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ORIGINAL ARTICLE

Esterification for butyl butyrate formation using *Candida cylindracea* lipase produced from palm oil mill effluent supplemented medium

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Abstract The ability of *Candida cylindracea* lipase produced using palm oil mill effluent (POME) as a basal medium to catalyze the esterification reaction for butyl butyrate formation was investigated. Butyric acid and *n*-butanol were used as substrates at different molar ratios. Different conversion yields were observed according to the affinity of the produced lipase toward the substrates. The *n*-butanol to butyric acid molar ratio of 8 and lipase concentration of 75 U/mg gave the highest butyl butyrate formation of 63.33% based on the statistical optimization using face centered central composite design (FCCCD) after 12 h reaction. The esterification potential of the POME based lipase when compared with the commercial lipase from the same strain using the optimum levels was found to show a similar pattern. It can be concluded therefore that the produced lipase possesses appropriate characteristics to be used as a biocatalyst in the esterification reactions for butyl butyrate formation.

1. Introduction

Lipase (EC 3.1.1.3) is an important industrial enzyme originally characterized by the ability to hydrolyze triglycerides at the oil–water interface, this enzyme also catalyzes a number of useful reactions, such as esterification, transesterification, acidolysis, alcoholysis, aminolysis and resolution of racemic mixtures (Gandhi, 1997; Reetz, 2002; Villeneuve et al., 2000). Microbial lipases are endowed with interesting characteristics such as action under mild conditions, stability in organic

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solvents as well as high substrate specificity (Snellman et al., 2002).

Lipases from different sources show different substrate specificities, however for commercial utilization of a specific microbial lipase, it is important to achieve high yield with higher activity and good stability in various solvent systems. Thus, good stability of the lipase helps in its exploitation in various fields of application, and also aids in extending its shelf half-life and its use cycles (Shu et al., 2010). Based on this, lipase catalyzed reactions do not require homogeneous lipase preparations; instead a certain degree of purity enables efficient and successful usage (Saxena et al., 2003).

Lipase-catalyzed reactions (e.g. esterification) have several benefits over the chemically catalyzed reactions. The most notable one is the transformation at moderate temperature and pressure under neutral pH conditions with no unwanted side reactions (Rajendran et al., 2009). Also, using lipases to carry out the esterification reaction lessens the need for several post-reaction separation processes, which can contribute in reducing the overall operating costs (Yahya et al., 1998). Lipases have been efficiently utilized for direct esterification and transesterification reactions in organic solvents to produce esters of glycerol (Chang et al., 1999), aliphatic alcohols (Abbas and Comeau, 2003), terpenic alcohols (Yadav and Lathi, 2004) such as amyl isobutyrate (Bezbradica et al., 2006), ethyl valerate (Salina et al., 2011), ethyl esters (Sun et al., 2009), glyceride of oleic acid (Kaushik et al., 2010), among others. Lipase from *Candida cylindracea* has been found to be among the most versatile biocatalysts owing to its high activity both in hydrolysis and synthesis (Linko and Wu, 1996; Wong et al., 2000). Thus, several studies proved that the enzyme based technology especially for the production of esters can be used to replace chemical processes both theoretically and practically (Babu and Divakar, 2001; Deng et al., 2003).

Esters of short chain fatty acids and alcohols such as butyl butyrate are important components of natural aromas that have been extensively used as flavor components in the food, beverage and pharmaceutical industries (Santos and de Castro, 2006). Based on these demands and benefits, this study explores the esterification reaction for butyl butyrate formation using *C. cylindracea* lipase that was produced using an inexpensive medium containing palm oil mill effluent. This can justify the development of this medium for the production of lipase if the produced enzyme has characteristics suitable for various applications, since most lipase catalyzed synthesis of different flavor components are performed by commercially available lipase preparations. Thus, attempt was made to optimize the *n*-butanol to butyric acid molar ratio and lipase concentration using face centered central composite design (FCCCD) in order to obtain the highest ester yield.

2. Materials and methods

All reagents used in this study were purchased from Sigma–Aldrich (USA) and were of analytical grade. Palm oil mill effluent (POME) was collected from West Oil Mill of Sime Darby Plantation Sdn. Bhd., Carey Island, Malaysia in clean containers and immediately brought to the laboratory and stored at 4 °C. *C. cylindracea* ATCC 14830 was obtained from American Type culture collection. The strain was grown on potato dextrose agar plates at 28 °C for 4 days and subcultured every 2 weeks. It was then maintained and preserved at 4 °C.

