Journal of Molecular Catalysis B: Enzymatic 90 (2013) 114–117

Contents lists available at SciVerse ScienceDirect

Journal of Molecular Catalysis B: Enzymatic

journal homepage: www.elsevier.com/locate/molcatb

Studies on the lipase-catalyzed esterification of alkyl oleates in solvent-free systems

Hui Zhong[a], Zheng Fang[b], Baohua Zou[b], Xin Li[a], Pingkai Ouyang[a,c], Kai Guo[a,*]

[a] College of Biotechnology and Pharmaceutical Engineering, Nanjing University of Technology, Nanjing 211816, PR China
[b] School of Pharmaceutical Sciences, Nanjing University of Technology, Nanjing 211816, PR China
[c] National Engineering Research Center for Biotechnology, Nanjing University of Technology, Nanjing 211816, PR China

ARTICLE INFO

Article history:
Received 11 October 2012
Received in revised form 10 January 2013
Accepted 14 January 2013
Available online 24 January 2013

Keywords:
Enzymatic esterification
Candida sp. lipase
Oleates

ABSTRACT

The alkyl oleates were prepared by esterification of oleic acid with alkyl alcohols catalyzed by the lipase from *Candida* sp. 99-125 in solvent-free system. The influence of several factors, including enzyme concentration, temperature, molar ratio between oleic acid and alkyl alcohols, the structure of alcohols and water content, was also investigated. The results indicated that the reactions catalyzed by *Candida* sp. lipase at 20 °C, in the presence of 5% (w/w) lipase, on the molar ratio of 1:1 between oleic acid and alcohols, afforded products in high yield and showed high selectivity to primary alcohols. The enzymatic synthesis gave purer products, compared with the conventional chemical synthesis. The lipase from *Candida* sp. 99-125 was identified to be an effective catalyst in the esterification of alcohol and oleic acid at low temperature.

1. Introduction

Recently, fatty acid esters, which have surfactant and combustible properties, have received widespread attention from scientists due to their wide applications in cosmetics, pharmaceutics as well as food and chemical industries [1]. Especially, biodiesels, which is defined as the mono-alkyl esters of long chain fatty acids, have received an increasing attention from researchers with the global shortage of fossil fuels, the increasing price of crude oil and environmental pollution concerns. Nowadays, the majority of biodiesel is produced by base-catalyzed trans-esterification of edible oils with methanol, which has some shortage such as the complicated procedure, more methanol content, hard to reclaim, and environmental pollution [2]. Therefore, the lipase-catalyzed synthesis has become a promising method to synthesize biodiesels owe to the advantages of mild conditions, simplified downstream processing, high region- and stereo-selectivity, low energy consumption, and environmental friend over the chemical catalysis [3].

The lipase-catalyzed syntheses of oleates were reported by several groups. In 1996, the lipase-catalyzed syntheses of fructose oleates and sucrose fatty acid esters have been reported by Ghoul's group and Vulfson's group, respectively [4,5]. Ferrer et al. described the region-selective synthesis of fatty acid esters of maltose, leucrose, maltotriose and *n*-dodecyl maltosides in 2000 [6]. In 2006, Tan et al. also synthesized 2-ethylhexyl palmitate via an immobilized lipase membrane reactor [7]. The most recently reported work of the enzymatic synthesis is the syntheses of fructose, sucrose and lactose esters from the corresponding sugars using *Candida antarctica* type B lipase immobilized in two different supports, namely acrylic resin and chitosan by Goncalves' group this year [8]. Due to the limitation of enzyme catalyzed reactions including high cost of enzyme, low yield, a long reaction time, the demand for investigation on the details of the enzymatic synthesis was still pressing. Herein, we report the esterification using one cheap lipase from *Candida* sp. 99-125 as an efficient catalyst at 1:1 reactant ratio at low temperature. In addition, the full investigation and optimization of the esterification are performed.

2. Experimental

2.1. Materials and reagents

The lipase from *Candida* sp. 99-125 was presented by Beijing University of Chemical Technology. Oleic acid was purchased from Shanghai Lingfeng Chemical Reagent Co., Ltd. All reagents, including methanol, ethanol, *i*-propanol, *n*-butanol, and *n*-octanol, were of analytical grade and purchased from Shanghai Lingfeng Chemical Reagent Co., Ltd.

Chromaticity was recorded on the PFX-i Series SpectroColorimeter from Tintometer Ltd., Amesbury, SP4 7SZ, UK.

* Corresponding author. Tel.: +86 25 58139926.
E-mail address: xinxin2019@hotmail.com (K. Guo).

