Immobilization of Lipozyme TL 100L for methyl esterification of soybean oil deodorizer distillate

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Abstract

An immobilization method for binding cross-linked enzyme aggregates of Lipozyme TL 100L on macroporous resin NKA (CLEA-TLL@NKA) was developed in this study. The esterification activity of CLEA-TLL@NKA reached 6.4 U/mg. The surface structure of immobilized lipase was characterized by scanning electron microscopy. Methyl esterification reaction of soybean oil deodorizer distillate (SODD) was catalyzed by CLEA-TLL@NKA, which the conversion rate reached 98% and its activity retained over 90% after 20 batches of reaction. Compared with the commercial enzyme Lipozyme TLIM, half-life ($t_{1/2}$) of CLEA-TLL@NKA increased by 25 times and the catalytic activity increased by approximate 10 times. Thus, CLEA-TLL@NKA had high catalytic activity, good operational stability, and potential industrial application in the field of oil processing.

Keywords Lipase · Immobilization · Cross-linked enzyme aggregate · Esterification · Soybean oil deodorizer distillate

Introduction

Lipases (EC 3.1.1.3) can catalyze various chemical reactions, such as ester hydrolysis, esterification, transesterification, and ammonolysis. Lipases play an important role in many commercial biocatalysis applications (Soto et al. 2017). Preparation of biodiesel using lipase as a biocatalyst becomes increasingly attractive because lipases can function under mild temperature conditions, resulting in low energy consumption and a wide variety of materials (Adlercreutz 2013; Rodrigues et al. 2016). Lipase from *Thermomyces*

lanuginosus (TLL) is an excellent biocatalyst widely used in oil ester processing and preparation of fine chemicals (Sun et al. 2017). TLL consists of 269 amino acids and its molecular weight is 31,700 g/mol. The three-dimensional structure and catalytic mechanism of TLL had been analyzed (Fernandez-Lafuente, 2010; Skjold-Jorgensen et al. 2014).

The immobilized enzyme is a system/formulation in which an enzyme molecule is confined in a space that retains its catalytic activity, which leads to an increase in stability and reusability (Gupta et al. 2013). The immobilization of enzyme aimed to improve its economic efficiency because immobilized enzyme can be used repeatedly for a longtime period, and biocatalyst can be easily separated from the product mixture. Immobilized enzymes are efficient, environmentally friendly and selective biocatalysts that catalyze reactions under extreme environmental conditions (Li et al. 2013; Koutinas et al. 2017; Bashir et al. 2018; Xia et al. 2019).

Cross-linked enzyme aggregates (CLEA) is a rapid and versatile method to produce immobilized enzymes, which have a number of outstanding properties, such as high volume activity, high structural stability preparation, low cost, and ease of synthesis (Sheldon 2007; Torres et al. 2013; Mahmood et al. 2015). However, the mechanically resistant of CLEA are too low for many industrial applications. The small pore size of CLEA can also reduce the diffusion rate

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of the substrate in a manner that affects enzyme activity (Garcia-Galan et al. 2011). The enzyme immobilization by adsorption and cross-linking method can result in higher stability and activity than other cross-linking methods, such as CLEA and cross-linked enzyme crystals (Gandhi et al. 2017). Therefore, the activity and stability of enzymes may be improved by combining CLEA technology with other immobilization methods.

Soybean oil deodorizer distillate (SODD) is a by-product of the soybean oil processing industry and important raw material for the production of tocopherol. Tocopherol is an essential fat soluble vitamin for human body. As an excellent antioxidant and nutrient, it is widely used in clinical, pharmaceutical, food, feed, health products, cosmetics and other industries (Kamal-Eldin et al. 1996; Gazis et al. 1999; Na et al. 2019). At present, the tocopherol in SODD is mainly obtained by molecular distillation, and the method faces problems such as low enrichment rate and harsh operating conditions (high temperature and low pressure). As alternatives, the chemical and physical treatment methods have been developed (Qin et al. 2017). However, the so-called supercritical and chemical-catalyzed methods usually require the use of high temperatures and pressures, which resulting in high operating costs and considerable investments. In traditional industry procedures, chemical-catalyzed esterification which requires acid or alkaline catalysts (e.g. H_2SO_4 , H_3PO_4 , KOH, and NaOH) is widely applied. However, homogeneous catalysts usually suffer from drawbacks such as corrosivity, separation difficulty, and serious pollution (Zhang et al. 2017).

