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Immobilized MAS1 lipase showed high esterification activity in the production of triacylglycerols with n-3 polyunsaturated fatty acids

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ABSTRACT

Immobilization of lipase MAS1 from marine *Streptomyces* sp. strain W007 and its application in catalyzing esterification of n-3 polyunsaturated fatty acids (PUFA) with glycerol were investigated. The resin XAD1180 was selected as a suitable support for the immobilization of lipase MAS1, and its absorption ability was 75 mg/g (lipase/resin ratio) with initial buffer pH value of 8.0. The thermal stability of immobilized MAS1 was improved significantly compared with that of the free lipase. Immobilized MAS1 had no regiospecificity in the hydrolysis of triolein. The highest esterification degree (99.31%) and TAG content (92.26%) by immobilized MAS1-catalyzed esterification were achieved under the optimized conditions, which were significantly better than those (82.16% and 47.26%, respectively) by Novozym 435. More than 92% n-3 PUFA was incorporated into TAG that had similar fatty acids composition to the substrate (n-3 PUFA). The immobilized MAS1 exhibited 50% of its initial activity after being used for five cycles.

1. Introduction

Long-chain n-3 polyunsaturated fatty acids (n-3 PUFA), especially eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), could reduce the risk of atherosclerosis (Abdukeyum, Owen, & Mclennan, 2008) and sudden death (Albert et al., 1998), contribute to reduce the incidence of cardiovascular disease (Cleland, Caughey, James, & Proudman, 2006), chronic inflammatory diseases (Giudetti & Cagnazzo, 2012) and cancer (Corsetto et al., 2011). In recent decades, EPA and DHA concentrates in triacylglycerols (TAG) form have attracted great interest due to their higher stability than those in free fatty acids (FFA) form and higher bioavailability than those in ethyl esters (EE) form (Valenzuela, Valenzuela, Sanhueza, & Nieto, 2005). Therefore, there is a growing demand for n-3 PUFA in the form of TAG with high purity in the pharmaceutical and food industry (Borg, Binet, Girardin, Rovel, & Barth, 2001).

n-3 PUFA-rich TAG can be synthesized chemically or enzymatically. Lipases are most commonly used in enzymatic process due to their high catalytic efficiency, mild reaction conditions, substrate specificity and positional selectivity (Akanbi, Adcock, & Barrow, 2013; Lyberg & Adlercreutz, 2008). Enzymes in immobilized form consume less enzyme, enable their reuse, increase stability, improve the ability to resist stress conditions and facilitate continuous operations (Zhang et al., 2014). Many researchers have reported the synthesis of n-3 PUFA-rich TAG in the solvent-free systems, but the resulting TAG content was low (Moreno-Perez, Luna, Señorans, Guisan, & Fernandez-Lorente, 2015; Sun et al., 2015). Other researchers used solvent systems to synthesize n-3 PUFA-rich TAG. Although high content of n-3 PUFA-rich TAG was obtained in solvent systems, these solvents could result in environmental pollution and food safety (Liu, Zhang, Hong, & Ji, 2007; Wang, Li, Ning, et al., 2012). Different lipases from various sources have been employed to synthesize n-3 PUFA-rich TAG. However, TAG content was less than 90% and maximum n-3 PUFA incorporated into TAG was reported to be 84.5% (Kosugi & Azuma, 1994). Therefore, it is necessary to develop new reaction systems as well as novel lipases with better catalytic properties.

In this paper, a thermostable lipase (named MAS1) from marine *Streptomyces* sp. strain W007 found in our laboratory (Yuan, Lan, Xin, Yang, & Wang, 2015) was immobilized on macroporous resins. The effects of lipase/support ratio and initial buffer pH on immobilization efficiency were studied. Then, the thermal stability, regiospecificity and catalytic ability of lipase MAS1 in free and immobilized form were investigated. Subsequently, immobilized MAS1 was employed in the esterification of glycerol with n-3 PUFA for the production of TAG and the effects of reaction conditions, including temperature, enzyme loading and glycerol/n-3 PUFA ratio were investigated. Furthermore, the reusability of

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immobilized MAS1 was evaluated. Finally, the catalytic properties of immobilized MAS1 were compared with those of Novozym 435 under the optimized conditions.

2. Materials and methods

2.1. Materials

The X33/MAS1 strain was stored in 50% glycerol solution (v/v) at −80 °C. Novozym 435 was obtained from Novo Nordisk (Denmark). DHA/EPA-rich EE were kindly provided by Sinomega Biotech Engineering Co., Ltd. (Zhejiang, China). Lauric acid was obtained from Aladdin Industrial Corporation (Shanghai, China). *n*-Hexane, 2-propanol and formic acid of chromatographic grade were purchased from Kermel Chemical Reagent Co., Ltd. (Tianjin, China). Three kinds of resins (Amberlite XAD1180, HP20 and HP2MGL) were obtained from Rohm and Haas Company (USA). The resins AB-8 and DA201 were purchased from Chemical Plant of Nankai University (Tianjin, China) and Zhengzhou Qinshi Technology Co., Ltd. (Henan, China), respectively. Bovine serum albumin (BSA) was purchased from Shanghai Bio Science & Technology Company (Shanghai, China). Bradford reagent was obtained from Sigma (Wuhan, China). All other chemicals were of analytical grade unless otherwise stated.

