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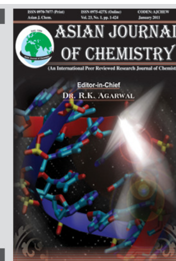

Fructose Laurate Enzymatic Synthesis by Resin D301 Immobilized *Candida* sp. 99-125 Lipase

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Sugar ester synthesis is catalyzed by the immobilized lipase of resin D301, which is obtained from immobilizing free *Candida* sp. lipase on the weakly basic anion exchange resin D301. This reaction requires butanone as a solvent, as well as fructose and lauric acid substrates. In this study, we designed and optimized an orthogonal immobilized enzyme catalyzed synthesis. The optimized reaction is incubated at 50 ºC for 24 h with 20 mL of methyl ethyl ketone as a solvent and a rotation speed of 180 rpm. The optimal molecular sieve concentration was 75 g/L, the substrate ratio was 1.5:1 and the enzyme concentration was 10 g/L. Under these optimal conditions, the maximum relative per cent conversion of lauric acid was 60 % with the di-fructose laurate substrate. The products were analyzed by TLC and ^{13}C NMR and the resulting products were 1-β-mono-D-fructopyranose laurate, which had an R_f of 0.44 and 1,6-β-di-D-fructopyranose laurate, which had an R_f of 0.79. Compared to traditional reactions in solvents, the solvent-free system was easier to separate and purify, which made the procedure both more cost and time effective.

Key Words: *Candida* sp. 99-125 lipase, Fructose laurate, Direct esterification, Immobilization.

INTRODUCTION

Fatty acid sugar esters are non-ionic surfactants which have highly emulsifying, stabilizing and detergent effect. These compounds are widely used in the food, cosmetic, detergent and pharmaceutical industries. These compounds are non-toxic, odorless, require no stimulation and are easily biodegradable[1]. Sugar esters are typically synthesized *via* chemical or biological enzymatic catalysis; chemical synthesis usually requires high temperature and high pressure conditions and there is a high incidence of by reactions. The disadvantage of ester bond elective, the number of ester difficult to control. Additionally, the high reaction temperature leads to coking and carbonation, which affect the final product colour. In contrast, enzyme catalysis requires mild reaction conditions and ester bond formation can be controlled[2,3]. Fructose is a type of fatty acid ester and fatty acids that have a fructose substrate can undergo sugar esterification[4]. The fructose ester has a high surface tension, as well as an increased emulsifying and foaming capacity compared to commercialized sucrose esters[5]. Fructose esters contribute to general non-ionic surfactant effects. Additionally, fructose is not dependent on insulin metabolism, which means that it can be safely metabolized by diabetics or patients with liver disease. In contrast, sucrose esters are not metabolically stable, especially in trauma and surgical patients and therefore do not produce as much energy and are not as safe as fructose. Moreover, fructose laurate mono-ester also has antibacterial activity, as studies have shown that it can effectively inhibit *Streptococcus* bacterial growth[6].

Currently, sugar esters are mainly synthesized by chemical methods[7-10]. Because reactions that involve sugars and sugar alcohols (*e.g.*, condensation and lactonization reactions) can result in strong reactions, chemical synthesis is preferred because the ester bond position can be controlled. In addition, chemical synthesis allows researchers to produce a variety of isomers and by-products, which can be controlled by the reaction conditions. Another problem frequently encountered in chemical synthesis of sugar esters is that the reaction requires high temperatures and results in coking, or a carbonization phenomenon, where the final colour of the product is often altered. Recently, enzyme catalysis[11,12] has become a popular method[13-16]; the studies that have explored enzyme catalysis include compound synthesis (especially for lipase), food processing, light industry, fermentation and traditional Chinese medicine. However, the poor stability of free lipase, its inability to be reused and additional problems have limited the industrial application of lipase. In recent studies, researchers have

endeavored to immobilize lipase to enhance its stability and increase the recycling rate to promote the industrial application of enzymatic catalysis[17].