To overcome the disadvantages of the process, lipase-catalyzed esterification method can be applied because of the characteristics, such as mild temperatures, no need of raw material pretreatment and tolerance of raw material with high acid content. The reaction system does not inactivate immobilized enzymes, resulting in a positive effect on the environment for the use of low quality and inedible oils (Brusamarello et al. 2010). Many studies have been conducted on the lipase-catalyzed methyl esterification of SODD. Torres et al (2007) attempted to develop a two-step enzymatic reaction system. The first step involves the esterification of sterols from SODD with free fatty acids (FFAs), and the second step involves the esterification of the remaining FFAs and glycerides (Watanabe et al. 2004; Torres et al. 2007). The problem of inefficient fractionation of FFAs and tocopherols can be solved by converting FFAs to their methyl esters. The development of highly efficient, environmental friendly and safe enzyme-catalyzed methyl esterification of SODD is an effective way for green and sustainable development.

In order to further improve the economy and practicability of immobilized enzyme, we developed a novel preparation method for the immobilization of CLEA-Lipozyme

TL100L on macroporous resins NKA (CLEA-TLL@NKA). Immobilization of CLEA-Lipozyme TL 100L on macroporous resin has important advantages over free enzyme, which include reusability, stability, expedience to uses, controllability to reactions, easier separation of the product. CLEA-TLL@NKA was applied to catalyze the methyl esterification of SODD in the present paper (Fig. 1).

Materials and methods

Materials

Lipozyme TL 100L (TLL, liquid lipase from *Thermomyces lanuginosus*), Novozym 435 (CAL-B, lipase B from *Candida antarctica* immobilized on macroporous polyacrylate resin), Lipozyme RM IM (RML, lipase from *Rhizomucor miehei* immobilized on ionic resin), and Lipozyme TL IM (TLL, lipase from *T. lanuginosus* immobilized on silica) were purchased from Novozymes A/S (Bagsvaerd, Denmark). Lipase PS immo (PCL, lipase from *Pseudomonas cepacia*) was purchased from Purolite Ltd. (Wales, UK). Ethylene glycol dimethyl ether (EGDME), t-butanol, isopropanol, acetone, glutaraldehyde (GA), and polyethyleneimine (PEI, molecular weight of 70,000) were from Aladdin Chemistry Co, Ltd. (Shanghai, China). Macroporous resins, including D3520, ESD-1, ESD-2, AB-8, and NKA, were purchased from Tianjin Nankai Hecheng Sci. &Tech. Co. Ltd. (Tianjin, China). Resins (DM130, D4020, NKA-9, HPD850, and AB-8) were purchased from Huayi Technology New Materials Co., Ltd. (Henan, China). Macroporous resins (DM11, CAD40, X-5, HZ-806, HZ-816, and HZ-818) were purchased from Anhui Sanxing Resin Technology Co., Ltd. (Anhui, China). Macroporous resins (LSA-21, XDA-8, and LSA-10) were purchased from Xi'an Lanxiao Technology New Materials Co., Ltd. (Shanxi, China). SODD was purchased from Cargill Group (USA). The acid value of SODD was 108 mg KOH/g, and the water content was below 3%.

Fig. 1 Schematic illustration of the preparation procedures of CLEA-TLL@NKA

Preparation of CLEA-TLL@NKA

Approximately 5 mL of Lipozyme TL100L (25 mg of protein per mL, pH 6.0 phosphate buffer) was added to a 50 mL centrifuge tube at 4 °C, and 20 mL of isopropanol was slowly added to the tube. After the mixed solution was left to settle for 30 min at 4 °C, it was centrifuged for 10 min with a high-speed centrifuge at 10,000 rpm and 4 °C. After removing the supernatant, the precipitate was sequentially added 2 mL of PEI solution (2.5%, w/v) and 2 mL of glutaraldehyde solution (0.2%, w/v), and 2.25 g of macroporous resin. The resulting solution was shaken at 35 °C and mixed at 200 rpm in a water bath shaker for 4 h. CLEA-TLL@NKA was obtained by suction filtration, then dried in a vacuum oven at 45 °C for 3 h and stored at 4 °C.

Lipase esterification activity assay

Reaction system: CLEA-TLL@NKA (0.02 g) was added to a mixed solution of oleic acid (1 mL, 3.15 mmol), t-butanol (0.5 mL), and methanol (0.5 mL, 12.36 mmol). The reaction was performed at 35 °C and 200 rpm in water bath shaker for 30 min. Subsequently, 10 µL of the reaction solution was added to 990 µL of ethyl acetate for gas chromatography (GC) analysis. The production of methyl oleate in the reaction system was detected. GC (Agilent 6890 N, Agilent Technologies USA) was conducted with a flame ionization detector and DB-23 column (30 m × 0.32 mm × 0.25 µm, Agilent Technologies). The carrier gas was nitrogen with an inlet flow rate of 2 mL/min and a split ratio of 1:20. The oven temperature was maintained at 220 °C.

Definition of immobilized lipase esterification activity: Under above mentioned reaction conditions, the amount of enzyme required to produce 1 µmol methyl oleate catalyzed by immobilized enzymes per minute were defined as an enzyme activity unit (U), namely U/mg.