2.1. Production of lipase in palm oil mill effluent (POME) based medium

The fermentation medium used in this study was based on our previous study (Salihu et al., 2011a), containing POME sample of 1.0% (w/v) total suspended solids (TSS) supplemented with 0.2% (v/v) olive oil, 0.65% (v/v) Tween-80 and 0.45% (w/v) peptone, adjusting the initial pH to 6.0. The fermentation was carried out in 2-L Biostat (Sartorius BBI Systems) bioreactor filled with 1-L of POME based medium and sterilized in situ at 121 °C and 15 psi for 15 min. The bioreactor was inoculated with 2.2% (v/v) of actively growing cells of *C. cylindracea* (10^8 cells/ml) from 48 h-Erlenmeyer flask cultures. Standard operating conditions (temperature, agitation and aeration) were set based on the developed process optimization by full factorial design using an incubation period of 36 h (Salihu et al., 2011b).

2.2. Determination of lipase activity and protein concentration

Lipase activity was carried out according to the method reported by Gopinath et al. (2005). The cell free extract (culture supernatant) was used as a source of crude extracellular lipase and assayed quantitatively using *p*-nitrophenyl palmitate (*p*NPP) as the substrate. One lipase unit (U) was defined as the amount of enzyme that liberated 1 μmol *p*-nitrophenol per milliliter per minute under the standard assay conditions. Extracellular protein was determined using the Bradford method (1976). Bovine serum albumin (BSA) standard curve was used in estimating the protein concentration.

2.3. Lipase-catalyzed butyl butyrate formation

Reaction mixture was prepared according to Kiran et al. (2000), which consists of butyric acid (160 mM) and *n*-butanol (320 mM) in *n*-heptane and incubated at 35 °C with a specific lipase concentration for definite periods of incubation. The mixture was stirred in an orbital shaker at 150 rpm. This was then followed by addition of methanol (2 ml) as a quenching agent. Decrease in butyric acid content was measured by titrating the reaction mixture against 20 mM NaOH using phenolphthalein indicator. The progress of esterification was monitored by determining the residual acid content and the yield was calculated as described by Deng et al. (2003) using the equation:

$$Y = \frac{M\alpha - M}{M\alpha - M\beta} \times 100 \quad (1)$$

where Y is the yield of butyl butyrate, Mα and M are amounts of NaOH consumed by titration of the mixture at the beginning (0 h) and the end of the reaction respectively, and Mβ is the amount of NaOH consumed by titration of the mixture without butyric acid. The conversion percentage obtained by gas chromatography (based on direct product formation) and titrimetric analyses (estimation based on residual acid content) were found to be in good agreement.

2.4. Effects of molar ratio, agitation and lipase concentration

One of the common ways of increasing the yield of the esterification process is to determine the appropriate molar ratio, effective mixing conditions of the substrates as well as the lipase concentration. Thus, the effects of *n*-butanol to butyrate molar ratio (1–8), agitation speed from 50 to 200 rpm and the enzyme concentrations (30, 45, 60, 75 and 90 U/mg) on final yield of the product were investigated.

2.5. Optimization of butyl butyrate formation using design of experiment

Response surface methodology (RSM) developed by the Design Expert software (Version 6.0.8, Stat-Ease Inc., Minneapolis, USA) was used to optimize the two significant factors (lipase concentration and molar ratio) in a set of 11 experimental runs with three center points (Table 1).

The ranges of the independent variables investigated in this study include lipase concentration (45–75 U/mg) and molar ratio of *n*-butanol to butyric acid of 4.0–8.0.

All the experiments were carried out in triplicate and the butyl butyrate yield (Y) was taken as the response of the experimental design.

The relationship between dependent and independent variables is explained by the second order polynomial equation:

$$Y = \beta_0 + \beta_1 X_1 + \beta_2 X_2 + \beta_{12} X_1 X_2 + \beta_{11} X_1^2 + \beta_{22} X_2^2 \quad (2)$$

where Y is the dependent variable (butyl butyrate formation); X_1 and X_2 are independent variables (molar ratio and lipase concentration respectively); β_0 is an intercept term; β_1 and β_2 are linear coefficients; β_{12}, is the interaction coefficient; and β_{11}, β_{22} are the quadratic coefficients.