In this study, we used D301 resin immobilized fructose laurate and assessed the ability of immobilized enzyme to catalyze an orthogonal reaction in a solvent-free system. Additionally, we optimized our reaction conditions to generate a protocol that results in optimal product yield. The general reaction scheme is shown in Fig. 1.

EXPERIMENTAL

The D301 resin immobilized enzyme (laboratory system) and *Candida* sp. 99-125 lipase (68.06 U/mg) was purchased from Beijing CTA New Century Biotechnology Co. Ltd. (PR China). Immobilized lipase Novozym 435 (*Candida Antarctica B* immobilized on an anion resin)was purchased from Novo Nordisk. The fructose, lauric acid, butanone were obtained from Sigma-Aldrich in the highest grade commercially available. Methanol, methyl ethyl ketone, *n*-propanol, *iso*-propanol, butanol, hexane, α-naphthol, 4 Å molecular sieve and other reagent were provided by Sinopharm Chemical Reagent Co. Ltd.(China). All reagents and solvents were of AR grade. D301 resin with lipase and Novozym 435 scanning electron microscope photographs shown in Fig. 2-1 and 2-2. Profile of the D301 resin with lipase and Novozym 435 in the comparison, shown in Fig. 2-3. The figure shows, D301 resin with lipase and Novozym 435 showed the overall appearance of the two spherical, the amplification can see the similarity with Novozym 435, D301 resin with lipase had smooth outer surface. There was a lot of criss-crossing channels inside, which was good for the enzyme molecule into the channels. Fig. 3 reports the 77 K nitrogen adsorption-desorption isotherms of the D301 resin with lipase and Novozym 435. The isotherms can be classified as type IV and exhibit an H3-type hysteresis loop at high relative pressure[17].

Orthogonal: The following three parameters were adjusted to optimize the reaction conditions *i.e.*, (a) the substrate ratio (acid/sugar); (b) the amount of molecular sieve added (zeolite/solvent) and (c) the amount of enzyme added (enzyme/solvent). These parameters were combined at a level of a 3 × 3 orthogonal factors.

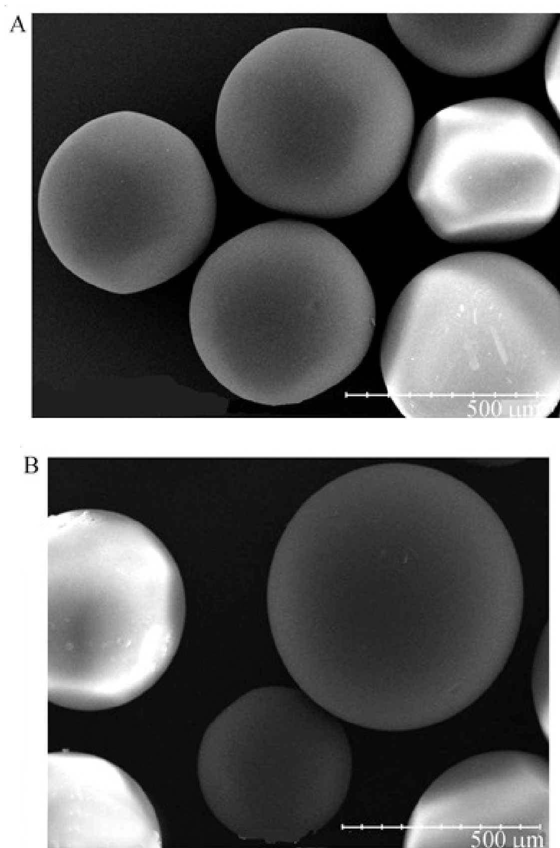
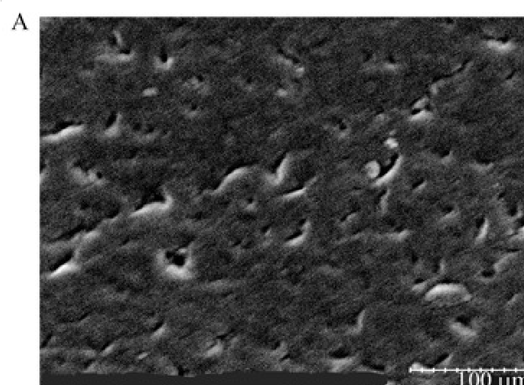