2.2. Production of crude lipase MAS1

The pPICZαA-MAS1-X33 expression strain was provided by the enzyme engineering group in our laboratory, and fermentation was carried out according to the method described previously (Wang et al., 2014). After fermentation, the broth was centrifuged (10,000*g* for 30 min, 4 °C) and filtered. Then, the resultant supernatant was concentrated and kept at 4 °C for subsequent immobilization. The hydrolytic activity of the concentrated crude extract containing lipase MAS1 was 940 ± 18 U/mL, according to the olive oil emulsion method described previously (Yang, Wang, & Yang, 2006). The specific activity of free lipase MAS1 was defined as units per mg of protein.

2.3. Immobilization of lipase MAS1 by physical adsorption

The pretreatment processes of macroporous resins were carried out as follows: each macroporous resin (10 g) was initially wetted with 30 mL of 95% (v/v) ethanol for 24 h. After decanting ethanol, the resins were washed with distilled water several times until no ethanol was detected in the supernatant. Then, the resins were mixed with 30 mL of 5% HCl (w/w) for 4 h. After that, the supernatant was filtered out and the resins were rinsed with distilled water repeatedly until the supernatant had a neutral pH value. Subsequently, the resins were immersed in 30 mL of 2% NaOH (w/w) for 4 h and recovered from the supernatant by filtration, washing with distilled water until the pH value of the filtrate was around 7.0. Subsequently, the resins were treated by 0.02 M sodium phosphate buffer (various pH values) for 4 h. Finally, the filtrate was removed and the resins were used for the immobilization of lipase MAS1. The purpose of using ethanol, acid and alkali was to remove bubbles, residual monomers and compounds in the resin pores, respectively.

The immobilization of lipase MAS1 on each macroporous resin was carried out as follows: lipase MAS1 solution (50 mg protein/g resin) was mixed with an equal volume of sodium phosphate buffer (0.02 M pH 8.0), and then 3 g of the pretreated each macroporous resin using sodium phosphate buffer (0.02 M pH 8.0) ahead of time was added to form a mixture. Then, the mixture was stirred by a shaking water bath at 30 °C with a speed of 200 rpm/min for 8 h. After that, the supernatant was filtered out and the obtained immobilized MAS1 was washed with 0.02 M pH 8.0 sodium phosphate buffer several times to remove the enzyme that loosely bound to the resins until no protein was detected in the filtrate. Finally, the immobilized MAS1 was vacuum-dried at 40 °C for 8 h and stored in closed vials at 4 °C before use.

The immobilization conditions such as initial pH (6.0, 7.0, 8.0, 9.0) of buffer, and lipase/support ratio (25, 50, 75, 100, 150 mg/g resin) were varied for optimization.

2.4. Determination of esterification activity and protein content of immobilized MAS1

The esterification activities of immobilized MAS1 and Novozym 435 were determined according to Novozymes Standard Method EB-SM-1069.02 (Basso, Froment, Hesseler, & Serban, 2013). The protein contents of free lipase MAS1 and immobilized MAS1 were determined according to the Bradford assay and a calibration curve using bovine serum albumin was used for protein quantification (Bradford, 1976). The specific activity of immobilized MAS1 was defined as units per mg of protein (U/mg). Protein amount in immobilized enzyme (mg/g) and protein recovery (%) was calculated according to Zhao, No, Kim, Garcia, and Kim (2015).

2.5. FT-IR spectroscopy of immobilized MAS1

Fourier transform infrared (FT-IR) analysis of immobilized MAS1 and XAD1180 resin was carried out using a Nicolet 8210E FT-IR spectrometer in the frequency range of 4000–400 cm^{-1}. The resolution was 2 cm^{-1} and 128 scans. The standard KBr pellet technique was applied for sample preparation. The free enzyme was lyophilized and then mixed with KBr in a mortar. Finally, the mixture was ground and pressed into a pellet for FT-IR analysis.

2.6. Thermal stability studies

The effect of temperature on the stability of free lipase and immobilized MAS1 was investigated by measuring the residual hydrolytic activities of lipases after incubation at 65 °C with continuous shaking for 3 h. Samples were withdrawn every 30 min and their hydrolytic activities were determined as described in Section 2.2. The hydrolytic activity at the beginning was set as 100% and the residual activity of the lipases after incubation was calculated accordingly.

