	30 (+ 1)	80 ± 3	77.9
2.	25 (-1)	3 (+ 1)	10 (-1)	30 ± 2	29.7
3.	45 (+ 1)	3 (+ 1)	10 (-1)	45 ± 5	47.3
4.	35 (0)	1 (-1)	20 (0)	45 ± 3	48
5.	25 (-1)	1 (-1)	10 (-1)	20 ± 3	17.1
6.	35 (0)	2 (0)	20 (0)	57 ± 1	58.7
7.	45 (+ 1)	3 (+ 1)	30 (+ 1)	98 ± 5	95.5
8.	25 (-1)	1 (-1)	30 (+ 1)	65 ± 3	65.3
9.	35 (0)	2 (0)	20 (0)	60 ± 4	58.7
10.	35 (0)	2 (0)	20 (0)	55 ± 3	58.7
11.	35 (0)	2 (0)	30 (+ 1)	80 ± 2	82.8
12.	25 (-1)	2 (0)	20 (0)	50 ± 3	50.9
13.	35 (0)	2 (0)	10 (-1)	37 ± 5	34.6
14.	35 (0)	2 (0)	20 (0)	60 ± 2	58.7
15.	45 (+ 1)	1 (-1)	30 (+ 1)	80 ± 5	78.9
16.	35 (0)	2 (0)	20 (0)	59 ± 3	58.7
17.	45 (+ 1)	1 (-1)	10 (-1)	30 ± 4	30.7
18.	35 (0)	3 (+ 1)	20 (0)	60 ± 2	62.6
19.	35 (0)	2 (0)	20 (0)	59 ± 1	58.7
20.	45 (+ 1)	2 (0)	20 (0)	70 ± 5	66.5

2.9 Product extraction and recycling of catalyst and solvent

The remarkable property of the used fluorous solvent was its temperature dependant solubility in the organic solvent [21]. Initially, it was observed that the reaction mixture contained two layers of solvent and acyl donor vinyl propionate at room temperature. Phenethyl alcohol is a more polar substrate mostly got dissolved in a fluorous solvent. As the reaction temperature increases, vinyl propionate gets dissolved in a solvent. On completion of the reaction, two layers again formed when reaction mixtures cooled down to around room temperature. The product phenethyl propionate and substrate vinyl propionate remained a separate phase above the fluorous solvent. The upper layer of product and un-reacted vinyl propionate was removed simply by decanting. The immobilized lipase and un-reacted phenethyl alcohol remained in the fluorous solvent. After removing the product from the first cycle, the fresh substrate was again added to the solvent and catalyst and kept for 45^{o}C for 3 h. Total four cycles of product removal and new substrate addition were carried out.

3. Result and discussion

3.1 Selection of immobilization support

The selection of immobilization support was made by determining the % loading of protein and activity retention after immobilizations. The % loading of protein was found to be > 90% for all four supports used for immobilization, which guaranteed that support matrix and lipase were compatible and could be used for immobilization (Table 2). Further immobilized lipase was screened by lipase activity assay. Lipase activity was highest when tylose was used as support for immobilization. The reason behind the increase in lipase activity after immobilization can be attributed to interfacial activation of lipase and resistance to aggregation [22]. As there was no catalyst aggregation, catalytic sites are readily available, and mass transfer becomes easy for catalytic activity. In the present study, Tyl-CRL gave the highest % loading of protein and lipase activity. Hence, Tyl-CRL was selected as a robust catalyst among all support compositions and utilized further experiments. A similar type of catalytic activity improvement was found by Badgujar et al. [23].

Table 2
Determination of percent of protein entrapment and lipase activity assay

No.	Support	Protein entrapped (%)	lipase activity (U/mg of support)	% Activity yield
1.	Tylose	98	4.04	178.94
2.	Chitosan	96	3.57	137.71
3.	Gum acacia	94	3.3	114.03
4.	Guar gum	90	3.1	96.49
5.	Crude lipase	--	3.04 (U/mg)	--

3.2 Stability of lipase in fluorous solvent

Immobilized Tyl-CRL was incubated in fluorous solvents for 5 h at 120 rpm at 45^{o}C temperatures. After incubation, hydrolytic activity assay of incubated and non incubated immobilized lipase using p-NPA as substrate was carried out. It was observed that lipase fairly retained its activity after incubation with all three fluorous solvents (Table 3). Activity retention of immobilized CRL is highest in PFO, which could be because of the higher log P value of PFO (5.92) than PFH (4.73) and PFMC (4.66). It was well demonstrated that lipase works well in non-polar solvents [24]. The non-polar solvents having a log P value of more than 4 keep the water around the microenvironment of lipase so that the activity of lipase gets conserved.

Table 3
Lipase stability in fluorous solvent

Sample	Solvent	Lipase activity (U/mg)	Relative activity (%)
Tylose-CRL (Incubated)	PFO	4.01	97.31
	PFH	3.801	92.28
	PFMC	3.174	77.51
Tylose-CRL Without incubation		4.12	

3.3 Determination of kinetic parameters

We have carried out a kinetic study using hydrolytic activity assay to determine the catalytic activity and efficiency. As expected, immobilized CRL showed higher activity. The value of *V*max for immobilized CRL (30.23 µm/min/mg) was found to be more than that of crude CRL (23.43 µm/min/mg). The value of *K*m for immobilized CRL was lower (5.5 mM) than that of crude CRL (6.6 mM), which showed that immobilized lipase had more affinity towards the substrate. Immobilization avoids the aggregation of lipase and keeps it evenly distributed all over the polymer matrix, which facilitates easy contact of the substrate and active site of lipase, which benefits the internal mass transfer [17, 25]. This could be the reason for increased *V*max and decreased *K*m for immobilized lipase. Moreover, immobilization could promote the open conformation of lipase, which provides easy entry of substrate into the catalytic site [26, 27].