Fig. 2-1. Scanning electron micrographs (100 ×) of (A) D301 and (B) Novozym 435



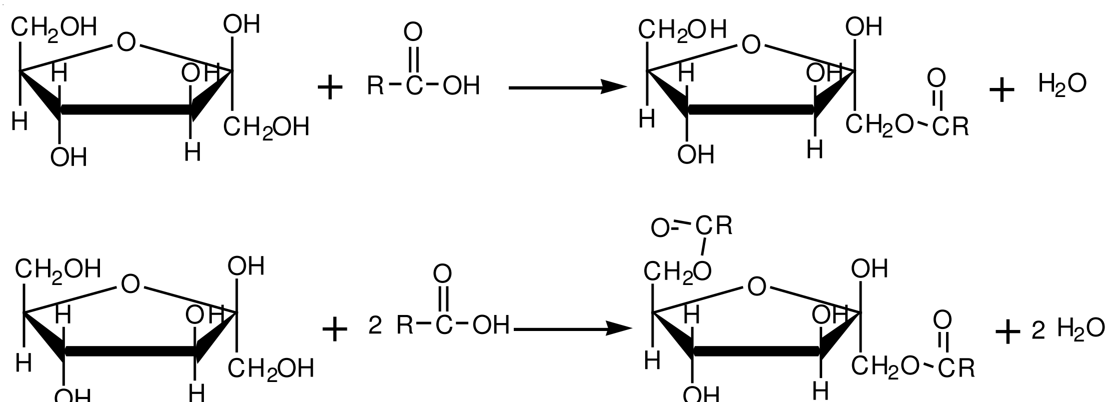


Fig. 1. An outline of the fructose ester synthesis reaction

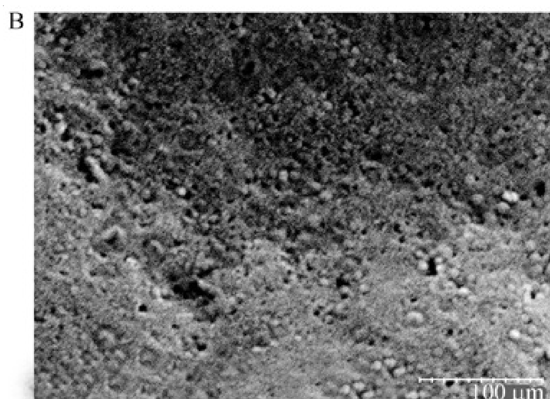
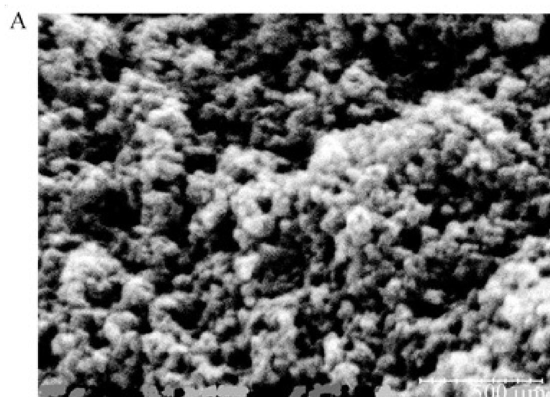