1381-1177/$ – see front matter © 2013 Elsevier B.V. All rights reserved.
http://dx.doi.org/10.1016/j.molcatb.2013.01.013

2.2. Reaction procedures

2.2.1. General procedures of esterification

All the esterification experiments were carried out in a 50 mL round bottom flask. The reaction procedure was described as follows: To a mixture of 0.05 mol oleic acid and corresponding alcohol, catalyst was added at the given temperature. Normally, the reactants mixture kept stirring for 24 h until the reaction completed. The samples were taken out every 1 h in the first 12 h, and the acid value was determined according to the standard GB1668-81. In the end, the chromaticity of the oleates was recorded on PFX-i Series from Tintometer Ltd.

2.2.2. Analytical methods

2.2.2.1. Determination of acid number. The ester content was quantified by calculating the residual fatty acid amount in the reaction mixture. Using a volumetric method (standard GB1668-81), a 0.2–0.3 g sample of the reaction mixture was diluted in 20 mL of 0.1% (w/w) phenolphthalein solution in absolute ethanol and titrated with standardized potassium hydroxide solution in water. The acid number (AN) was calculated from the equation:

$$\mathrm{AN} = \frac{56.1 \times V \times N}{W}$$

V, volume of NaOH, (mL); N, molarity concentration of titrant (mol/L); W, weight of the sample in grams.

2.2.2.2. Calculation of esterification rate.

$$\text{esterification rate}\,(\%) = \frac{\mathrm{AN}_{\mathrm{org.}} - \mathrm{AN}_{\mathrm{eq}}}{\mathrm{AN}_{\mathrm{org.}}} \times 100\%$$

$\mathrm{AN}_{\mathrm{org.}}$, acid number at the starting point; $\mathrm{AN}_{\mathrm{eq}}$, acid number at the checking point.

3. Results and discussion

3.1. Effect of lipase concentration

The investigation of the lipase concentration influence on the esterification rate was performed by varying the concentration of lipase in the range between 0.5% and 10% (w/w of substrates) in the reaction bulk of 0.05 mol oleic acid and 0.15 mol ethanol at 30 °C, with magnetic stirring rate of 400 rpm. As shown in Fig. 1, the lipase concentration affected the initiate rate as well as the final conversion. This could be explained by the increase in amount of the substrate bonded to the enzyme with the increase in enzyme amount. When the lipase concentration was higher than 5%, the increase of conversion was not remarkable as not all the enzyme particles were exposed to the substrates and the excess of enzyme present in the reaction mixture was not actively involved in the reaction, consistent with other reports [9]. The conversion of esterification completed in 4 h and 2 h, at the lipase concentration of 5% (w/w) and 10% (w/w) respectively. Considering the price of lipase, it was concluded that the 5% (w/w of substrates) was the optimal concentration for the esterification. Operating at this condition a total esterification percentage of about 55% after 24 h of bioconversion was attained. Therefore, all further studies in this work were performed at this enzyme concentration.

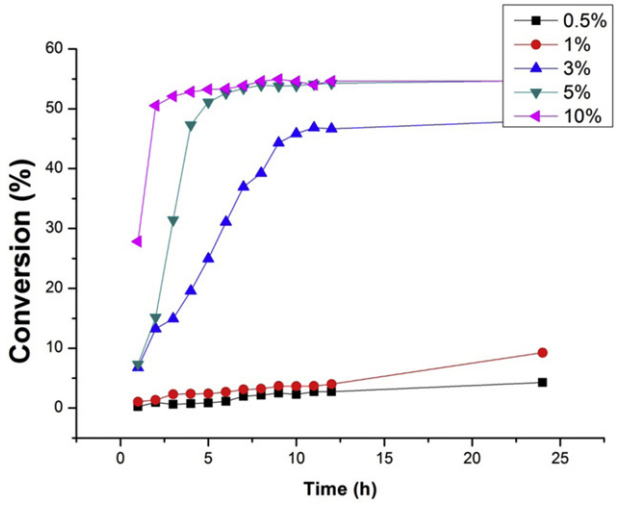

Fig. 1. Effect of lipase concentration on esterification rate. (■) 0.5% (w/w); (●) 1% (w/w); (▲) 3% (w/w); (▼) 5% (w/w); (◄) 10% (w/w).