3.4 Lipase screening for the synthesis of phenethyl propionate

Various lipases, namely CRL, MJL, and ANL, were screened to synthesize phenethyl propionate under similar reaction conditions. These lipases are readily available and commonly utilized for biotechnological and biocatalytic applications [1, 2, 15, 28]. Immobilized CRL gave 99% yield in 3 h. In comparison, other immobilized lipases MJL and ANL provided 6.2% and 25.7% yield in 3 h. It was noticed crude lipases MJL, ANL and CRL gave 3, 8, and 28% yield at the same reaction conditions (Fig. 2). Accumulation of free lipases in nonaqueous media may be the explanation for the lower activity of crude

lipases contrasted with immobilized lipases[25]. Immobilized lipases remained consistently dispersed all through the polymer grid, bringing about surface activation and better catalysis.

Molecular docking analysis was carried out to see the interactions between lipases and substrates. The docking study noticed that the acyl substrate-CRL complex has lower binding energy than the MJL and ANL- acyl substrate complex (Data shown in the supplementary datasheet). The lower free energy implies that CRL has a greater affinity for both substrates, giving it a better catalytic efficiency. The conclusions produced using the docking study was predictable with experimental results, so CRL was utilized for remaining experiments.

3.5 Instrumentation

Surface characterization of immobilized Tyl-CRL film and plain tylose film was carried out using SEM. Both CRL-loaded and plain tylose films looked similar, suggesting that CRL is nicely blended in polymer and distributed uniformly all over the tylose film (Fig. 3) [6].

FTIR spectrometry analysis was done to check the conservation of the secondary structure of a protein in the process of immobilization (Fig. 4a). In the secondary structure of the protein, amide I is the major adsorption band, was found. It is mainly because of C = O and C-N stretching vibrations [29]. The characteristic band of amide I was seen at 1645 in Tyl-CRL.

Determination of stability of enzymes under the various temperatures is necessary as the enzymes are prone to denaturation at elevated temperatures [30]. The degradation pattern of immobilized CRL was studied using the TGA. All three samples, namely crude lipase, Tyl-CRL, and plain Tylose films, were analyzed (Fig. 4b). It was noticed that immobilized lipase showed increased stability than crude lipase. It is demonstrated that a small amount of water in the microenvironment of lipase is necessary for its conformational flexibility and activity [31]. Water loosely present around the lipase gets evaporated around 100^{o}C, but tightly bound water gets removed above 200^{o}C [32]. Figure 4b showed that weight loss due to the removal of tightly bound water around 200^{o}C was more in crude lipase than immobilized lipase. That showed immobilization had increased the water holding capacity of lipase, which helped the lipase retain its stability even at elevated temperatures

3.6 Process optimization and model development

In literature, one factor at a time design is generally used to optimize reaction parameters which require more time, chemicals, and energy for the experiment as a number of runs are more in this type of design. In contrast, the response surface methodology (RSM) used with the three-factor-three-level method used in this study is more efficient. It reduces the number of experiments, so time and energy to perform these experiments. RSM allows understanding the correlation between various variables involved in synthesizing phenethyl propionate by immobilized lipase. The lowest yield obtained was 20% when the ratio of vinyl propionate: phenethyl alcohol was 1, temperature 25^{o}C, and the catalyst was 10 mg (Table 1). The maximum yield was 98%. Furthermore, the data from 20 experiments were used to figure out the suitable model among linear, two-factor interaction, quadratic and cubic models. Analysis of

variance (ANOVA) (Table 4) showed that the quadratic model was highly suitable to establish the correlation between a response that is % yield and other significant variables like concentration of phenethyl alcohol and vinyl propionate, immobilized CRL amount, and temperature with significantly lower p-value and acceptable R^2 value. The equation for the quadratic model is as follows.

Table 4
ANOVA model fit summary table for % yield

Source	Sum of Squares	df	Mean Square	F-value	p-value	
Model	7015.20	5	1403.04	221.20	< 0.0001	Significant
A-Temperature	608.40	1	608.40	95.92	< 0.0001	
B-Substrate mole ratio	532.90	1	532.90	84.02	< 0.0001	
C-Catalyst amount	5808.10	1	5808.10	915.69	< 0.0001	
AB	8.00	1	8.00	1.26	0.2803	
B^2	57.80	1	57.80	9.11	0.0092	
Residual	88.80	14	6.34			
Lack of Fit	69.47	9	7.72	2.00	0.2311	not significant
Pure Error	19.33	5	3.87			
Cor Total	7104.00	19				
R^2						0.98
R^2 Adjusted						0.98
Predicted R^2						0.97

% Yield = 58.70 + 7.80*A + 7.30*B + 24.10*C + 1.00*A*B-3.40*B^2

3.7 Mutual effect of various reaction parameters

In the present work, we have tried to find out the mutual effect of reaction parameters for phenethyl propionate synthesis by lipase. Stoichiometrically 1:1 ratio of substrate amount is required for lipase transesterification reaction. But the literature reveals that using a higher concentration of acyl donor increases reaction rate and yield [33]. Moreover, keeping the higher amount of acyl donor concentration also prevents the hydrolysis of the product. So we studied the effect of the increasing amount of vinyl propionate (Fig. 5). It was noticed that as mmol of vinyl propionate increased from 1 to 3 mmol, keeping

the amount of phenethyl alcohol to 1 mmol, % yield also increased. Likewise, with an increase in temperature increase in % yield was observed. As temperature increases, the solubility of reactants increases, molecular collisions between substrates and biocatalyst also increase, which together avoids mass transfer limitation and accelerates the catalysis [8, 34].