Fig. 2-2. Scanning electron micrographs (100 ×) of (A) D301 and (B) Novozym 435



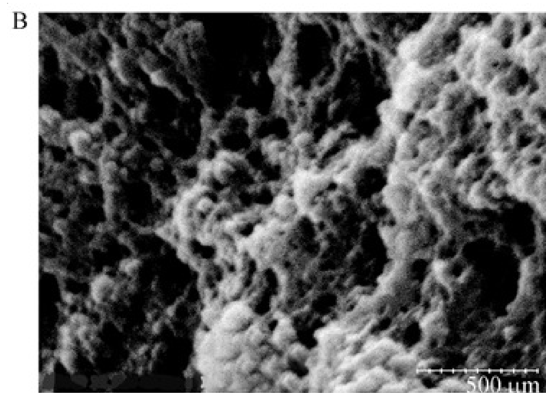
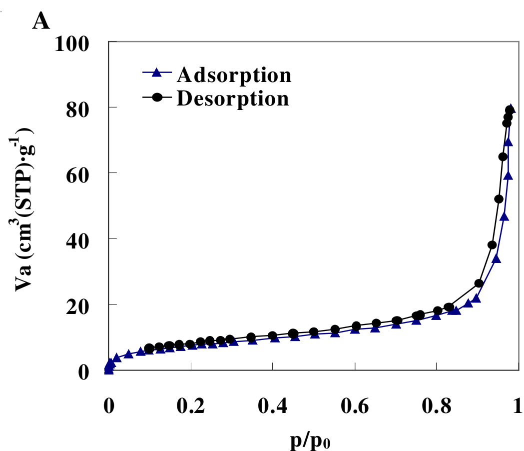


Fig. 2-3. Scanning electron micrographs (30000 ×) of (A) D301 and (B) Novozym 435



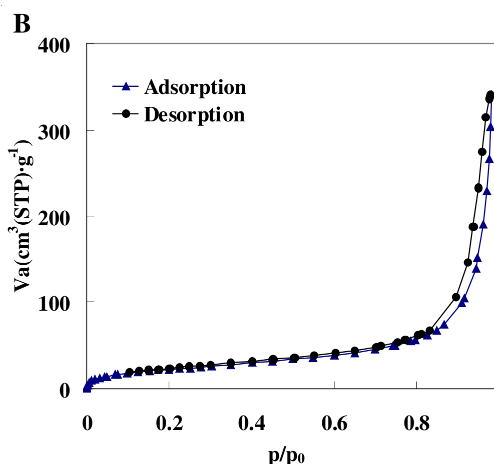


Fig. 2-4. N_2 adsorption-desorption isotherms of (A) D301 and (B) Novozym 435

Fructose laurate synthesis: A plug was placed into a 100-mL conical flask and 20 mL of methyl ethyl ketone was added to the solvent (Table-1); the corresponding amounts of lauric acid, fructose, molecular sieve and D301 resin immobilized enzyme were added (Table-2) for the 9 experiments. The reactions were incubated at 50 ºC in a water bath at 180 rpm for 24 h.

TABLE-1
ORTHOGONAL EXPERIMENTAL CONDITIONS

Level	Factor		
	Substrate ratio	Molecular sieve conc. (g/L)	Enzyme conc. (g/L)
1 level	1.5	50	10
2 levels	2.0	75	20
3 levels	2.5	100	30

TABLE-2
ORTHOGONAL EXPERIMENTAL PROGRAM

Serial number	Factor		
	Substrate ratio	Molecular sieve conc. (g/L)	Enzyme conc. (g/L)
1	Level 1	Level 1	Level 1
2	Level 1	Level 2	Level 2
3	Level 1	Level 3	Level 3
4	Level 2	Level 1	Level 2
5	Level 2	Level 2	Level 3
6	Level 2	Level 3	Level 1
7	Level 3	Level 1	Level 3
8	Level 3	Level 2	Level 1
9	Level 3	Level 3	Level 2

Determination of the relative conversion rate: After the esterification reaction, the D301 resin immobilized enzyme and molecular sieve were filtered out, the solvent was evaporated and the pH was adjusted with 30 mL of 1:1 (v/v) ether:ethanol mixture to terminate the reaction and dissolve the remaining lauric acid. Phenolphthalein was used as an indicator and the solution was titrated with 0.1 mol/L of NaOH. The remaining free lauric acid and the amount of lauric acid that was reduced was calculated according to the relative conversion rate of lauric acid (lauric acid to fructose diester) according to the following equations:

Conversion (%)

$$= \left(1 - \frac{\text{Remained titration laurate after reaction}}{\text{Blank titrtion}}\right) \times 100\ \%$$

Relative conversion (%)

$$= \frac{\text{Conversion of lauric acid}}{\text{Theoretical maximum conversion of lauric acid}} \times 100\ \%$$

Thermal stability of the free lipase and the D301 resin immobilized lipase: We incubated free or immobilized enzyme at the typical incubation temperature, then heated the reactions to 40 ºC to allow the enzyme to generate *p*-nitrophenyl ester. The amount of *p*-nitrophenol was subsequently measured with time and compared for the two enzymes to assess the enzymatic thermostability.

Operational stability of the free lipase and the D301 resin immobilized lipase: Free and immobilized enzyme were incubated at 37 ºC under the conditions described in reaction number 5 (Table-2) and the amount of *p*-nitrophenol product was recorded with time. *p*-Nitrophenol production formation was measured to assess enzyme activity over time and the data were compared for the two different forms of the enzyme.

Storage stability of free lipase and the D301 resin immobilized lipase: We incubated free and immobilized enzyme with *p*-nitrophenyl esters over a period of several days and measured product formation with time to assess enzymatic activity.

Product extraction: The products were separated by silica gel chromatography columns with a mobile phase of 9:1 (v/v) ethyl acetate:methanol; product chromatography was monitored by TLC.

TLC and ^{13}C NMR analyses: The products were analyzed by TLC. The fructose solution, lauric acid solution and fructose laurate were developed on a G silica gel plate with toluene:ethyl acetate:methanol:water (10:5:4.5:0.2). The plates were sprayed with a colour display reagent (1.59 g α-naphthol dissolved in 51 mL of ethanol, followed by the addition of 6.5 mL of 18 M sulfuric acid and 4 mL water) and heated to 105 ºC for 5 min to develop the sugar and sugar ester spots. The samples were analyzed by ^{13}C NMR ($CDCl_3$, 500 MHz; TMS was used as an internal standard reagent).

RESULTS AND DISCUSSION

Orthogonal experiment analysis: The orthogonal experimental results are listed in Table-3.

TABLE-3
ORTHOGONAL EXPERIMENT RESULTS

	Substrate ratio	Molecular sieve conc. (g/L)	Enzyme conc.(g/L)	Relative conversion (%)
1	1.5	50	10	55
2	1.5	75	20	64
3	1.5	100	30	39
4	2.0	50	20	35
5	2.0	75	30	47
6	2.0	100	10	40
7	2.5	50	30	33
8	2.5	75	10	63
9	2.5	100	20	32

The data from the analyses of the experimental results are listed in Table-4.

TABLE-4
STATISTICAL ANALYSIS OF THE EXPERIMENTAL RESULTS

	Substrate ratio	Molecular sieve conc. (g/L)	Enzyme conc. (g/L)
K_1	158	123	158
K_2	122	174	131
K_3	128	111	119
$k_1 (= K_1/3)$*	52.67	41	52.67
$k_2 (= K_2/3)$	40.67	58	43.67
$k_3 (= K_3/3)$	42.67	37	39.67
Extreme difference	12	21	13

*k_1, k_2, k_3 are 1 level, 2 levels, or 3 levels corresponding to the average.

The data listed in Table-4 for the extreme differences in the various factors indicate that the amount of molecular sieve is the most important factor that affects the relative per cent conversion.

The statistical analyses of the orthogonal experiments were performed with an ANOVA (analysis of variance) test, as well as a Pareto test (primary and secondary factors that order). The results of these statistical analyses are shown in Table-5 and Fig. 2.