Determination of kinetics and thermal stability of CLEA-TLL@NKA

Most enzymes lose their activity due to structural changes caused by thermal denaturation. Therefore, we determined the kinetics and thermal stability of CLEA-TLL@NKA. The kinetics constant of CLEA-TLL@NKA catalyzed esterification of oleic acid and methanol was calculated by Lineweaver-Burkplot method. The initial reaction rates were measured in the presence of the concentration of oleic acid (0.4–1.4 mol/L) at 35 °C in t-butanol.

The half-life ($t_{1/2}$) of CLEA-TLL@NKA at 50 °C was determined, which coincided with a first-order curve. The half-life ($t_{1/2}$) of CLEA-TLL@NKA at 50 °C was calculated by linear fitting of $\ln$ (residual activity) versus incubation time, and the rate constant (k_d) was calculated by linear

fitting of $\ln$ (residual activity) versus incubation time using the following equation: $\ln$ (residual activity) = $k_d \times t$ (Feng et al., 2018). At 50 °C, 0.02 g of CLEA-TLL@NKA was incubated in t-butanol for a period of time, and then quickly taken out, and the corresponding enzyme activity assay system was added to determine the relative activity of the immobilized enzyme. The relative activity of CLEA-TLL@NKA before incubation is defined as 100%.

General procedure for CLEA-TLL@NKA-catalyzed methyl esterification of SODD

10 g of SODD was added into a liquid synthesizer (Synthesis 1, Heidolph Germany), and then 5 mL of t-butanol and 4 mL of methanol were mixed and added to the above reactor. Finally, 0.5 g of CLEA-TLL@NKA was added to the above mentioned reaction system. The entire reaction process was performed at 50 °C and 550 rpm for 8 h.

Calculation of methyl esterification rate

Determination of the acid value of SODD: The reaction solution was measured following the method specified in the national standard of the People's Republic of China (GB/T5530-2005). The method determining reduction in acid content of SODD was used for assaying lipase activity in methyl esterification of SODD.

Method for calculating the methyl esterification rate of SODD: The methyl esterification rate was calculated as follows: methyl esterification rate = (the acid value of SODD after reaction/the initial acid value of SODD) × 100%.

Reusability of CLEA-TLL@NKA in the methyl esterification of SODD

The batch stability of CLEA-TLL@NKA in the methyl esterification of SODD was determined. CLEA-TLL@NKA was added to the reaction system containing SODD and methanol; t-butanol was used as solvent at 50 °C and 200 rpm for 8 h. The reaction system was filtered through filter paper, and the recovered immobilized enzyme was washed with t-butanol for 3 times and reused in the next batch of reaction. The methyl esterification rate for each batch was calculated for comparison.

Analysis of fatty acid ester, tocopherol, and sterol composition

We analyzed the reaction product composition using gas chromatography mass spectrometry (GC–MS; Agilent7890A/5975C, Agilent Technologies USA).

The analysis method of fatty acid esters comprised the following steps: GC column model, HP-5 MS; injection

port temperature, 250 °C; injection volume, 1 µL; split ratio, 100:1; column flow rate, 1 mL/min; column oven temperature, 100 °C for 2 min at a rate of 10 °C/min; temperature rise to 250 °C; retention time, 8 min; auxiliary heating zone temperature, 250 °C; MS quadrupole temperature, 150 °C; ion source temperature, 230 °C; scan quality range, 30–500 amu; emission current, 200 µA; and electron energy, 70 eV.

The analysis method of tocopherols and sterol comprised the following steps: GC column model, HP-5 MS; injection port temperature, 300 °C; injection volume, 1 µL; split ratio, 100:1; column flow rate, 1 mL/min; column oven temperature, 250 °C for 1 min at a rate of 10 °C/min; temperature rise to 280 °C; retention, 16 min; auxiliary heating zone temperature, 250 °C; MS quadrupole temperature, 150 °C; ion source temperature, 230 °C; scan quality range, 30–500 amu; emission current, 200 µA; and electron energy: 70 eV.

The relative contents of fatty acid ester, tocopherol and sterol were calculated as follows: Relative content of fatty acid esters = area of a fatty acid methyl ester peak/area of all fatty acid methyl ester peaks × 100%.

Results and discussion

Optimization of CLEA-TLL@NKA preparation process

Selection of precipitation agent

The preparation of CLEAs involves the precipitation of the enzyme from the aqueous phase, followed by cross-linking with a functional reagent. In the first step of preparing CLEAs, aggregation is a crucial process. Organic solvent or nonionic polymer can induce the aggregation of the protein molecule by changing the hydration state of the molecule or the electrostatic constant of the solution (Torres et al. 2013). The pH value has a significant effect on the activity of enzyme, because the dissociation state and behavior of the enzyme are affected by the pH in the system. The activity of the immobilized enzyme prepared by precipitating the enzyme solution under different pH conditions is quite different (Kartal et al. 2011). The activity of the immobilized enzyme is the highest under the pH condition of the commercial enzyme solution (Fig. 2), so the pH value of enzyme solution is not changed in the following experiment.