3.8 Obtaining optimal reaction parameters and model verification

One can find the combination of optimal reaction parameters using the response surface method. Table 5 lists the variety of parameters to obtain the highest yield (%). Some additional experiments were carried out at suggested reaction parameters to check the validity of the model obtained by the RSM study. Table 5 gave predicted and actual responses at suggested reaction parameters for the synthesis of phenethyl propionate. The predicted response and response obtained from actual experiments are very similar, which depicted that the model obtained from RSM was appropriate and the suggested model can be used as a reference model for phenethyl synthesis.

Table 5
Model solution and verification

No.	Substrate ratio Vinyl propionate: Phenethyl alcohol	Biocatalyst (mg)	Temperature (°C)	Predicted yield (%)	Actual Yield (%)
1.	3	30	42	92.86	95 ± 3
2.	3	30	40	91.1	90 ± 5
3.	3	25	45	83.45	83 ± 2
4.	1.5	28	45	80.78	84 ± 2
5.	2.5	25	45	81.85	83 ± 5

3.9 Substrate study

In order to study the scope of developed protocol, immobilized CRL was used to synthesize different propionate esters important in fragrance, flavors, pharma, and home care products (Fig. 6). The alcohol with a small chain like propanol, butanol, and isobutanol gave good yield up to 70–80%, slightly less yield is because of polar nature of these alcohols which might interfere with enzyme [6]. Long-chain alcohol like octanol gave a very poor yield of 26% because the long chain of these alcohols is hydrophobic and may interact with hydrophobic side chains of enzymes, giving rise to a steric hindrance [35]. Cyclohexanol is secondary alcohol and showed very poor yield (10%) because of a steric hindrance at adjacent α-carbon atoms [36]. Branched alcohol like geraniol provided yields up to 50%. Aromatic alcohols like cinnamyl alcohol and benzyl alcohol gave 90% and 70% yield, respectively. Heteroaromatic furfuryl alcohol showed 77% yield.

3.10 Recycling of biocatalyst and fluorous solvent

To make the protocol economical, it is essential to reuse the resources as much as possible. In this protocol, we have reused the catalyst as well as the solvent. After completion, the reaction product was removed simply by decanting, as explained in the experimental section, and the remaining solvent and immobilized CRL were used for the next cycle. It was observed that the catalyst could be used for up to four cycles (Fig. 7). After four cycles, the decrease in % yield may be due to the leaching of the enzyme from support and prolonged exposure of the enzyme to the alcoholic substrate [37].

4. Molecular Docking Study to Understand Reaction Mechanism

In the present study, we have also tried to see the interactions between lipase and reactants at the molecular level. Molecular docking study helps to check such interactions between macromolecules like protein and DNA and small molecules usually referred to as ligands. Such interaction studies can inform us about the confirmation of molecules and their preferred binding site in the macromolecules here in the lipase. The 3-dimensional protein structure of CRL was downloaded from the RCSB PDB website.1CRL PDB ID was used for downloading and docking. The structures of substrate were drawn in MarvinSkech software. The substrate structures of minimum energy were selected for docking. Chimera 1.15 and AutoDock Vina software were used for docking. Before docking with the CRL, the previously attached ligands were removed, and protein was prepared for docking using the DockPrep function in Chimera 1.15. The active site for CRL was found to be His449, ser209, glu341, and gly124 [26]. So the grid region was drawn around the active residues, and docking was carried out using AutoDock Vina [38, 39]. The default setting for all other parameters was kept as it is while running Autodock Vina Software.

Docking of phenethyl alcohol, vinyl propionate, and phenethyl propionate was carried out against CRL. Phenethyl alcohol showed binding energy of -5 kcal/mol and hydrogen bonding with Ser209 (Fig. 8A). Vinyl propionate forms the hydrogen bond with Ser209 and Gly124 with a binding energy of -3.6 kcal/mol Fig. 8B). The serine oxygen attacks the substrates carbonyl carbon, forming serine esters which react with alcohol to complete the transesterification. The histidine and glycine helps in formation and stabilization of a serine oxyanion. Gly124 is a part of an oxyanion hole that formed a hydrogen bond with the carbonyl group of vinyl propionate and stabilized it. Though the binding energy of phenethyl alcohol with CRL is less than vinyl propionate, the hydrogen bond length was more than that of vinyl propionate (Fig. 8). The short hydrogen bonding of vinyl propionate showed a stronger interaction with the lipase. As both substrate phenethyl alcohol and vinyl propionate showed a good binding affinity with CRL, the reaction may follow the complex ternary mechanism in which both substrates bind to the lipase.

In contrast, in the ping pong bi bi mechanism first substrate binds with the lipase, and the first product is released, then the second substrate binds to the modified enzyme, so it doesn't show great binding affinity with the original enzyme. A systematic kinetic study should support the conclusion drawn through docking observation. Furthermore, product phenethyl propionate showed hydrogen bonding with active sites Ser209, His449, and Gly124 with a binding energy of -5.3 kcal/mol (Fig. 8C).