3.2. Effect of temperature

It is well-known, that temperature is an important parameter affecting reaction rate, enzyme activity and chemical equilibrium. The effect of temperature was studied by varying the temperature in the range between 10 °C and 60 °C, with the lipase concentration of 5% (w/w) and acid/alcohol ratio of 1:3, at the rate of 400 rpm stirrer speed. The results are illustrated in Fig. 2. In contradiction to the generally reported literatures [7,10,11], the lipase from *Candida* sp. 99-125 had high catalytic activity at 20 °C instead of 40 °C. However, the results were in good agreement with the Tan's report [12] in which the optimal temperature for the immobilized lipase from *Candida* sp. 99-125 ranged from 15 °C to 25 °C. All the experiments here were carried out three times to show the reproducibility. The result suggested that the lipase from *Candida* sp. 99-125 had high catalytic activity at room temperature, which means it would have a wider application in industry. Additionally, the esterification at 10 °C was also conducted, which resulted in a low conversion due to the coagulation of oleic acid. Therefore, the further reactions were operated at 20 °C.

3.3. Effect of the molar ratio between oleic acid and alcohols

Acid/alcohol molar ratio is one of the most important parameters in enzymatic esterification. Since the reaction is reversible, an increase in the alcohol concentration should result in higher ester yields and shift the chemical equilibrium toward the ester

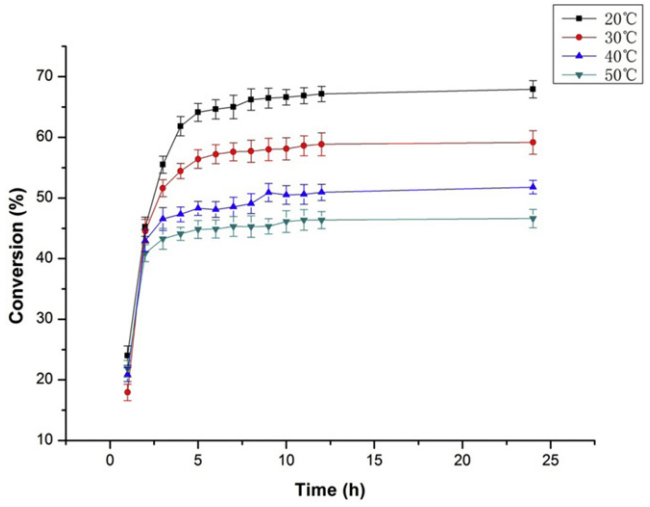

Fig. 2. Effect of temperature on the esterification. Oleic acid, 0.05 mol/L; ethanol alcohol, 0.15 mol/L; lipase, 5%; speed, 400 rpm. (■) 20 °C; (●) 30 °C; (▲) 40 °C; (▼) 50 °C.

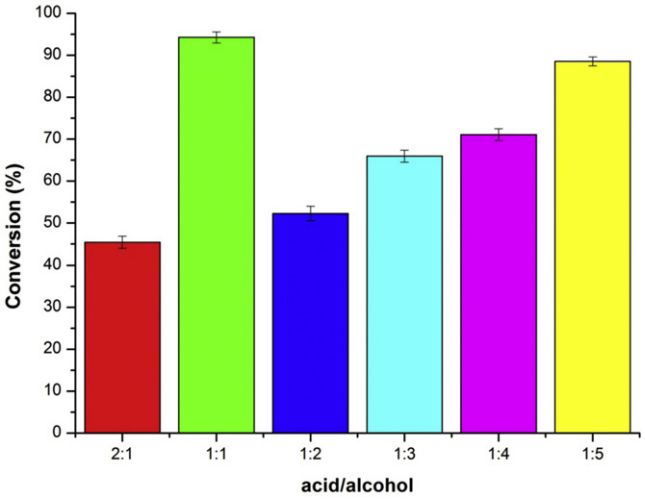

Fig. 3. Effect of the ratio of acid/alcohol on the esterification. Lipase, 5%; temperature, 20 °C; speed, 400 rpm.

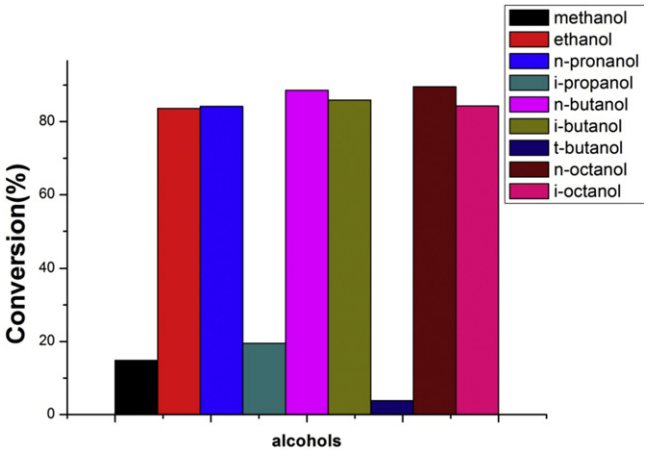

Fig. 4. Effect of alcohol structure on the esterification.

synthesis. However, high substrates concentration may slow down the reaction rates due to inhibition to the enzyme.