TABLE-5
ANOVA STATISTICAL ANALYSIS OF THE RELATIVE CONVERSIONS

	SS	df	MS	F	*p*
(1) Substrate ratio (L+Q)	708.223	2	708.2223	1593.5	0.009774
(2) Molecular sieve conc. (L + Q)	326.889	2	326.8889	735.5	0.005772
(3) Enzyme conc. (L + Q)	344.889	2	344.8889	776.0	0.020794
Error	0.889	2	0.4444	–	–
Total SS	1380.889	8	–	–	–

ANOVA analysis indicates that the conversion rate is relatively small. (An orthogonal experimental *p*-value < 0.05 was considered to be statistically significant and smaller *p*-values indicate greater effects).

Based on the above analyses, the substrate ratio and the amount of enzyme added have a small effect on the per cent conversion. The key parameter is the amount of zeolite added. The optimized conditions for $B_2C_1A_1$ were 75 g/L of the molecular sieve, a substrate ratio of 1.5:1 and 10 g/L of enzyme. The experimental results that were obtained under optimal conditions are shown in Fig. 3.

Under optimal conditions, the maximum relative per cent conversion of lauric acid into fructose laurate was 60 % with the D301 resin immobilized enzyme. For the solvent-free system and the solvent system with butanone, respectively, the addition of the Novozym 435 lipase yielded maximum relative conversions of 75.6 %[17] and 68 % under optimal conditions, compared to the D301 resin. However, the relative conversion reaction catalyzed by the immobilized enzyme was decreased, eventhough the overall relative conversion rate was relatively large. The D301 resin immobilized enzyme costs

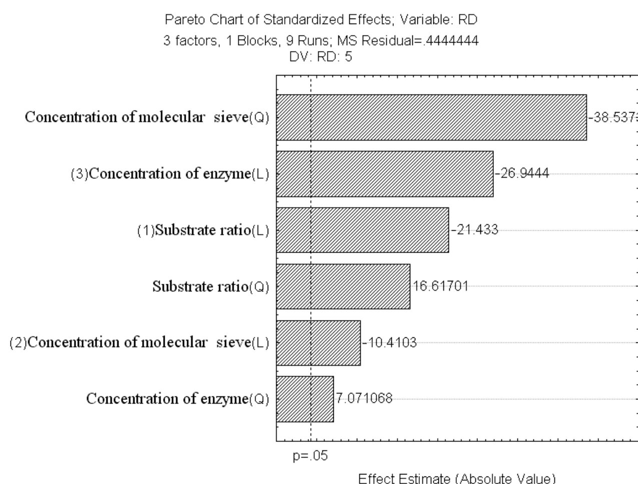


Fig. 3. A Pareto chart illustrating the relative conversion rate

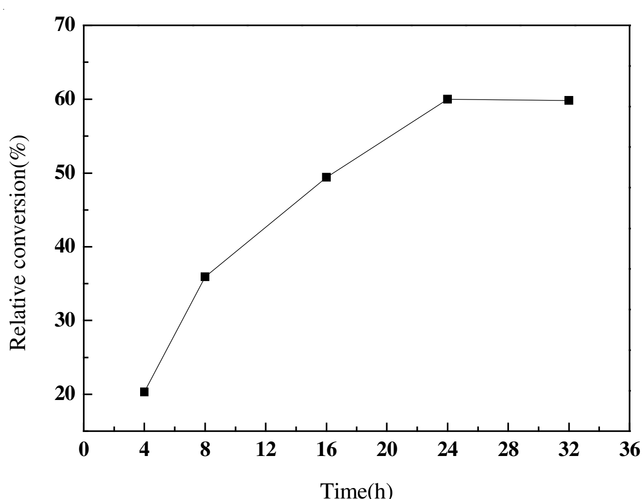


Fig. 4. Relative per cent conversion vs. time under optimal reaction conditions

much less than the Novozym 435 lipase, which is widely used in industrial catalytic synthesis of D301 resin immobilized fructose esters.

Thermal stability: The reactions with either free or immobilized enzyme were incubated at 50 ºC for 0-6 h and then quickly cooled to 40 ºC. The residual enzyme activity was determined and the results are shown in Fig. 5.