In this experiment, five organic solvents (isopropanol, acetone, EGDME, t-butanol, and ethanol) were selected as the precipitants for the preparation of CLEA-TLL in accordance with the method described in the section of preparation of CLEA-TLL@NKA. The protein content of the precipitated supernatant was determined by Coomassie blue staining to obtain the protein precipitation rate. The esterification activity of CLEA-TLL@NKA was measured to optimize the most suitable organic precipitation agent. As shown in

Fig. 2 Effect of pH value of enzyme solution on the activity of CLEA-TLL@NKA

Table 1 Effects of organic solvents precipitating agent on CLEAs-TLL@NKA

Organic solvent	Protein precipitation rate (%) ^a	Esterification activity (U/mg)
Isopropanol	99.3 ± 0.5	3.1 ± 0.8
Acetone	99.0 ± 0.3	1.9 ± 0.3
EGDME	99.1 ± 0.6	2.3 ± 0.5
t-Butanol	95.0 ± 0.4	1.9 ± 0.6
Ethanol	99.4 ± 0.2	1.4 ± 0.1

^aPrecipitation rate was calculated by this formula, precipitation rate (%) = (Quality of enzyme in solution after precipitation/Quality of free enzyme in the initial enzyme solution) × 100%

Table 1, the precipitation rate of each organic solvent for the enzyme protein reached more than 90%, but the esterification activity of CLEA-TLL@NKA prepared from isopropyl alcohol-precipitated enzyme protein was high and reached 3.1 U/mg. Therefore, isopropanol was selected as the organic precipitant in the subsequent experiment.

Effect of resin type and added amount of resin

Macroporous resin is a cross-linked polymer with a relatively large specific surface and a suitable pore size. Different types of resins have different physical and chemical properties, such as polarity, pore size and specific surface area, which affect lipase activity linked with enzyme aggregation. Thus, the type of resin was screened to obtain the optimal resin type.

The esterification activity of CLEA-TLL@resin prepared with different resin types was compared. As shown in Fig. 3a, CLEA-TLL@NKA which prepared with the resin

Fig. 3 Effect of resin types **a** and the addition amount of resin **b** on the activity of CLEA-TLL@resin. Symbols: (●) for the activity of CLEA-TLL@NKA, (▲) for total activity

NKA demonstrated the highest esterification activity. The results obtained in this work showed that NKA resin was an effective resin for TLL immobilization. Therefore, NKA resin was selected during the subsequent experiment. Many studies have reported that immobilization on a hydrophobic carrier enhances lipase stability and activity, which used macroporous resin NKA as the support (Gunawan et al. 2008). This kind of resin is not only inexpensive but also exhibits excellent immobilization performances with a variety of lipases, such as *Burkholderia cepacia* and *Yarrowia lipolytica* lipases (Feng et al. 2014). Resin NKA has a large pore size and specific surface area. Thus, CLEA-TLL@NKA demonstrated a large specific surface area, thereby increasing the contact area between lipase protein and substrate and improving its catalytic activity.

In the preparation of immobilized enzyme, the enzyme loading greatly affects the properties of the immobilized enzyme. The excessive enzyme loading will reduce the immobilization efficiency. Crowding can prevent the mobility of the enzyme during incubation of the enzyme. If these hydrophobic groups may interact with other hydrophobic

Fig. 4 Lineweaver-burk plots of CLEA-TLL@NKA

pockets of other enzymes, the reversibility of these conformational movements is impaired, which in turn reduces the stability of the enzyme (Yang et al. 2012).

To investigate the effect of the added amount of resin on the esterification activity of CLEA-TLL@NKA, we altered the added amount of resin during the procedure of immobilization. After analyzing the relationship between the total enzyme activity and esterification activity of CLEA-TLL@NKA, the optimal addition amount of resin was obtained. As illustrated in Fig. 3b, when the added amount of resin increased, the esterification activity of CLEA-TLL@NKA gradually decreased, and the total activity gradually increased at first and then decreased. When the addition amount of resin was 3.75 g, the total activity was the highest and reached 13,300 U, and its esterification activity also reached 3.5 U/mg. We chose the amount of resin 2.25 g during the subsequent experiment on comprehensive consideration of the esterification activity and total activity, and its esterification activity reached 5.0 U/mg.

The kinetics and thermal stability analysis of CLEA-TLL@NKA

The kinetics (apparent Michaelis constant (K_m), K_{cat} and V_{max}) for the transesterification between oleic acid and methanol with CLEA-TLL@NKA was calculated by Lineweaver-Burkplot method as shown in Fig. 4. The K_m , K_{cat} and V_{max} of CLEA-TLL@NKA were 1280.7 mM, $6.3 \times 10^3 \text{ min}^{-1}$ and 243.9 $\mu\text{mol/min}$, respectively. The kinetic constant indicated that the CLEA-TLL@NKA has a good catalytic effect on the transesterification between oleic acid and methanol.