To investigate the effect of molar ratio between oleic acid and alcohols, the reactions were run by varying the amount of ethanol, which was supposed to be an inhibitor to the lipase from *Candida* sp. 99-125, whilst keeping a constant amount of oleic acid of 0.05 mol, with magnetic stirring rate of 400 rpm at 20 °C, in the presence of 5% (w/w) lipase. The results are demonstrated in Fig. 3. The esterification of an equimolar solution of oleic acid and ethanol reached to the chemical equilibrium fast and afforded the highest conversion of 94%, compared with 66% conversion of the esterification at the reported optimum ratio of 1:2 [13]. It could be explained as following description. As we know, an increase in amount of alcohol will shift the chemical equilibrium to the product point as well as increase the reaction rate. These manifest themselves as an increased conversion of esterification over a specified reaction time. Meanwhile, an increased amount of ethanol will decrease the reaction rate due to the inhibition on the enzyme. It manifests itself as the decreased conversion of esterification for a specified period of reaction. At the low ethanol concentration, inhibition of alcohol to the enzyme predominate the reaction. While the ethanol concentration increased, the contribution of alcohol to the reaction equilibrium dominated the reaction. The esterification at 1:1 the acid/alcohol ratio gave the highest conversion, because the enzyme had the highest activity without inhibition from excess alcohol. As the acid/alcohol ratio decreased till to 1:4, the conversion decreased due to the inhibition. When ratio of acid/alcohol decreased to 1:5, the conversion was similar with that of 1:1 acid/alcohol. However, it is obvious that reaction with 1:1 acid/alcohol ratio has huge advantages for industrial cost saving.

3.4. The effect of alkyl chain of alcohols

The influence of alcohol structure on the esterification was studied via the esterification of oleic acid and alkyl alcohols, including methanol, ethanol, propanol, *i*-propanol, *n*-butanol, and *n*-octanol, at the ratio of 1:1, with 5% lipase at 20 °C. The results are demonstrated in Fig. 4. It was found that straight long-chain contributed to the reaction well. As shown in Fig. 4, the esterification gave much lower conversion starting from *i*-propanol and *t*-butanol compared with the others. It is deduced that the primary alcohols, which have less hindrance to the nucleophilic hydroxyl group, benefited the esterification. Also, the activity of the enzyme was elevated with the increasing length of carbon chain, as shown in Fig. 4. As we know, short chain alcohols, such as methanol and ethanol are toxic to the lipase. However, the synthesis of ethyl oleate catalyzed by the lipase from *Candida* sp. 99-125 was reported by Tan's group [12]. It is deduced that ethanol mere inhibits the lipase from *Candida* sp. 99-125 but no deactivate, so does methanol. The lipase from *Candida* sp. 99-125 was higher sensitive to methanol compared with ethanol. This could explain why the esterification between oleic acid and methanol afford a low conversion while that between oleic acid and ethanol gave a high conversion.

3.5. The effect of water content

Water plays an important role in the enzyme-catalyzed esterification because of the following reasons: Firstly, proper protein hydration state contributes to the enzyme structural integrity, active site polarity and protein stability [14,15]. Secondly, water produced in the esterification affects the reaction equilibrium position since it is one of the two products in the reaction. The comparison of free powdered-lipase-catalyzed reaction in the optimal condition above with that in the presence of molecular sieve was performed, with the results as shown in Fig. 5. It indicated that the molecular sieves removed the water produced in the reaction and contributed the esterification.

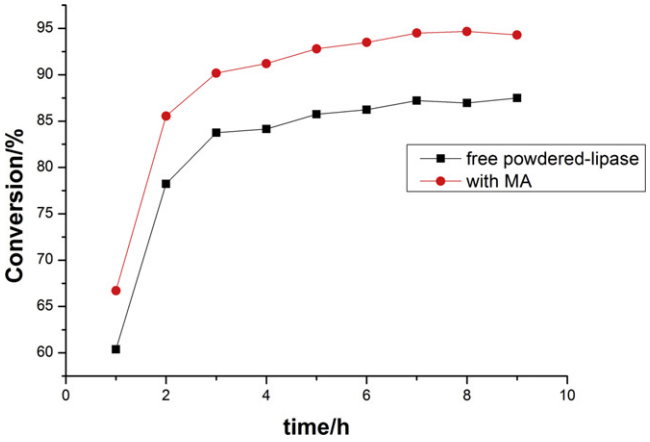

Fig. 5. Effect of water content on esterification between oleic acid and *n*-butanol with 1:1 acid–alcohol molar ratio, 5% enzyme, 20 °C.