2.7. Regiospecificity of immobilized MAS1

The determination of the regiospecificity of free lipase and immobilized MAS1 was carried out in the hydrolysis of triolein under the conditions of enzyme amount of 30 U/g triolein (U/w, with respect to oil mass), sodium phosphate buffer (0.1 M, pH 7.0) of 20% (w/w, with respect to oil mass) and reaction temperature of 65 °C according to the method described previously (Li et al., 2015). A commercial non-regiospecific enzyme (lipase AYS) was also used to catalyze the hydrolysis of triolein at a temperature of 40 °C. Samples were withdrawn at selected times for high-performance liquid chromatography (HPLC) analysis. The ratio of 1,2(2,3)-DAG to 1,3-DAG was used as an indicator to evaluate the regiospecificity of the lipases. The hydrolytic activities of the lipases were determined as described in Section 2.2.

2.8. Comparison of the catalytic ability of immobilized MAS1 and its free form in the esterification of glycerol and n-3 PUFA

The esterification activities of immobilized MAS1 and lyophilized free lipase MAS1 were measured, respectively, according

to the method described by Qin, Huang, Lan, Wang, and Yang (2014). Lipase activity recovery (%) was calculated according to Liu et al. (2011). Free n-3 PUFA were prepared from EE according to the method described by Senanayake and Shahidi (1999). Then, immobilized MAS1 and free lipase MAS1 were used to catalyze esterification of glycerol with n-3 PUFA, respectively. Reaction mixture in a 100-mL glass vessel contained glycerol (0.8832 g, 9.6 mmol) and n-3 PUFA (9.1168 g, 28.9 mmol). The immobilized MAS1 and lyophilized free lipase MAS1 with equal esterification activity (1500 U) were added to the mixture, respectively. Reactions were immediately performed at a temperature of 65 °C and a speed of 200 rpm/min for 24 h under vacuum. Samples were withdrawn at selected times for HPLC analysis. The esterification degree represents the percentage of initial FA consumed in the reaction mixture, which was calculated according to He and Shahidi (1997).

2.9. Synthesis of n-3 PUFA-rich TAG by immobilized MAS1-catalyzed esterification under vacuum

For the synthesis of n-3 PUFA-rich TAG, the reaction mixtures consisted of 10 g substrates (1:5, 1:4, 1:3, 1:2, 1:1 of molar ratio of glycerol to n-3 PUFA) in a total volume of 10.8 mL. The reactions catalyzed by different amounts of immobilized MAS1 [750, 1125, 1500 (1 g), 1875, 2250 U] were incubated at various temperature (50, 55, 60, 65, 70 °C) and agitated on magnetic stirrer at 200 rpm/min for 24 h under vacuum. In order to evaluate the catalytic properties of immobilized MAS1, Novozym 435 (1500 U, 0.2 g) was used to catalyze the same reaction under the optimized conditions. Samples were withdrawn periodically to monitor the production of glycerides and their fatty acid profiles by HPLC and gas chromatography (GC) analysis, respectively.

2.10. Reusability of immobilized MAS1

The reactions were carried out under the conditions of enzyme loading of 150 U/g (U/w, with respect to the total substrates), substrate molar ratio of 1:3 (glycerol: n-3 PUFA) at a temperature of 65 °C under vacuum for 24 h. The experiments about the reusability of free lipase MAS1 were carried out as a control. At the end of each reaction, the enzyme (immobilized MAS1 or free enzyme solution) was recovered from the reaction product by centrifugation, washing the enzyme three times with *n*-hexane and placed at room temperature to remove residual *n*-hexane. Then, the glycerol layer containing free lipase MAS1 or immobilized MAS1 was added for the next reaction cycle with the introduction of fresh n-3 PUFA. A certain amount of fresh glycerol was also added to keep the constant molar ratio of glycerol to n-3 PUFA in each run. TAG content in the first reaction was defined as 100% and TAG content in the subsequent reactions was calculated accordingly. The reusability of immobilized MAS1 and free lipase MAS1 was evaluated by measuring the differences in TAG content obtained from different reaction cycles.

2.11. Analysis of the composition of the reaction mixture by HPLC and FA composition analysis by GC

Analysis of the composition of the reaction mixture was performed using HPLC according to the method described by Li et al. (2015). Acquisition and processing of data were made using Waters 2695 integration software. For analysis of FA composition of TAG in the reaction product, TAG was firstly separated from other lipids using thin-layer chromatography method according to Qin et al. (2014). Then, the scraped TAG and the substrate (n-3 PUFA) were separately methylated to fatty acids methyl esters (FAME) according to the method of ISO 5509:2000(E) (Wang et al., 2010). Finally, the FAME were analyzed using an Agilent 7890A GC equipped with a capillary column CP-Sil 88 (60 m × 0.25 mm × 0.2 μm) according to the method of Qin, Wang, Wang, Huang, and Yang (2011). In this study, the composition of n-3 PUFA was the sum of the composition of EPA, DPA and DHA.

2.12. Statistical analysis

All experiments were carried out in triplicate. Significant differences among mean values were evaluated through significant differences test and variance analysis of SPSS for Windows 13.0. The results were reported as the means ± standard deviations (SD).