Statistical analysis of the model was performed to evaluate the analysis of variance (ANOVA). This analysis includes Fisher's *F*-test (overall model significance), its associated probability values and coefficient of determination R^2 which measures the goodness of fit of regression model.

2.6. Validation and comparison of the esterification process

The statistical model was validated with respect to the variables within and outside the design space. These sets of experimental combinations were also used to compare the produced lipase with the commercial lipase (62316-Sigma USA) from the same strain.

3. Results and discussion

3.1. Effects of molar ratio, agitation and lipase concentration

One of the most important variables affecting the percentage conversion of reactants during the esterification process is the nature of substrates. In the solvent-free system, the esterification was carried out with various molar ratios of *n*-butanol to butyric acid (ranging from 1 to 8) at a fixed enzyme concentration of 30 U/mg. As shown in Fig. 1, the esterification yield continued to increase with increasing molar ratio and the highest formation of butyl butyrate (29%) was obtained at *n*-butanol to butyric acid molar ratio of 8 after 12 h of incubation. Although several researches show some inhibitory effects of high concentration of *n*-butanol during the esterification reactions (Dossat et al., 2002; Leitgeb and Knez, 1990; Ghamgui et al., 2004), in this study high molar ratio resulted in the highest conversion percentage. This is because the nature of each ester to be produced determines the extent of molar ratio of acid to alcohol to be used. Similarly, during the synthesis of cetyl oleate, excess of alcohol accelerated the ester synthesis and the highest rates were observed at acid/alcohol ratio of 1:10 (Garcia et al., 2000). However, in the case of amyl acetate, excess of acid drastically decreased the yield of esters, while the acid to alcohol ratio of 1:2 was found to be the best in terms of amyl acetate formation (Bezbradica et al., 2006). Also, Baron et al. (2005) showed the esterification reaction of lipase produced by *P. corylophilum* for *n*-butyl oleate synthesis, where a molar ratio of 3:1 of *n*-butanol and oleic acid respectively was found to be appropriate for the reaction.

Agitation insures efficient contact of the enzyme–substrate mixture during the esterification process. Thus, agitation speeds of 50–200 rpm were investigated and the maximum esterification yield was found at 150 rpm (Fig. 2). Linko and Wu (1996) reported the use of 200 rpm in a reaction mixture containing deionized water, l-butanol, oleic acid (molar ratio of acid to alcohol was 1:5) and *Candida rugosa* lipase (980 U) at 37 °C for l-butyl oleate synthesis. When *Candida* sp. 99-125 was used for fatty acid alkyl ester synthesis,

Table 1 Experimental design using FCCCD of two independent variables showing the actual and coded values with three center points and the yield of butyl butyrate as the response.

Run	Molar ratio	Enzyme concentration (U/mg)	Esterification yield (butyl butyrate formation, %)	
			Experimental	Predicted
1	8 (+1)	45 (−1)	14.43	12.37
2	4 (-1)	45 (−1)	28.65	27.29
3	4 (-1)	60 (0)	40.32	44.05
4	6 (0)	60 (0)	51.01	50.10
5	6 (0)	60 (0)	54.88	50.10
6	6 (0)	75 (+1)	48.50	53.94
7	8 (+1)	60 (0)	42.19	47.33
8	6 (0)	45 (−1)	20.81	24.24
9	4 (−1)	75 (+1)	41.15	38.97
10	8 (+1)	75 (+1)	63.33	60.26
11	6 (0)	60 (0)	53.28	50.10

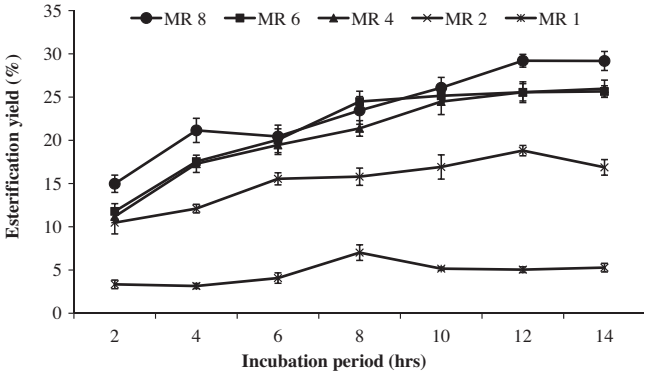

Figure 1 Effects of molar ratio on butyl butyrate formation using the produced lipase (30 U/mg) at different incubation times.