In the methylation reaction of soybean deodorized distillate, the immobilized enzyme has the best catalytic activity at 50 °C. So we analyzed the thermal stability and measured

Fig. 5 Thermal stability analysis of CLEA-TLL@NKA at 50 °C

Fig. 6 The linear fitting of ln (residual activity) versus incubation time

the $t_{1/2}$ of CLEA-TLL@NKA at 50 °C (Figs. 5, 6). As a result, we calculated that the half-life value of the CLEA-TLL@NKA was 1564.8 h. It can be seen from the above

data that CLEA-TLL@NKA prepared by the method has good thermal stability.

Scanning electron microscopy (SEM) analysis of CLEA-TLL@NKA

After the sputtering of platinum with NKA resin and CLEA-TLL@NKA, the surface morphology of NKA resin and CLEA-TLL@NKA were observed by SEM (FESEM, Hitachi S-4800). The scanning voltage was set at 5 kV; the dry samples were evenly spread on the conductive adhesive tape and were coated with gold using an ion sputtering instrument prior to determination. Figure 7a shows the SEM micrograph of NKA at a magnification of 5×10^4 . The surface of the NKA resin had many cavities, which provided a large space for the cross-linked immobilized protein. This space allowed readily available access of substrate molecules to the enzyme catalytic sites, thereby improving mass transfer for the reaction to proceed (Pöhnlein et al. 2015). As shown in Fig. 7b, the surface structure of NKA resin was covered with a layer of CLEAs. Thus, this method could bind the enzyme protein on the resin surface effectively.

Comparison of CLEA-TLL@NKA with the commercial lipase

To compare with the activity of CLEA-TLL@NKA in the methyl esterification of SODD, we selected the commercially available immobilized lipases (Novozym 435, Lipozyme RMIM, Lipozyme TLIM, and Lipase PS immo) for the methyl esterification reaction. Under the methyl esterification reaction conditions of the SODD was described in the section of general procedure for CLEA-TLL@NKA-catalyzed methyl esterification of SODD. As illustrated in Fig. 8, CLEA-TLL@NKA can achieve the same or better catalytic effect compared with the selected commercial enzyme. (i.e., Novozym 435, Lipozyme RMIM, Lipozyme TLIM, and Lipase PS immo)

Fig. 7 SEM images of NKA resin (a) and CLEA-TLL@NKA (b)

Fig. 8 Methyl esterification rates of soybean oil deodorizer distillate catalyzed by Novozymes 435, Lipozyme TLIM, Lipozyme RMIM, Lipase PS immo, and CLEA-TLL@NKA

in the methyl esterification of SODD. The methyl esterification rate of CLEA-TLL@NKA was approximate 10 times that of Lipozyme TLIM. The immobilization method of combining resin adsorption and CLEA exhibited a good technical advantage.

Optimization of CLEA-TLL@NKA-catalyzed methyl esterification

Effect of reaction temperature

In general, the reaction temperature plays an important role in the catalytic reaction. The reaction temperature affects the activity and stability of the biocatalyst. High temperature can reduce the viscosity of the reaction medium, enhance mutual solubility, improve the diffusion process of the substrate, and enhance the interaction between enzyme and substrate. However, high temperatures destroy the active conformation of the enzyme and resulting in loss of the activity in the biocatalytic process (Yadav and Dhoot 2009; Zheng et al. 2011; Fu et al. 2014).

Using CLEA-TLL@NKA to catalyze the methyl esterification of SODD at the temperature range of 35–55 °C, we determined the acid value of the above mentioned reaction solution and calculated the corresponding methyl esterification rate. As shown in Fig. 9a, the maximum methyl esterification rate of CLEA-TLL@NKA-catalyzed methyl esterification of SODD was obtained at 50 °C. Therefore, the subsequent methyl esterification reaction was carried out at 50 °C.

Effect of organic solvent

Due to the surface properties of lipase, such as hydrophobicity and charge distribution, it is a major factor contributing to the stability of lipase in organic solvents. In addition, the organic solvent reduces the viscosity of the lipase in the transesterification reaction, thereby improving the stability of the lipase and the mass transfer of the substrate (Chakravorty et al. 2012; Nasaruddin et al. 2014). Therefore, the hydrophobic solvent is superior to the hydrophilic solvent to maintain the amount of bound water in the surrounding lipase and to avoid inactivation due to moisture loss in the enzyme structure necessary for lipase activity (Royon et al. 2007; You et al. 2013; Duarte et al. 2015).