3. Results and discussion

3.1. Immobilization of the crude lipase MAS1

3.1.1. Screening of immobilization supports

The immobilized enzyme showed good advantages such as reusability, inexpensive, lower protein-contaminant on product and easier separation of enzyme from products. The type of support is a crucial factor in the enzyme immobilization process. In this study, five kinds of macroporous resins (AB-8, XAD1180, HP20, HP2MGL, and DA201) with various properties such as polarity and specific surface area were used to immobilize lipase MAS1 directly from the crude extract. Table 1 shows the effect of the selected supports on protein loading, esterification activity and specific activity of immobilized lipases. XAD1180 (~24 U/mg) and DA201 (~22 U/mg) as carriers resulted not only in significantly higher protein loading but also in higher specific activity, compared with the other supports tested. However, lower protein loading did not necessarily result in low specific activity of immobilized MAS1. For example, although AB-8 resin showed low protein loading (~68 mg/g), the immobilized enzyme exhibited similar specific activity, compared with HP20 and HP2MGL resins that showed higher protein loading. Except for DA201 resin, protein loading and esterification activity of immobilized lipases increased with the increasing specific surface area and pore diameter of the resins. Among the carriers, XAD1180 gave the highest protein loading, esterification activity and specific activity, which is due to its biggest specific surface area and pore diameter being

Table 1
Protein loading, esterification activity and specific activity of immobilized MAS1 on different macroporous resins.

Resin	Polarity	Particle size (mm)	Specific surface area (m^2/g)	Pore diameter (nm)	Protein loading (mg/g immobilized lipase)	Esterification activity (U/g immobilized lipase)	Specific activity of immobilized lipase (U/mg protein)
AB-8	Weak polar	0.3-1.25	480–520	13–14	68 ± 0.74	1287 ± 15	19 ± 0.21
XAD1180	Non-polar	0.4	700	40	106 ± 1.02	2546 ± 13	24 ± 0.72
HP20	Non-polar	0.3-1.25	600	26	90 ± 0.26	1699 ± 9	19 ± 0.15
HP2MGL	Middle polar	0.3	570	24	85 ± 0.72	1586 ± 14	19 ± 0.56
DA201	Polar	0.3-1.25	⩾200	10–13	99 ± 0.91	2195 ± 14	22 ± 0.53

able to facilitate the enzyme immobilization in the matrix and on the surface (Ramachandran, Narayanan, Gandhi, Sethuranman, & Krishnan, 2015). Moreover, the enzyme (<5 nm) with far less diameter than resin XAD1180 made it freely absorbed into the matrix. Therefore, resin XAD1180 was selected as a suitable support for the further experiments.

FT-IR spectroscopy was used to check whether or not lipase MAS1 had been absorbed onto XAD1180 resin, and the results are shown in Fig. 1. The absorption band in the distinctive region associated with C—O stretching (1050–1150 cm^{-1}) was observed only in the spectra of immobilized MAS1 and free lipase MAS1 but not in the spectra of XAD1180 resin. The results suggested that lipase MAS1 was successfully immobilized onto XAD1180 resin.

3.1.2. Effect of initial pH of buffer

Effect of initial pH of buffer on immobilization efficiency was evaluated and the results are shown in Fig. 2a. There were no significant changes in protein loading when the initial pH of buffer was increased from 6.0 to 9.0. The esterification activity (2713 U/g) and specific activity (24 U/mg) of immobilized MAS1 reached maximum values at pH 8.0. Lower esterification activities and specific activities were obtained when initial pH of buffer was above or below 8.0. The results showed that immobilized MAS1 had the highest protein loading, esterification activity and specific activity at pH 8.0. Therefore, an initial buffer pH value of 8.0 was selected for the further immobilization of lipase MAS1.

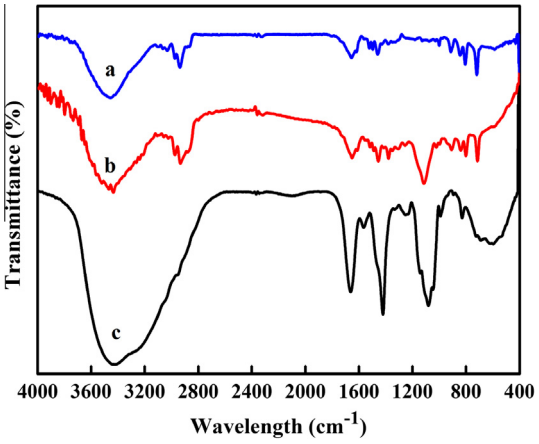

Fig. 1. FT-IR spectra of XAD1180 resin (a), immobilized MAS1 (b), and free lipase MAS1 (c).