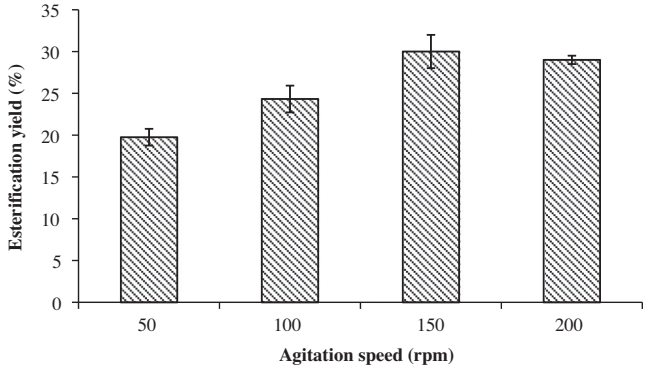

Figure 2 Effects of agitation speed on butyl butyrate formation using the produced lipase (30 U/mg) at molar ratio of 8.

180 rpm was found to be adequate for achieving the highest yield (Deng et al., 2003). However, higher agitation speed of 300 rpm was reported during the esterification reaction of glycerol and glycerol analogs (*n*-propanol, isopropanol, 1,2- and 1,3-propanediols) in organic media by immobilized lipases from *Pseudomonas cepacia* and *C. antarctica* (Chang et al., 1999).

Effect of different lipase concentrations (30, 45, 60, 75 and 90 U/mg) was studied based on the established conditions (agitation of 150 rpm and molar ratio of *n*-butanol to butyric acid of 8), the results showed that the ester yield was found to increase as the concentration of the enzyme increased up to a certain limit as indicated in Fig. 3. The highest yield of butyl butyrate formation was obtained at the enzyme concentration of 75 U/mg, indicating that a further increase in enzyme concentration did not result in higher ester yield. This could be attributed to the saturation of all the binding sites of the enzyme at this level. Also, this is in agreement with what has been observed during the esterification of butyl oleate by an immobilized *Rhizopus oryzae* lipase, where a remarkable increase was observed when the amount of lipase was 60 U; suggesting that the amount of enzyme is the rate determining factor, since a further increase in enzyme concentration above 60 U did not improve the esterification process (Ghamgui et al., 2004). Similarly, Santos and de Castro (2006) studied the effects of temperature (37–50 °C), substrate molar ratio of butyric acid to *n*-butanol (0.6–2.0) and enzyme amount (0.2–0.4 g with specific

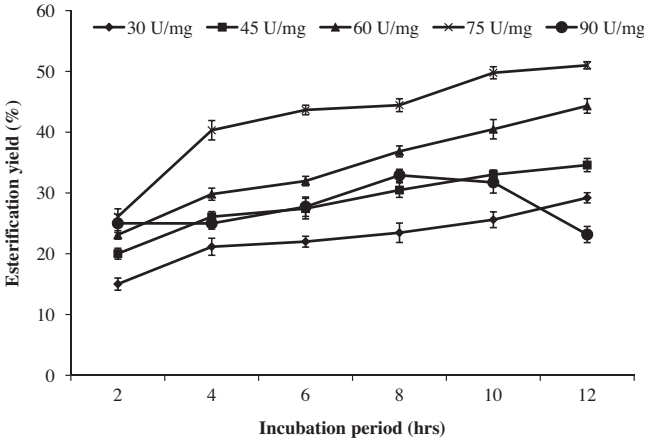

Figure 3 Effects of enzyme concentration on butyl butyrate formation based on the established molar ratio of 8, agitation speed of 150 rpm and incubation period of 12 h.

activity of 75 U/mg) on the ester yield. The mathematical model obtained based on factorial design showed that 41 °C and 0.4 g (30,000 U) of lipase were the optimal conditions that led to a yield of 75% of the ester (butyl butyrate). Thus, in most cases having attained the optimum no significant improvement was observed.