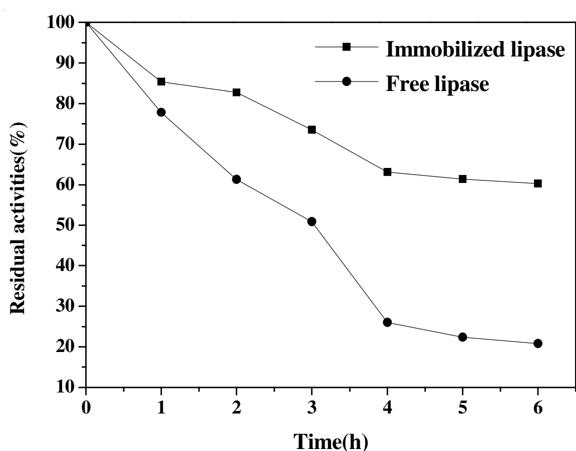


Fig. 5. Thermal stability of the free lipase and immobilized lipase

Free enzyme that was incubated at 50 ºC had almost no activity after 6 h, while the immobilized enzyme activity remained above 60 %, indicating that the immobilized lipase has a higher thermostability.

Operational stability: The reaction was incubated at 37 ºC under the conditions listed for reaction 5 (Table-2) and the experimental results are shown in Fig. 6.

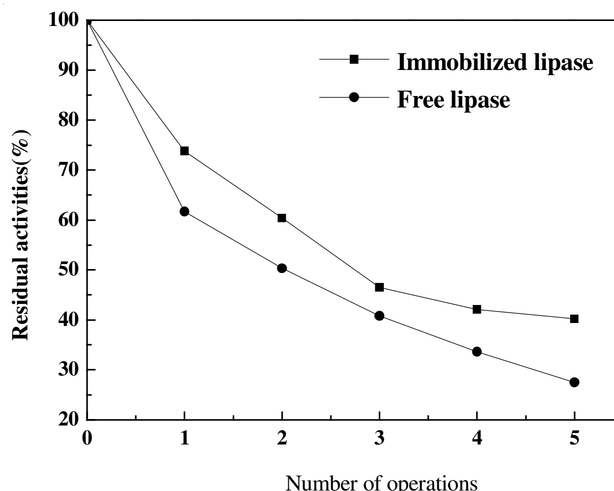


Fig. 6. Operational stabilities of the free lipase and immobilized lipase

The figure shows that the activity of both types of enzyme decreased with the number of operations, although the rate of decrease was different for the two enzymes. For both of the enzymes, the first three cycles of activity declined similarly and the residual activity of the free and immobilized enzymes after the third cycle was 46.5 and 40.8 %, respectively. However, the D301 resin immobilized lipase showed a further decrease in activity after the third cycle and the residual activity after five cycles was 40.2 %; in contrast, the free enzyme activity continued to decline rapidly and was 27.5 % after five cycles. Thus, the D301 resin immobilized lipase was more stable over a repeated number of reactions.

Storage stability: The free enzyme and immobilized enzyme were incubated at 4 ºC and the enzymatic activity was measured every 10 days. The change in the lipase activity with storage time is shown in Fig. 7.