3.1.3. Effect of lipase/support ratio

Lipase/support ratio (w/w) was also an important factor to be considered for the immobilization efficiency. Lipase MAS1/support ratio ranging from 25 to 150 mg/g was tested to determine its effect on the immobilization of lipase MAS1 (Fig. 2b). The protein loading increased with increasing lipase/support ratio, while different behaviors were exhibited for esterification activity and specific activity of immobilized MAS1. It could be seen that the esterification activity and specific activity of immobilized MAS1 increased with increasing lipase/support ratio from 25 to 75 mg/g. Esterification activity and specific activity reached their corresponding maximum values (2732 U/g and 27 U/mg, respectively) at 75 mg/g and then decreased with further increasing lipase/support ratio. This may be probably because of the inhibition of the substrate accessibility to active sites of the lipases when lipase/-support ratio was too high. Therefore, lipase/support ratio was fixed at 75 mg/g and the resulting immobilized MAS1 was used for the subsequent esterification reactions.

Low lipase activity recovery ratio (21.5%) and high protein recovery ratio (82%) were obtained under the optimized conditions of 75 mg/g (lipase/support ratio) and initial pH 8.0 of buffer. The specific activity of the immobilized MAS1 (24 U/mg) was therefore lower than that of the free lipase (125.3 U/mg). These could be explained that the interaction between the support and the lipase by physical absorption may be weak, resulting in enzyme leakage from the resins during the washing step of the immobilization process. In addition, losses of specific activity may be associated with the drying process of the resulting immobilized MAS1. More researches are needed to study the improvement of immobilization efficiency in future.

3.2. Properties of immobilized MAS1

3.2.1. Thermal stability

The thermal stability of free lipase MAS1 and immobilized MAS1 is shown in Fig. S1. The results indicated that the activity of immobilized MAS1 decreased more slowly than that of free lipase MAS1 when the two lipases were incubated at 65 °C for 3 h. The activity of free lipase MAS1 decreased very quickly at initial 30 min and its residual activity was 13% of its initial activity. After that, the activity of free lipase MAS1 decreased gradually and linearly as time prolonged. However, immobilized MAS1 was found to be more stable to heat. The immobilized MAS1 retained 88% and 45% of its initial activity after incubation at 65 °C for 30 min and 3 h, respectively. It could be concluded that the thermal stability of lipase MAS1 was greatly enhanced after immobilization.

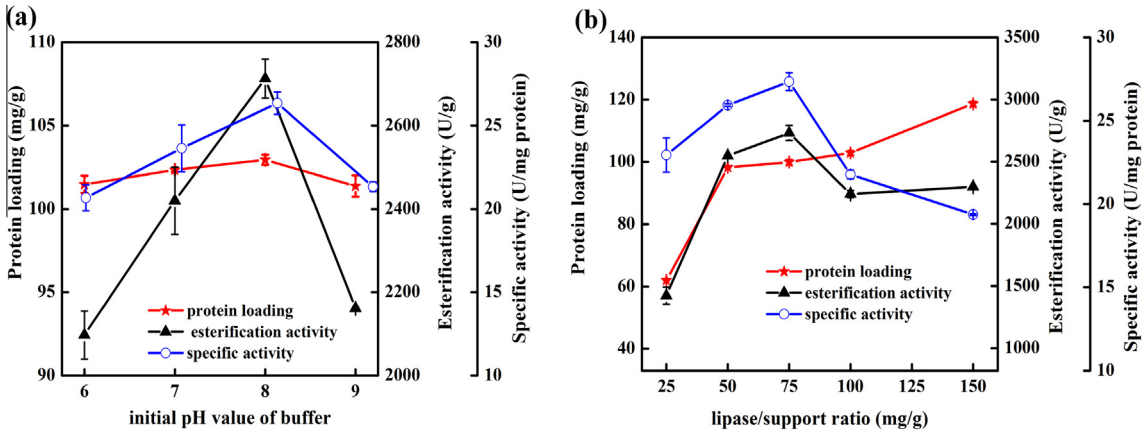

Fig. 2. Effects of initial pH of buffer (a) and lipase/support ratio (b) on immobilization efficiency.

3.2.2. Regiospecificity of immobilized MAS1

The results of free lipase MAS1 and immobilized MAS1-catalyzed hydrolysis of triolein are shown in Fig. S2. The ratio of 1,2(2,3)-DAG to 1,3-DAG in the immobilized MAS1-catalzyed reaction decreased from 0.79 to 0.68 when reaction time was prolonged from 1 to 10 min (Fig. S2b), which was not significantly ($p > 0.05$) higher than that (1,2-DAG/1,3-DAG ratio of 0.55–0.67) in the free lipase MAS1-catalzyed reaction (Fig. S2a). The results showed that immobilization process has no significant effect on the regiospecificity of lipase MAS1. The ratio of 1,2-DAG to 1,3-DAG in a non-regiospecific lipase AYS-catalyzed reaction ranged from 5.12 to 3.24 when reaction time was prolonged from 1 to 10 min (Fig. S2c). In contrast, the ratio of 1,2-DAG to 1,3-DAG in 1,3-regiospecific lipase rProROL-catalyzed reaction found to be 26.71 after 10 min of reaction (Li et al., 2015). These results indicated that immobilized MAS1 showed no regiospecificity towards triacylglycerols in the hydrolysis reaction.