Table 1
Comparison between enzymatic esterification and chemical esterification.

Method	Chemical method				Enzymatic method	
Catalyst	*p*-TsOH	$MeSO_3H$	$NaHSO_4{\cdot}H_2O$	Tf-OH	Powdered lipase	Novozyme 435
Conversion of acid, %	87	88	54	94	96	81
Chroma	87.7	>505	120.7	133.5	67.6	70.2

3.6. Comparison of the chemical synthesis and enzymatic synthesis of ethyl oleate

As known, the enzymatic catalysis has advantages of mild condition, high selectivity and high conversion. In order to compare the acid-catalyzed synthesis of ethyl oleate with the lipase-catalyzed synthesis in the optimal condition above, the chromaticity of the alkyl oleate produced were determined. The results were shown in Table 1, the chromaticity of ethyl oleate by lipase-catalyzed synthesis was much lower than that by chemical synthesis, which means that some impurities were introduced in downstream of chemical synthesis. And the esterification catalyzed by Novozyme 435 was also performed, employing a 5% lipase loading, with 1:1 acid/alcohol ratio at 20 °C, which is equal to the optimal condition of lipase from *Candida* sp. 99-125. As shown in Table 1, esterification catalyzed by the lipase from *Candida* sp. 99-125 afforded high conversion and purer oleates.

4. Conclusions

Alkyl oleates were successfully produced via enzymatic synthesis over the commercially available lipase from *Candida* sp. 99-125. The effects of several parameters on the reaction, including lipase concentration, substrate ratio, temperature, the structure of alcohols, and water content, were investigated. The optimal conditions were described as follows: 5% (w/w) lipase with molecular sieves, acid/alcohol molar ratio 1:1, 20 °C. The mild reaction condition means the effective cost-saving in further industrial production. Additionally, it was found that the lipase from *Candida* sp. 99-125 preferred primary alcohols and molecular sieves contributed the reaction conversion. Finally, the comparison of chromaticity of the oleates produced by chemical synthesis with that by enzymatic synthesis indicated that the enzymatic catalysis afforded purer oleates. Further investigations on the immobilized-lipase-catalyzed synthesis of oleates will be performed to gain deeper insight into esterification catalyzed by lipase from *Candida* sp. 99-125.

Acknowledgements

This work was supported by a grant from the Major State Basic Research Development Program of China (1073 Program) (Nos. 2012CB725204 and 2011CB710803) and the Program for Changjiang Scholars and Innovative Research Team in University (No. IRT1066).

References

[1] X.L. He, B.Q. Chen, T.W. Tan, J. Mol. Catal. B: Enzym. 18 (2002) 333.
[2] D. Li, T.W. Tan, F. Wang, Chin. J. Biotechnol 19 (2003) 97.
[3] T. Kobayashi, Biotechnol. Lett. 33 (2011) 1911.
[4] D.B. Sarney, M.J. Barnard, D.A. MacManus, E.N. Vulfson, J. Am. Oil Chem. Soc. 73 (1996) 1480.
[5] R.H. Valivety, G.A. Johnston, C.J. Suckling, P. Hailing, J. Biotechnol. Bioeng. 38 (1991) 1137.
[6] M. Ferrer, M.A. Cruces, F.J. Plou, M. Bernabé, A. Ballesteros, Tetrahedron 56 (2000) 4053.
[7] T. Tan, B. Chen, H. Ye, Biochem. Eng. J. 29 (2006) 41.
[8] Gonçalves, Food Hydrocolloids 27 (2012) 324.
[9] N.N. Gandhi, S.B. Sawant, J.B. Joshi, Biotechnol. Bioeng. 46 (1995) 1.
[10] Y. Tao, B. Chen, L. Liu, T. Tan, J. Mol. Catal. B: Enzym. 74 (2012) 151.
[11] X. He, B. Chen, T. Tan, J. Mol. Catal. B: Enzym. 18 (2002) 333.
[12] W. Li, B. Chen, T. Tan, Appl. Biochem. Biotechnol. 163 (2011) 102.
[13] N. Dörmö, K. Bélafi-Bakó, L. Bartha, U. Ehrenstein, L. Gubicza, Biochem. Eng. J. 21 (2004) 229.
[14] C. Laudani, M. Habulin, M. Primožič, Ž. Knez, G.D. Porta, E. Reverchon, Bioprocess. Biosyst. Eng. 29 (2006) 119.
[15] M. Leitgeb, Z. Knez, J. Am. Oil Chem. Soc. 67 (1990) 775.