Under the methyl esterification reaction conditions of the SODD described in the section of general procedure for CLEA-TLL@NKA-catalyzed methyl esterification of SODD, the organic solvents (toluene, dichloromethane, ethyl ether, petroleum, t-butanol, *n*-hexane, and cyclohexane) were selected as the reaction solvents. As shown in Fig. 9b, the methyl esterification rate in the organic solvent was significantly higher than that in the absence of the solvent. In addition, the methyl esterification rate of CLEA-TLL@NKA could reach 93% with t-butanol as the organic solvent. With a certain amount of t-butanol as the reaction medium, both methanol and glycerol by-product were mutually soluble, and the negative effect caused by methanol and glycerol on lipase catalytic activity was eliminated completely (Wang et al. 2007). Therefore, we selected t-butanol as the organic solvent.

Effect of reaction time

To inspect the time course of CLEA-TLL@NKA catalyzed methyl esterification of SODD, we measured the methyl esterification rate at 1 h intervals. As shown in Fig. 9c, the methyl esterification rate reached over 98% at 10 h, and the reaction reached the equilibrium of esterification and hydrolysis. The time course of enzymatic process also was affected by the shaking speed, the added amount of enzymes and the molar concentration of substrates.

Batch stability of CLEA-TLL@NKA

Immobilization of the enzyme within the porous structure of a solid may permit to have better complete dispersion of the enzyme molecule. The immobilization process allows the enzyme molecule to avoid interaction with the molecules, prevent protease aggregation, autolysis or proteolysis, and without the possibility of interacting

Fig. 9 Effect of temperature (a), organic solvents (100% v/v) (b), reaction time (c) and operation batch (d) on CLEA-TLL@NKA catalyzed methyl esterification of soybean oil deodorizer distillate

with any external interface, thus making the enzyme more stable (Roddy and Shanke 1993).

Compared with the common chemical catalysts, biocatalysts are usually expensive. Thus, the reusability of immobilized enzyme plays an important role in reducing the service cost. The batch stability of CLEA-TLL@NKA catalyzed methyl esterification was investigated. After 20 batches of reaction, the methyl esterification rate of CLEA-TLL@NKA still exceeded 90%, thereby it indicated that CLEA-TLL@NKA had good operational stability (Fig. 9d). However, Lipozyme TL IM is essentially inactive after 3 batches of the methyl esterification reaction due to Lipozyme TL IM absorb water in the reaction and lead to self-cleavage. Compared with the immobilized enzymes reported by Yücel et al. (2014), the catalytic activity and batch stability have been greatly improved. The CLEA-TLL@NKA had high stability and esterification activity, making it ideal biocatalyst for large-scale applications.

Analysis of reaction product components

In this enzymatic process, the function of lipase is to transform free fatty acid into fatty acid methyl ester and transform triglycerides and sterol fatty acid ester into fatty acid methyl ester and sterol, while tocopherol does not react. Lipase catalyzed methyl esterification of free fatty acid is the main reaction. We analyze the composition of the reaction products by GC–MS. As shown in Table S2, SODD contained seven fatty acid esters. The highest relative content of methyl linoleate was 42.5%, followed by methyl oleate (28.2%). This result was similar to the findings reported by Gunawan et al. (2008). As shown in Table S3 and Table S4, SODD contained three kinds of natural tocopherol and sterol. The main content of tocopherol was γ -tocopherol, accounting for 65.2%, whereas α -tocopherol accounted for only 9.3%. As mentioned by Wang et al., the tocopherol content of SODD was 3–12% (Wang et al. 2007). The main sterol was sitosterol, which accounted for 48.3% of the total sterols.

Table 2 The advantage/novelty of the current immobilized lipase

Entry	Advantage/novelty
Methods	Combining CLEAs method with macroporous resin adsorption to improve the reusability and stability
Activities	Esterification activity is 6.4 U/mg, which is superior to commercial immobilized lipase
Operational stability	CLEA-TLL@NKA retained over 90% after 20 batches of the methyl esterification reaction of SODD
Preparation process and cost	Preparation process is simple, the used materials are cheap, and the preparation cost of immobilized lipase is low

Conclusions

The preparation of CLEA-TLL@NKA which expected to overcome the lack of mechanical resistance to CLEA and problems faced in industrial applications was reported in this work. The CLEA-TLL@NKA prepared by a novel method has the advantages of high catalytic activity, strong tolerance to organic solvents, good stability and low price and its esterification activity reaches 6.4 U/mg (as shown in Table 2). The surface structure of immobilized lipase was characterized by scanning electron microscopy. Compared with the commercial enzyme Lipozyme TLIM, the half-life ($t_{1/2}$) of CLEA-TLL@NKA increased by 25 times, the catalytic activity increased by approximate 10 times. CLEA-TLL@NKA showed excellent batch stability in the methyl esterification of SODD. The composition of the reaction product (fatty acids, tocopherol and sterol) was determined by GC-MS. The CLEA-TLL@NKA can be well applied in batch stirred-tank reactors (BSTRs), packed-bed reactors (PBRs), fluidized-bed reactors (FBRs) and membrane reactors. We hope that this new method of preparing immobilized lipase can promote the application of lipase in the oil processing industry.