3.2.3. Comparison of the catalytic properties of free lipase MAS1 and immobilized MAS1

The catalytic properties of free lipase MAS1 and immobilized MAS1 were compared in esterification of glycerol with n-3 PUFA for the synthesis of TAG and the results are given in Fig. S3. After 24 h of reaction, higher esterification degree (97.54%) and TAG content (92.37%) were obtained by immobilized MAS1 (Fig. S3b) than those (83.56% and 72.84%, respectively) by free lipase MAS1 (Fig. S3a). It could be also observed that DAG and MAG content (5.06% and 0.1%, respectively) synthesized by immobilized MAS1 were lower than those (10.41% and 0.29%) by free lipase MAS1. Therefore, the catalytic efficiency of lipase MAS1 was enhanced after immobilization.

3.3. Esterification of glycerol with n-3 PUFA using immobilized MAS1

3.3.1. Effect of temperature

The effect of temperature on the esterification reaction is shown in Fig. 3a. The esterification degree and TAG content initially increased with an increasing reaction temperature from 50 to 65 °C at 24 h. The maximum TAG content and esterification degree (65.3% and 75.6%, respectively) were observed at 65 °C. Then, the esterification degree and TAG content decreased when temperature was further increased from 65 to 70 °C. This may be explained by the protein denaturation of lipase MAS1 and the loss of its catalytic function when the reaction temperature was over 65 °C. There were no significant differences in DAG and MAG contents between the test temperatures. Therefore, 65 °C was selected for the subsequent experiments.

3.3.2. Effect of enzyme loading

Fig. 3b shows the effect of enzyme loading on the esterification reaction. The esterification degree increased from 75.6% to 95% after 24 h of reaction with increasing enzyme loading in the range of 750–1500 U. However, the esterification degree decreased slightly when enzyme loading further increased from 1500 to 2250 U. TAG content increased with increasing esterification degree. The maximum TAG content (89.4%) and highest esterification degree (95%) were achieved at 1500 U of enzyme loading. DAG and MAG contents kept almost constant at the test enzyme