5. Conclusion

The present work successfully immobilized CRL on tylose polymeric film by entrapment method with 98% loading efficiency. Immobilized CRL showed good hydrolyzing activity than crude lipase as well as it remained stable in fluorous solvent up to 4 h. Immobilized CRL was further used to synthesize phenethyl propionate in a fluorous solvent. Reaction conditions were optimized using RSM, a three-factor- three-level central composite design method. Optimized reaction conditions were substrate ratio 1:3, immobilized lipase quantity 30 mg, and temperature 45^{o}C. The catalytic activity decreased by 77% up to the fifth recycle run. The optimized protocol was used to synthesis other propionate esters. A molecular docking study was carried out to see substrate interactions with the catalytic site.

Declarations

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Statements and Declarations

The authors declare that they have no conflict of interest.

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Figures

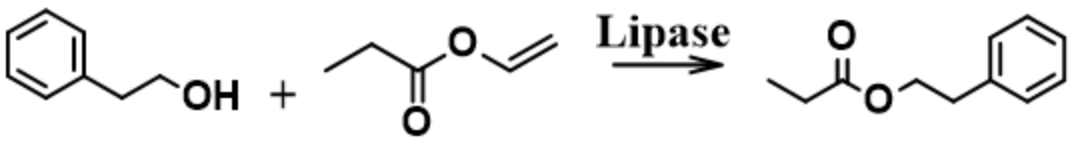

Figure 1

Synthesis of phenethyl propionate using lipase as catalyst

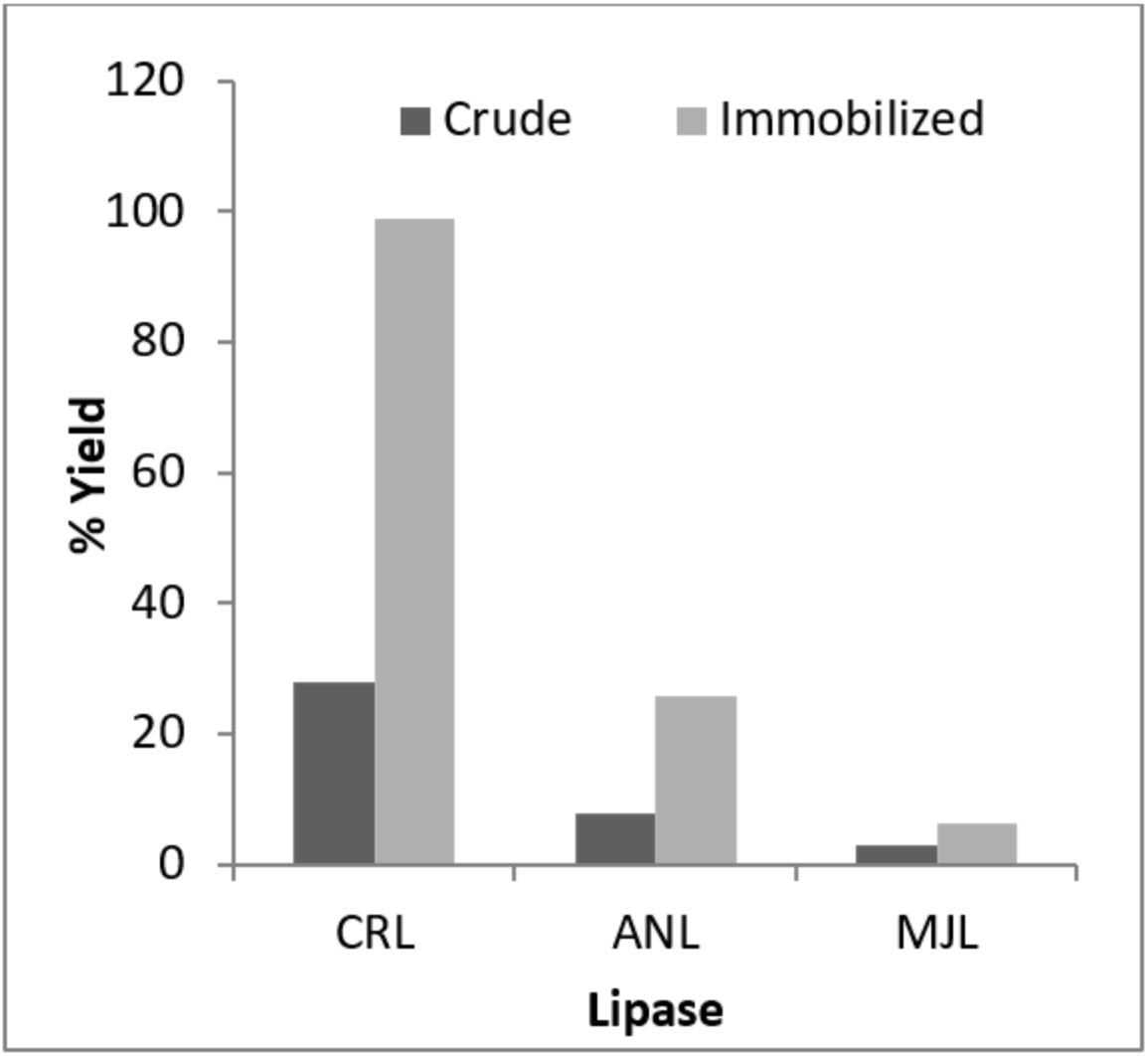

Figure 2

Lipase screening for the synthesis of phenethyl propionate (Reaction conditions: Phenethyl alcohol 1 mmol, Vinyl propionate 3 mmol, Lipase 30 mg, Time 3h, Temperature 45^{o}C, Solvent PFO up to 1mL)