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Compliance with ethical standards

Conflict of interest The authors declared that they have no conflicts of interest to this work.

Ethical approval This article does not contain any studies with human participants or animals performed by any of the authors.

Informed consent The authors confirm that the manuscript has been read and approved by all named authors. The authors further confirm that the order of authors listed in the manuscript has been approved by all of us.

References

- Adlercreutz P (2013) Immobilisation and application of lipases in organic media. *Chem Soc Rev* 42:6406–6436
- Bashir F, Asgher M, Hussain F, Randhawa MA (2018) Development and characterization of cross-linked enzyme aggregates of thermotolerant alkaline protease from *Bacillus licheniformis*. *Int J Biol Macromol* 113:944–951
- Brusamarello CZ, Rosset E, Treichel H, De OD, Mazutti MA, Di LM, Oliveira JV (2010) Kinetics of lipase-catalyzed synthesis of soybean fatty acid ethyl esters in pressurized propane. *J Bio Technol* 147:108–115
- Chakravorty D, Parameswaran S, Dubey VK, Patra S (2012) Unraveling the rationale behind organic solvent stability of lipases. *Appl Biochem Biotechnol* 167:439–461
- Duarte SH, del Peso Hernández GL, Canet A, Benaiges MD, Maugeri F, Valero F (2015) Enzymatic biodiesel synthesis from yeast oil using immobilized recombinant *Rhizopus oryzae* lipase. *Biores Technol* 183:175–180
- Feng S, Li GL, Zhang HJ, Yan YJ, Rials T, Rooney W (2014) Enhanced performance of *Rhizopusoryzae* lipase immobilized on hydrophobic carriers and its application in biorefinery of rapeseed oil deodorizer distillate. *Bioenerg Res* 7:935–945
- Feng Y, Liu S, Jiao Y, Wang YL, Wang M, Du GC, Chen J (2018) Improvement of L-asparaginase thermal stability by regulating enzyme kinetic and thermodynamic states. *Process Biochem* 71:45–52
- Fernandez-Lafuente R (2010) Lipase from *Thermomyces Lanuginosus*: Uses and prospects as an industrial biocatalyst. *J Mol Catal B Enzyme* 62:197–212
- Fu XF, Zheng JY, Ying XX, Yan HD, Wang Z (2014) Investigation of lipozyme TL IM-catalyzed transesterification using ultraviolet spectrophotometric assay. *Chin J Catal* 35:553–559
- Gandhi A, Vania Anisya A, Muhammad S, Heri H (2017) Effect of chitosan's amino group in adsorption-crosslinking immobilization of lipase enzyme on resin to catalyze biodiesel synthesis. *Energy Procedia* 136:47–52
- Garcia-Galan C, Berenguer-Murcia Á, Fernandez-Lafuente R, Rodrigues RC (2011) Potential of different enzyme immobilization strategies to improve enzyme performance. *Adv Synth Catal* 353:2885–2904
- Gazis A, White DJ, Page SR, Cockcroft JR (1999) Effect of oral vitamin e (α -tocopherol) supplementation on vascular endothelial function in type 2 diabetes mellitus. *Diabet Med* 16:304–311
- Gunawan S, Kasim NS, Ju YH (2008) Separation and purification of squalene from soybean oil deodorizer distillate. *Sep Purif Technol* 60:128–135
- Gupta S, Bhattacharya A, Murthy CN (2013) Tune to immobilize lipases on polymer membranes: techniques, factors and prospects. *Biocatal Agric Biotechnol* 2:171–190
- Kamal-Eldin A, Appelqvist L (1996) The chemistry and antioxidant properties of tocopherols and tocotrienols. *Lipids* 31:671–701
- Kartal F, Janssen MHA, Hollmann F (2011) Improved esterification activity of *Candida rugosa* lipase in organic solvent by