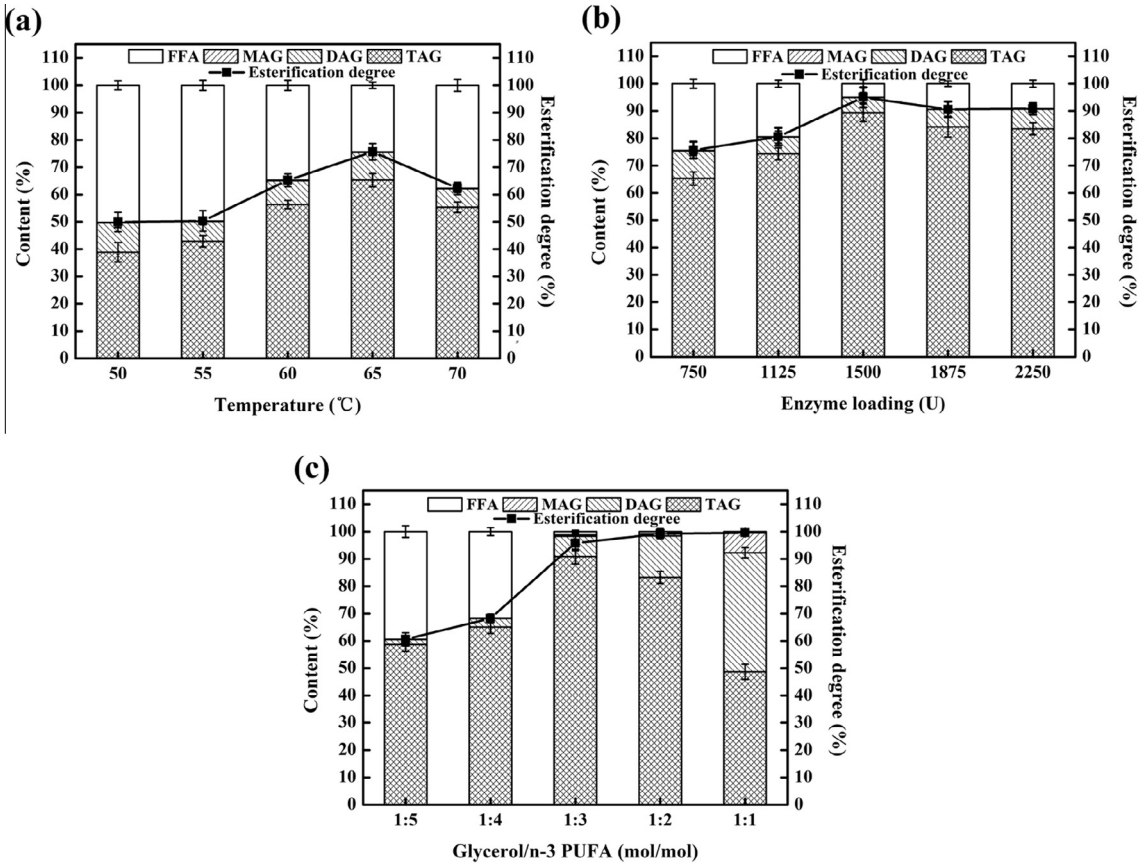

Fig. 3. Effects of temperature, enzyme loading and glycerol/n-3 PUFA molar ratio on esterification reaction. (a) Effect of temperature on esterification reaction. Reaction conditions: substrate molar ratio (glycerol: n-3 PUFA) of 1:3, 750 U of immobilized MAS1, 24 h of reaction under vacuum; (b) effect of enzyme loading on esterification reaction. Reaction conditions: substrate molar ratio (glycerol: n-3 PUFA) of 1:3, 65 °C of reaction temperature, 24 h of reaction under vacuum; (c) effect of glycerol/n-3 PUFA molar ratio on esterification reaction. Reaction conditions: 1500 U of immobilized MAS1, 65 °C of reaction temperature, 24 h of reaction under vacuum.

loading. This was probably because partial enzyme reached saturation and the reactions reached equilibrium when enzyme loading was high. Moreover, the reaction was reversible and a large number of the reaction products would inhibit the reaction when higher enzyme loading was used. Bhandari, Chaurasia, and Dalai (2015) reported that when the enzyme concentration was over 50 mg per 0.49 g fatty acids, TAG content decreased. Therefore, 1500 U was selected as the optimal enzyme loading for the subsequent experiments.

3.3.3. Effect of glycerol/n-3 PUFA molar ratio

Fig. 3c shows the effect of glycerol/n-3 PUFA molar ratio on esterification reaction. The esterification degree increased with increasing amount of glycerol. The maximum esterification degree (99.6%) was obtained at a molar ratio of 1:1 (glycerol to n-3 PUFA). However, TAG content initially increased when the glycerol/n-3 PUFA molar ratio varied from 1:5 to 1:3, then decreased when the molar ratio changed from 1:3 to 1:1. The maximum TAG content (90.8%) was achieved at a molar ratio of 1:3. Besides, DAG content increased with increasing amount of glycerol. However, there was no significant difference in MAG content between the test substrate molar ratio. This was probably because MAG and DAG were produced initially, and then TAG was synthesized in the esterification reaction. When the molar ratio of glycerol to n-3 PUFA was below 1:3, fatty acids were not enough to synthesize TAG. Similar results were also observed in some reports (Cerdán, Medina, Giménez, González, & Grima, 1998; Ergan, Trani, & André, 1990) where Lipozyme IM-20 and Novozym 435 were used to catalyze esterification of glycerol with FA. Therefore, the optimum molar ratio of glycerol to n-3 PUFA for the synthesis of TAG was 1:3.

3.4. Evaluation of the reusability of immobilized MAS1

Fig. S4 shows the results about the reusability of immobilized MAS1 and free lipase MAS1 for the production of TAG by esterification of n-3 PUFA with glycerol. TAG content synthesized by immobilized MAS1 reached 92.95% after the first run and still retained approximately 50% of its initial TAG content after five cycles. However, TAG content after the first reaction catalyzed by free lipase MAS1 was 72.29%. It declined to 5.94% after five runs, which was approximately 8.21% of its initial yield. The results indicated that immobilized MAS1 showed better reusability than free lipase MAS1 in esterification of n-3 PUFA with glycerol.

3.5. Comparison of the catalysis properties of immobilized MAS1 and Novozym 435

Novozym 435 is a commercial immobilized lipase with relatively high esterification activity. Although Novozym 435 may perform more efficiently under other conditions which has been optimized, esterification of glycerol with n-3 PUFA catalyzed by immobilized MAS1 and Novozym 435 were carried out under the conditions optimized in this study, respectively. The results are given in Fig. 4. After 24 h of reaction, esterification degree (99.31%) and TAG content (92.26%) obtained by immobilized MAS1 (Fig. 4a) were higher than those (82.16% and 47.26%, respectively) by Novozym 435 (Fig. 4b). It could be also observed that less DAG content (6.8%) and MAG content (0.23%) were obtained by immobilized MAS1 than those (33.31% and 1.41%, respectively) by Novozym 435. Besides, the composition of glycerides synthesized by immobilized MAS1-catalyzed esterification was similar to that of natural fish oils containing major proportion of TAG