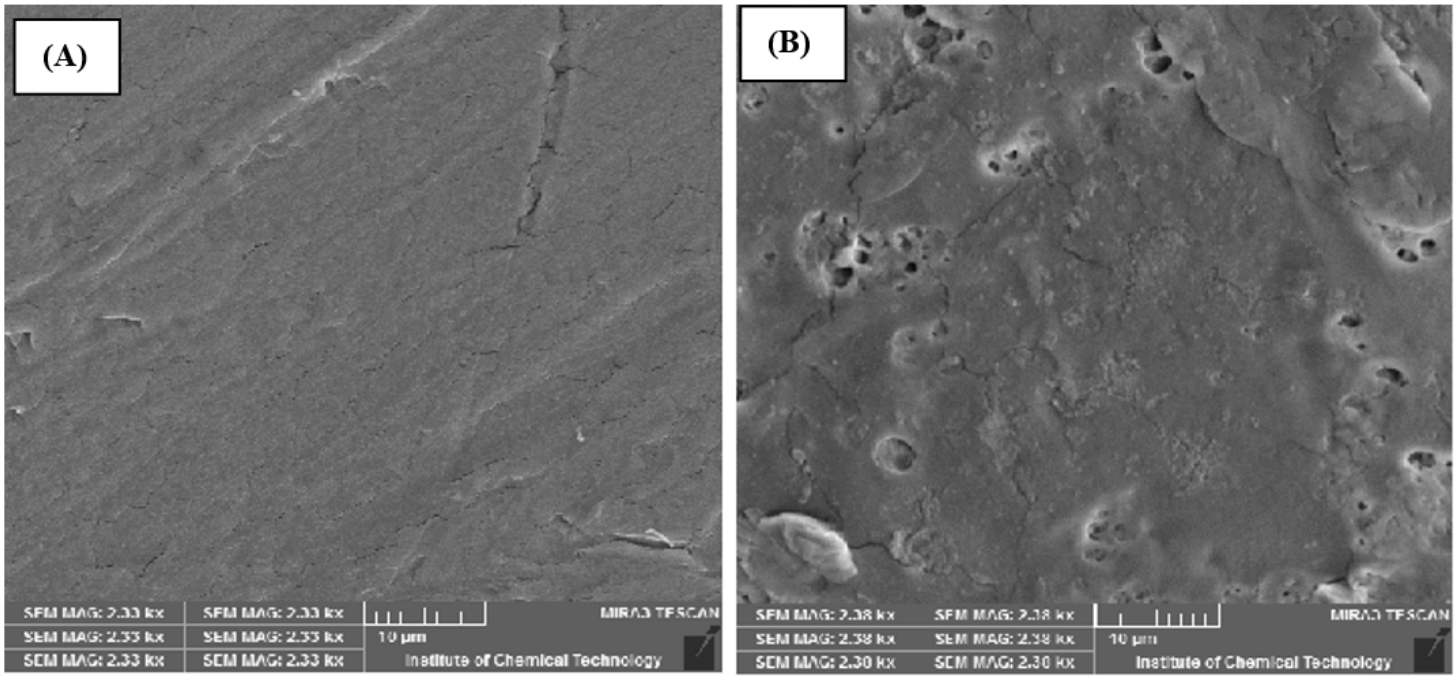

Figure 3

Surface characterization using scanning electron microscopy

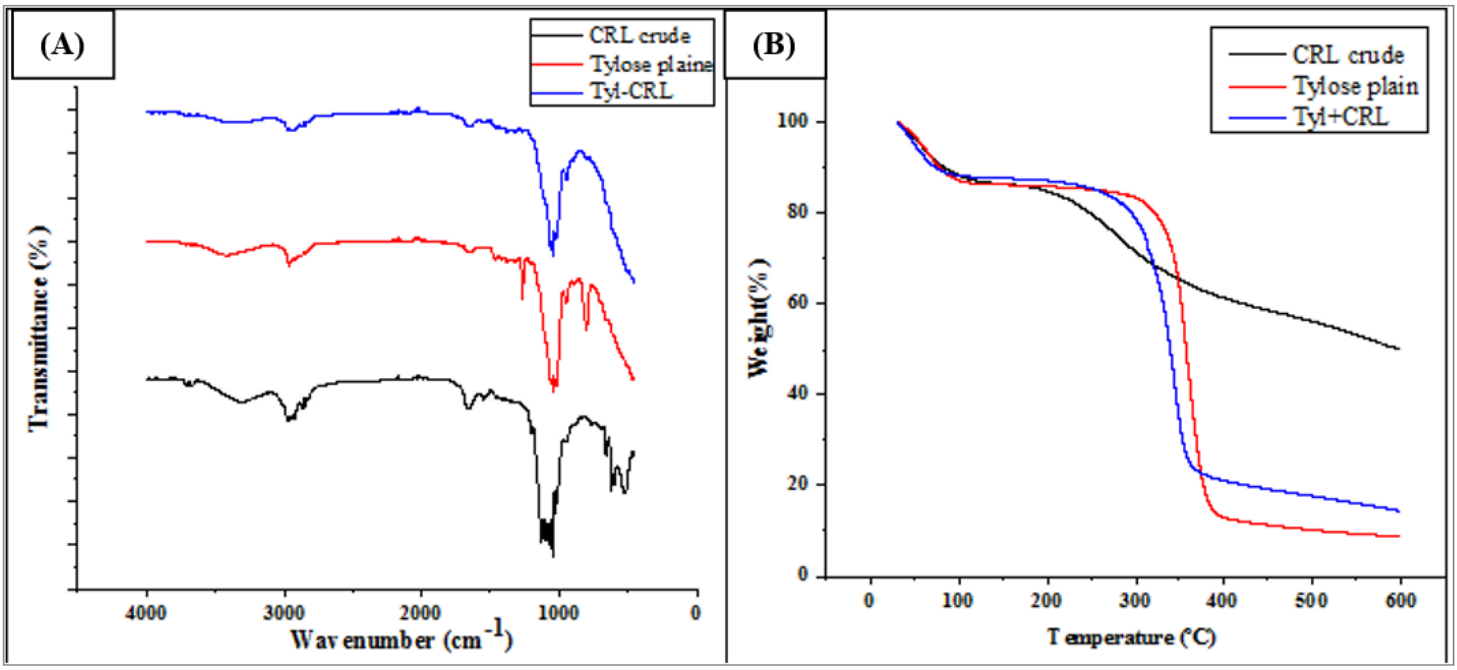

Figure 4

FTIR **(a)** and TGA **(b)** characterization of immobilized and crude lipase

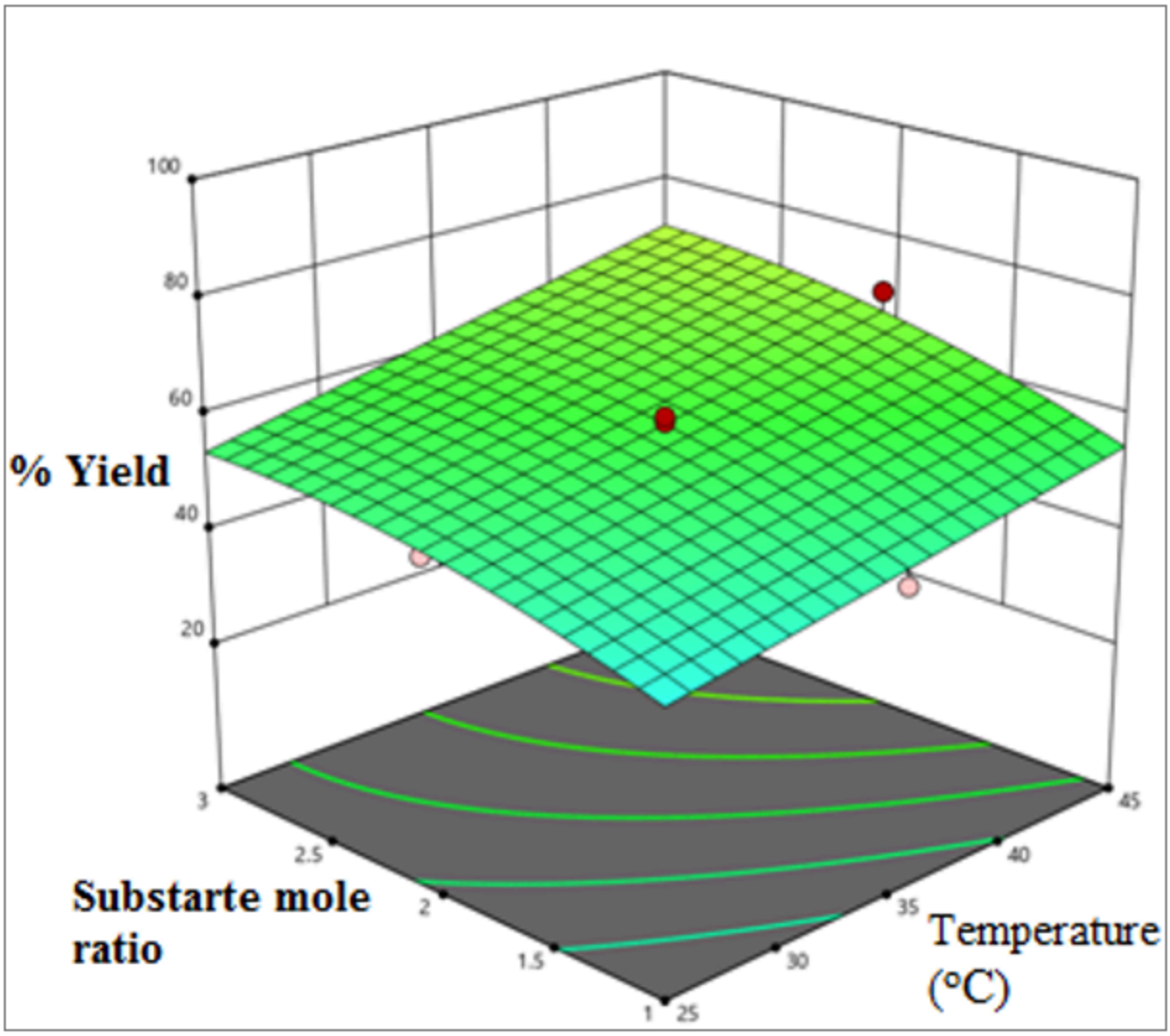

Figure 5

Effect of substrate concentration and temperature on the % yield

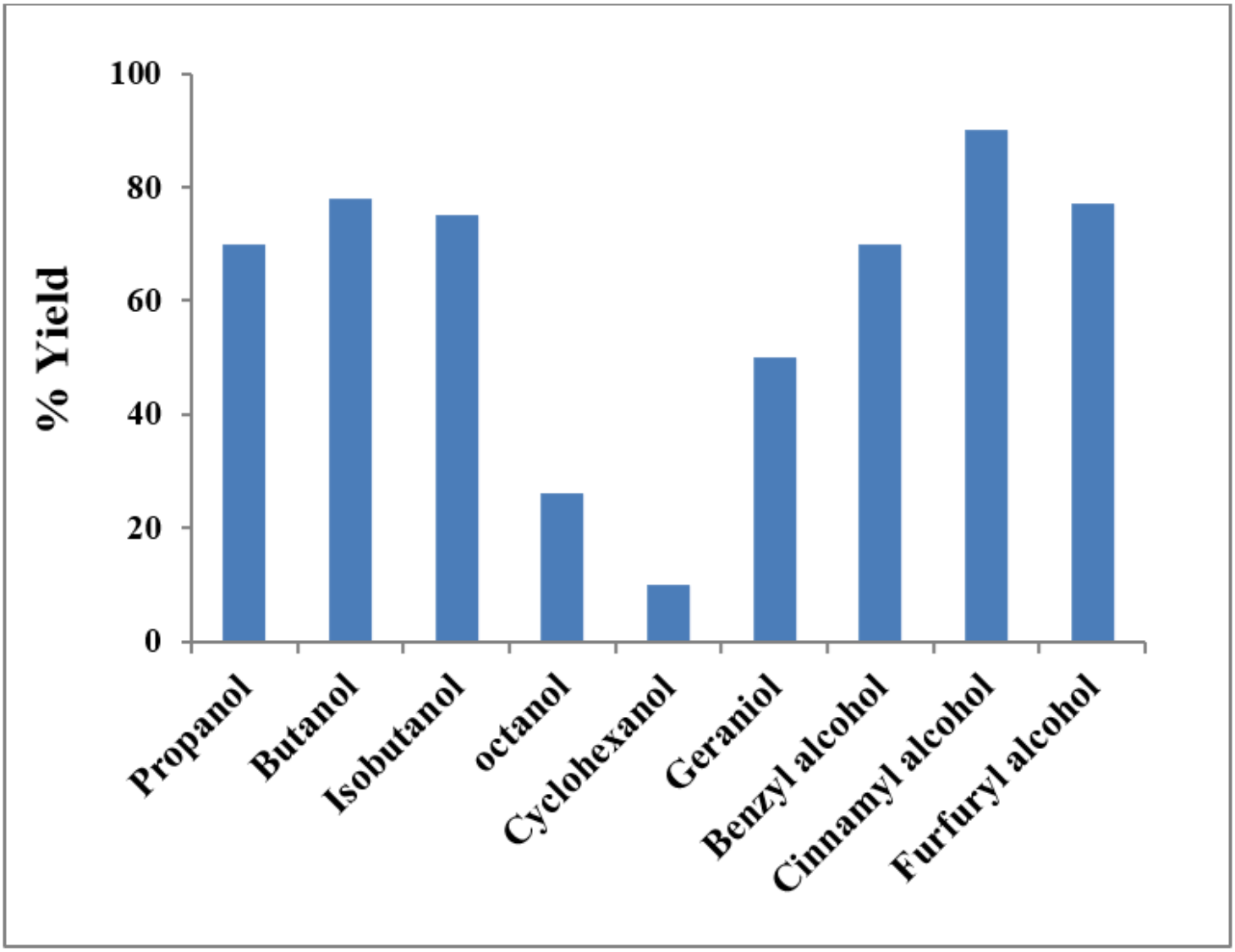

Figure 6

Substrate scope of immobilized lipase (Reaction conditions: alcohol 1 mmol, Vinyl propionate 3 mmol, Lipase 30 mg, Time 3h, Temperature 45^{o}C, Solvent PFO up to 1mL)