- immobilization as cross-linked enzyme aggregates (CLEAs). *J Mol Catal B Enzyme* 71:85–89
- Koutinas M, Yiangou C, Osório NM, Ioannou K, Canet A, Valero F, Ferreira-Dias S (2017) Application of commercial and non-commercial immobilized lipases for biocatalytic production of ethyl lactate in organic solvents. *Biores Technol* 247:496–503
- Li ZX, Zhang YF, Lin MM, Ouyang PK, Ge J, Liu Z (2013) Lipase-catalyzed one-step and regioselective synthesis of clindamycin palmitate. *Org Process Res Dev* 17:1179–1182
- Mahmod SS, Yusof F, Shah NH, Jami MS, Khanahmadi S (2015) Development of an immobilized biocatalyst with lipase and protease activities as a multipurpose cross-linked enzyme aggregate (multi-clea). *Process Biochem* 50:2144–2157
- Na X, Anuj GS, Bo L, Thamonwan A, Eric AD (2019) Impact of phospholipid-tocopherol combinations and enzyme-modified lecithin on the oxidative stability of bulk oil. *J Agric Food Chem* 67:7954–7960
- Nasaruddin RR, Alam MZ, Jami MS (2014) Evaluation of solvent system for the enzymatic synthesis of ethanol based biodiesel from sludge palm oil (SPO). *Biores Technol* 154:155–161
- Pöhnlein M, Hausmann R, Lang S, Syltatk C (2015) Enzymatic synthesis and modification of surface-active glycolipids. *Eur J Lipid Sci Technol* 117:145–155
- Qin L, Zeng Q, Zhang J, Cheng H, Chen L, Qi Z (2017) Integrated process for extracting vitamin E with high purity from the methylated oil deodorizer distillate. *Sep Purif Technol* 196:229–236
- Roddy LG, Shanke V (1993) Immobilised nucleases. *Crit Rev Biotechnol* 13:255–273
- Rodrigues J, Canet A, Rivera I, Osório NM, Sandoval G, Valero F, Ferreira-Dias S (2016) Biodiesel production from crude jatropha oil catalyzed by non-commercial immobilized heterologous *Rhizopus oryzae* and *Carica papaya* lipases. *Biores Technol* 213:88–95
- Royon D, Daz M, Ellenrieder G, Locatelli S (2007) Enzymatic production of biodiesel from cotton seed oil using t-butanol as a solvent. *Biores Technol* 98:648–653
- Sheldon RA (2007) Enzyme immobilization: the quest for optimum performance. *Adv Synth Catal* 38:1289–1307
- Skjold-Jorgensen J, Vind J, Svendsen A, Bjerrum MJ (2014) Altering the activation mechanism in *Thermomyces lanuginosus* Lipase. *Biochem* 53:4152–4160
- Soto ID, Escobar S, Mesa M (2017) Study of the physicochemical interactions between *Thermomyces lanuginosus* lipase and silica-based supports and their correlation with the biochemical activity of the biocatalysts. *Mat Sci Eng C Mater* 79:525–532
- Sun J, Ding C, Zheng JY, Yu XJ, Wang Z (2017) Improved enantioselective esterification of DL-menthol catalyzed by immobilized TL 100L lipase. *J Mol Catal B Enzyme* 133:S271–S276
- Torres CF, Torrelo G, Señorans FJ, Reglero G (2007) A two steps enzymatic procedure to obtain sterol esters, tocopherols and fatty acid ethyl esters from soybean oil deodorizer distillate. *Process Biochem* 42:1335–1341
- Torres MPG, Foresti ML, Ferreira ML (2013) Effect of different parameters on the hydrolytic activity of cross-linked enzyme aggregates (CLEAs) of lipase from *Thermomyces lanuginosus*. *Bio Chem Eng J* 72:18–23
- Wang L, Du W, Liu D, Li L, Dai N (2007) Lipase-catalyzed biodiesel production from soybean oil deodorizer distillate with absorbent present in tert-butanol system. *J Mol Catal B Enzyme* 43:29–32
- Watanabe Y, Nagao T, Hirota Y, Kitano M, Shimada Y (2004) Purification of tocopherols and phytosterols by a two-step in situ enzymatic reaction. *J Am Oil Chem Soc* 81:339–345
- Xia H, Zhong X, Li ZX, Jiang YB (2019) Palladium-mediated hybrid biocatalysts with enhanced enzymatic catalytic performance via allosteric effects. *J Colloid Interf Sci* 533:1–8
- Yadav GD, Dhoot SB (2009) Immobilized lipase-catalysed synthesis of cinnamyl laurate in non-aqueous media. *J Mol Catal B Enzyme* 57:34–39
- Yang X, Zheng P, Ni Y, Sun Z (2012) Highly efficient biosynthesis of sucrose-6-acetate with cross-linked aggregates of lipozyme TL 100L. *J Biotechnol* 161:27–33
- You QH, Yin XL, Zhao YP, Zhang Y (2013) Biodiesel production from jatropha oil catalyzed by immobilized *Burkholderia cepacia* lipase on modified attapulgate. *Biores Technol* 148:202–207
- Yücel Y, Demir C, Dizge N, Keskinle B (2014) Methods for lipase immobilization and their use for biodiesel production from vegetable oil. *Energ Source Part A* 36:1203–1211
- Zhang X, Yu J, Zeng A (2017) Optimization and modeling for the synthesis of sterol esters from deodorizer distillate by lipase-catalyzed esterification. *Biotechnol Appl Bioc* 64:270–278
- Zheng GW, Yu HL, Li CX, Pan J, Xu JH (2011) Immobilization of *Bacillus subtilis* esterase by simple cross-linking for enzymatic resolution of dl-menthyl acetate. *J Mol Catal B Enzym* 70:138–143