3.2. Statistical optimization of the esterification reaction for butyl butyrate formation

In order to determine the effectiveness of the esterification reaction for butyl butyrate formation using the produced enzyme, FCCCD was employed in the present work to optimize the molar ratio and enzyme concentration for better ester yield.

The experimental matrix of the design was conducted in 11 experimental runs as shown in Table 1, and the formation of butyl butyrate was found to be strongly dependent on the selected factors. The degree of esterification (Y) widely varied from 14.43 to 63.33, where the lowest and highest yields were realized in run 1 and 10 respectively. The experimental results of FCCCD were fitted into a quadratic polynomial equation which showed the dependence of the esterification yield as a function of the molar ratio and the enzyme concentration. The degree of esterification for butyl butyrate formation could be predicted by the model equation:

$$Y(\text{butyl butyrate formation}) = +50.10 + 1.64A + 14.85B - 4.41A^2 - 11.02B^2 + 9.10AB \quad (3)$$

where A and B are the molar ratio of *n*-butanol to butyric acid and the enzyme concentrations respectively.

The summary of analysis of variance for the model is represented in Table 2. The values of F-statistic and p-value were found to be 15.57 and 0.0045 respectively. This implies that the quadratic model is significant. The predicted R^2 of 0.9397 indicated a good agreement between the experimental and predicted values for the ester formation. The adjusted R^2 corrects the R^2 values for the sample size and for the number of terms in the model. In this case, the adjusted R^2 value

Table 2 ANOVA of quadratic model for the esterification (butyl butyrate formation).

Source	Sum of squares	*F*-value	*p*-value	
Model	2124.61	15.57	0.0045	Significant
Molar ratio, *A*	16.10	0.59	0.4771	
Enzyme amount, *B*	1322.61	48.47	0.0009	
A^2	49.35	1.81	0.2365	
B^2	307.43	11.27	0.0202	
AB	331.09	12.13	0.0176	
Lack of fit	128.87	11.36	0.0820	Not significant

R^2 = 0.9397; Adj R^2 = 0.8793; CV = 12.53 and adequate precision = 12.41.

(0.8793) was found to be close to the R^2 value. Adequate precision ratio of 12.41 indicates an adequate signal. A ratio greater than 4 is desirable. Adequate precision measures the signal to noise ratio. A relatively lower value of the coefficient of variation (CV = 12.53) indicated a good precision and reliability of the experiment.

To determine the optimal levels of each variable, the graphical representation of the regression equation in the form of three dimensional response surface plots was constructed by plotting the response on the *Z*-axis against the two independent variables (molar ratio and lipase concentration) as shown in Fig. 4. An increase in esterification yield was observed when the lipase concentration or molar ratio increased, indicating the interaction of the two in enhancing the yield up to a certain limit.

The esterification yield (butyl butyrate formation) obtained in this study was found to be promising based on the literature reports of different lipase-catalyzed synthesis of esters using free and immobilized enzyme systems. Babu and Divakar (2001) studied the synthesis of esters of anthranilic acid using

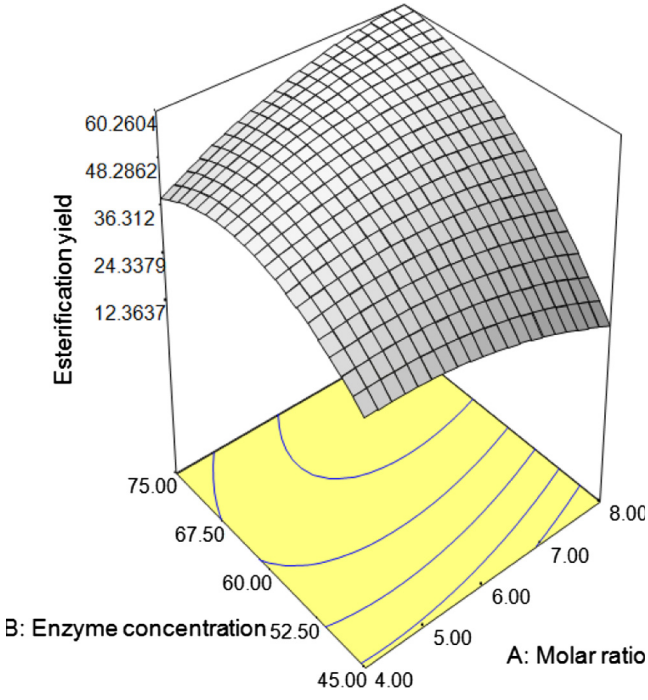

Figure 4 3D response surface plot showing the interaction of the lipase concentration and molar ratio on butyl butyrate formation.