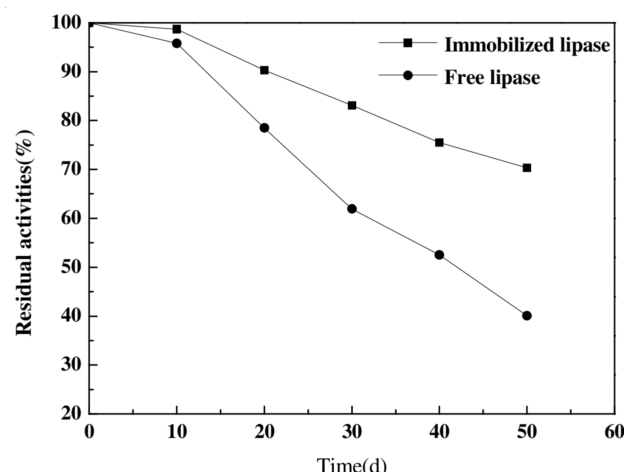


Fig. 7. Storage stability of the free lipase and immobilized lipase

TABLE-6
^{13}C NMR DATA (δ) FOR THE RECOVERED PRODUCTS

Compound	C-1	C-2	C-3	C-4	C-5	C-6	C=O	C=O
1-β-Mono-D-fructopyranose laurate	66.7	98.1	69.8	71.4	70.8	64.6	171.6	–
Product	66.5	96.9	69.3	77.5	77.3	65.6	171.2	–
1,6-β-di-D- fructopyranose laurate	66.8	101.7	78.0	77.1	80.2	65.3	174.9	175.4
Product	65.1	100.7	76.4	77.3	79.2	64.9	174.0	174.4

The figure shows that with time, free enzyme and immobilized enzyme activity gradually decreased over a period of 50 days. After 50 days, the free enzyme and immobilized enzyme activities were 40.1 and 70.3 % of the original activity, respectively. The data indicate that immobilized enzyme is more stable at 4 ºC compared to the free enzyme.

Qualitative analysis of the reaction products: The final products were filtered and separated by silica gel column chromatography and two pale yellow oil compounds were recovered. The products were analyzed by TLC and ^{13}C NMR. The results, shown in Fig. 8 and Table-6, indicate that the products were 1-β-mono-D-fructopyranose laurate, with an R_f of 0.44 and 1,6-β-di-D-fructopyranose laurate, with an R_f of 0.79.

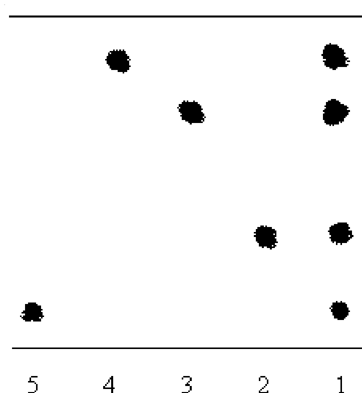


Fig. 8. Thin layer chromatography of the recovered reaction products. (1) Products; (2) mono-fructose laurate; (3) di-fructose laurate; (4) lauric acid; (5) fructose

According to the data presented in Table-6, the ^{13}C NMR data for 1-β-mono-D-fructopyranose laurate and 1,6-β-di-D-fructopyranose laurate agree with the data previously published by Arcos[16].

Conclusion

Lipase-catalyzed esterification of fructose with a fatty acid was performed with a small amount of water adjuvant at atmospheric pressure. Because fructose and fatty acids cannot form a symmetrical phase, sugar ester synthesis is difficult to perform. Additionally, we developed a three-factor orthogonal esterification reaction and optimized the conditions for product formation. The parameters that were manipulated include the substrate ratio (acid/sugar), the amount of molecular sieve (zeolite/solvent) and the amount of enzyme (enzyme/solvent). The reaction was run in 20 mL of methyl ethyl ketone as solvent at 50 ºC with a rotation speed of 180 rpm for 24 h. Our results indicate that the optimal conditions are as follow: 75 g/L of zeolite, a 1.5:1 substrate ratio and 10 g/L of enzyme. The maximal relative conversion rate of lauric acid was 60 % (calculated by fructose laurate diester) and our data indicate that the D301 resin immobilized lipase was less active than the Novozym 435 enzyme. However, the immobilized lipase is economically cheaper, has an increased stability and tolerates storage conditions better than the free enzyme. Thus, the immobilized enzyme may be a more cost-effective approach that can be applied to a wide range of applications. The reaction products were analyzed by TLC and ^{13}C NMR and the results indicate that the products were 1-β-mono-D-fructopyranose laurate (R_f of 0.44) and 1,6-β-di-D-fructopyranose laurate (R_f of 0.79).

ACKNOWLEDGEMENTS

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