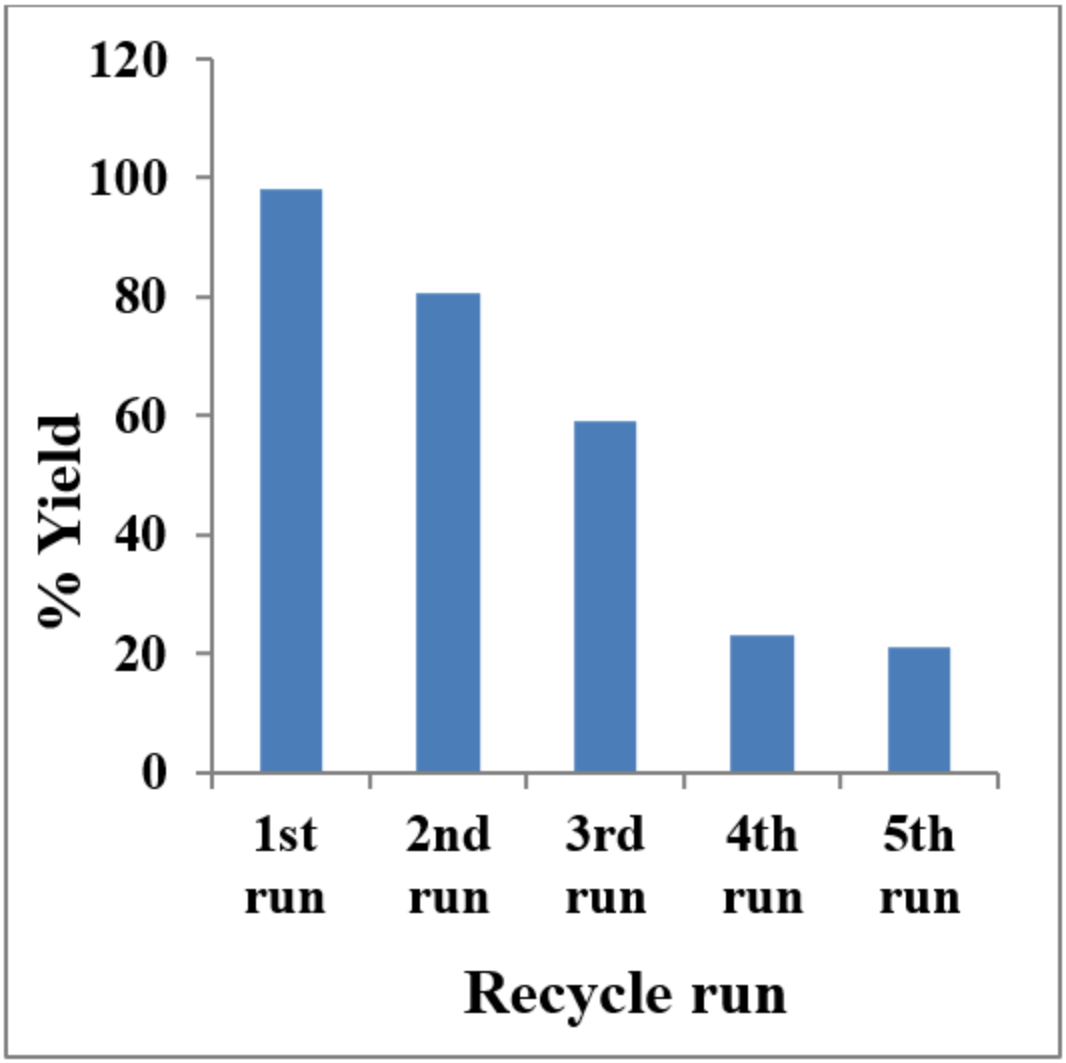

Figure 7

Recycling of biocatalyst (Reaction conditions: Phenethyl alcohol 1 mmol, Vinyl propionate 3 mmol, Lipase 30 mg, Time 3h, Temperature 45^{o}C, Solvent PFO up to 1mL)

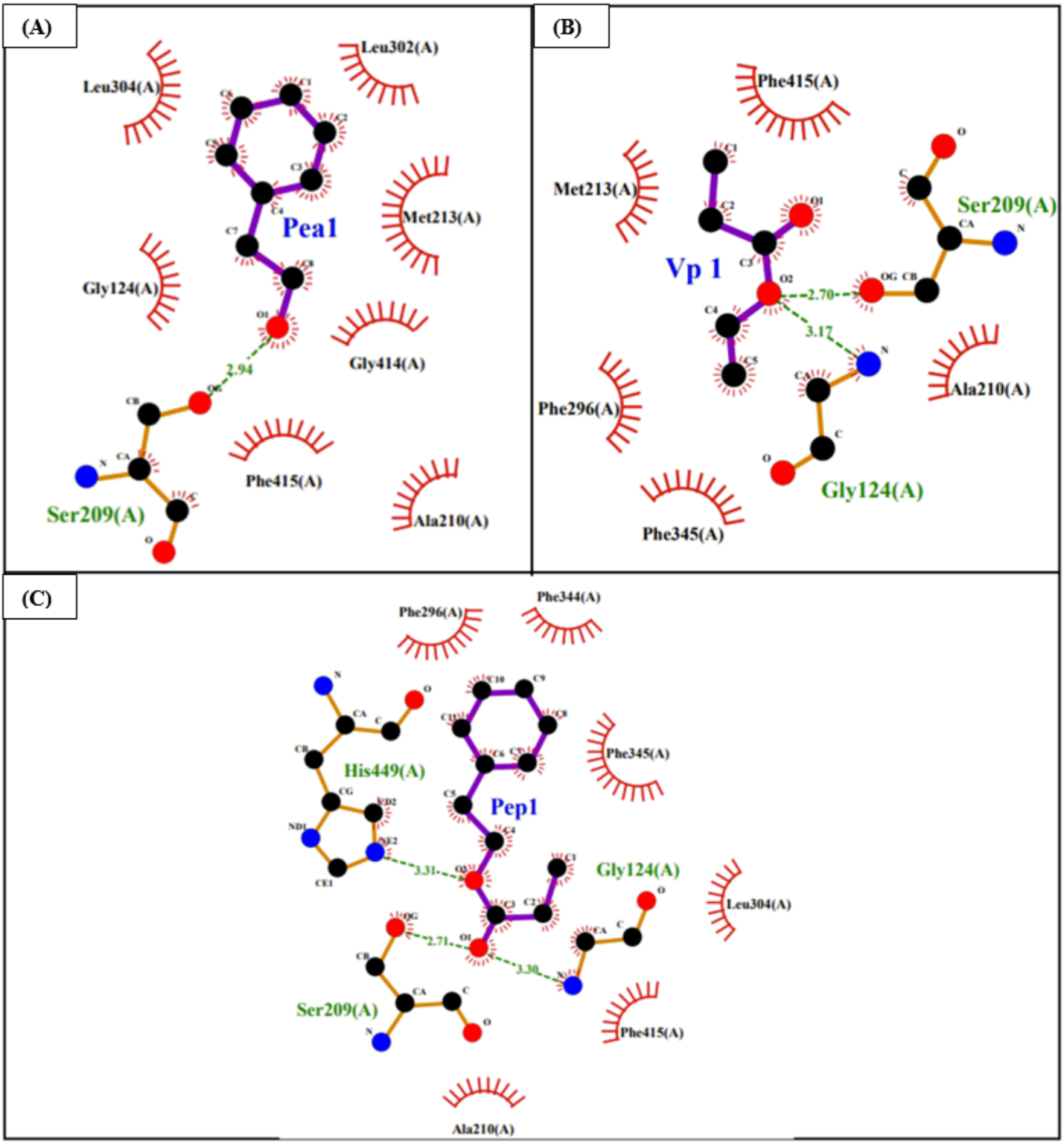

Figure 8

Docking of CRL against a) Phenethyl alcohol b) Vinyl propionate, and c) Phenethyl propionate

Supplementary Files

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