the Plackett–Burman experimental design. Among all alcohols (C_1–C_{18}), the esterification with methanol gave the highest ester yield at 45.6%. In case of ethyl valerate (a green apple flavor), statistical optimization using RSM was carried out. The highest yield of ethyl valerate was found to be 84.28% under the optimum conditions of temperature, enzyme amount (Novozym 435 from *C. antarctica* lipase) and agitation speed of 30 °C, 25% (w/w) and 51 rpm respectively (Salina et al., 2011). During the synthesis of citronellol laurate, 53% conversion was achieved using immobilized lipases (Novozym SP 435) following the optimization of process conditions (Yadav and Lathi, 2004). Using lyophilized lipase from *Rhizopus chinensis*, high yields of caprylic acid esters was achieved in the presence of ethanol, *n*-propanol and *n*-hexanol, with conversion of 92%, 93% and 92% respectively after 20 h of incubation (Sun et al., 2009). Also, *Aspergillus terreus* lipase showed higher potential for solvent free synthesis of partial glycerides of oleic acid with 96% efficiency based on incubation period of 12 h (Kaushik et al., 2010).

3.3. Validation and comparison of the butyl butyrate formation of the produced and the commercial lipase (62316-Sigma USA)

To check the adequacy of the model equation, some sets of confirmation experiments were carried out within and outside the design space based on the established optimum conditions by the FCCCD. Also, comparison of esterification yields was made between the produced and commercial lipases through five sets of experiments (Table 3).

Based on this, it was found that an increase in molar ratio in the case of the produced enzyme did not result in higher esterification yield, instead it resulted in lower yield while the commercial enzyme showed higher esterification yield (butyl butyrate formation) in all the experiments considered in this study. The highest esterification yield of 84.22% was realized using the commercial lipase at molar ratio and enzyme concentration of 10 and 75 U/mg respectively compared to the yield of 60.34% by the produced enzyme under the same condition (Table 3).

Thus, Yadav and Lathi (2003) proposed a mechanism during the esterification reaction that a nucleophilic substrate may act as an inhibitor by forming a non-reactive dead-end complex between an enzyme and an alcohol. This could likely be the effect that resulted in decreased esterification yield of the produced enzyme as a result of the increase in molar ratio.

It is not surprising that the yield of butyl butyrate was found to be lower using the produced enzyme; this is because the lipase catalytic efficiency in organic solvents is in most

Table 3 Validation and comparison of the experimental model.

Run	Molar ratio	Enzyme concentration (U/mg)	Esterification yield (butyl butyrate formation, %)	
			Produced lipase	Commercial lipase
1	8	67.5	54.02	68.34
2	8	75	63.33	80.06
3	7	60	57.13	62.41
4	10	75	60.34	84.22
5	9	75	60.57	83.98

cases, in the orders of magnitude lower than in aqueous systems. This behavior can be related to different causes as described by Liu et al. (2010), such as diffusion limitations, high saturating substrate concentrations, restricted protein flexibility, low stabilization of the enzyme substrate intermediate and even partial enzyme denaturation which become irreversible in anhydrous solvents.

4. Conclusion

In this study, the major parameters affecting the esterification reaction of the produced lipase for butyl butyrate formation were investigated. Among the three parameters analyzed, *n*-butanol to butyric acid molar ratio and lipase concentration were subjected to statistical optimization using the FCCCD. The highest esterification yield of 63.33% was obtained at molar ratio of 8 and lipase concentration of 75 U/mg by the produced lipase. In case of the commercial lipase, molar ratio of 10 and enzyme concentration of 75 U/mg led to the highest conversion of 84.22%. Although in all cases the commercial lipase showed to some extent slightly higher conversion percentages but overall the produced lipase was found to be promising in terms of the esterification reaction in solvent free system. Based on this, it is expected that the produced enzyme may exhibit a higher esterification potential when immobilized and/or lyophilized.

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