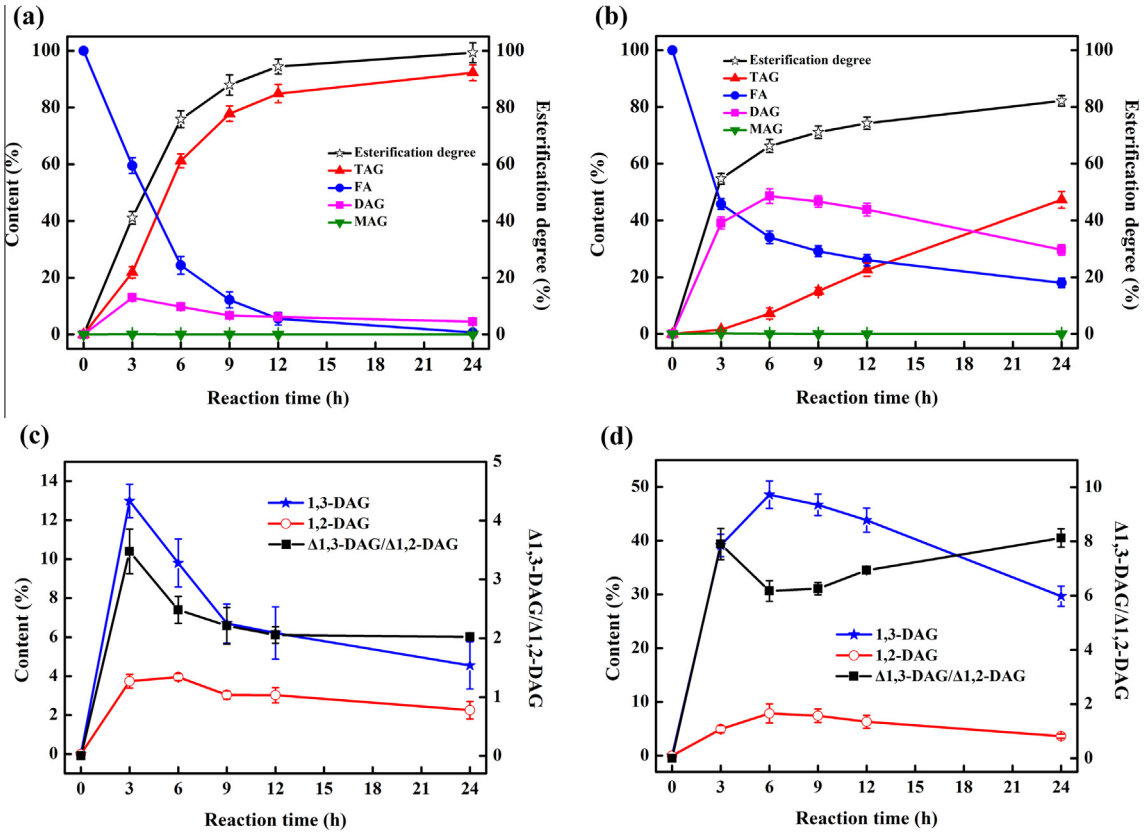

Fig. 4. Time course of esterification of glycerol with n-3 PUFA by (a) immobilized MAS1 and (b) Novozym 435. Time course of 1,3-DAG and 1,2-DAG content synthesized by (c) immobilized MAS1 and Novozym 435 (d). Reaction conditions: substrate molar ratio (glycerol: n-3 PUFA) of 1:3, 1500 U of immobilized MAS1 and Novozym 435, 65 °C of reaction temperature, 24 h of reaction under vacuum.

Table 2
FA composition of n-3 PUFA and n-3 PUFA-rich TAG synthesized by immobilized MAS1 and Novozym 435-catalyzed esterification.

Fatty acids	Free n-3 PUFA	n-3 PUFA-rich TAG	
		Immobilized MAS1	Novozym 435
C12:0	0.1 ± 0.02	0.42 ± 0.03	0.15 ± 0.10
C14:0	0.13 ± 0.01	0.12 ± 0.01	0.10 ± 0.09
C16:0	0.19 ± 0.04	0.91 ± 0.03	0.58 ± 0.10
C18:0	0.22 ± 0.10	0.53 ± 0.10	0.41 ± 0.12
C18:1	1.87 ± 0.08	1.69 ± 0.21	1.78 ± 0.10
C18:2	0.52 ± 0.17	0.55 ± 0.12	0.53 ± 0.20
C18:3	0.94 ± 0.05	0.69 ± 0.01	0.93 ± 0.01
C20:5	38.81 ± 0.43	38.80 ± 0.34	38.67 ± 0.23
C22:5	6.37 ± 0.21	6.40 ± 0.17	6.32 ± 0.09
C22:6	45.19 ± 0.31	45.02 ± 0.22	44.98 ± 0.27
Other FAs	5.66 ± 0.41	4.87 ± 0.21	5.55 ± 0.33
Total n-3 PUFA	90.37 ± 0.32	90.22 ± 0.56	89.97 ± 0.45

and minor proportions of DAG and MAG. More importantly, the esterification degree of the reaction catalyzed by immobilized MAS1 reached 99.31%, which indicated that the complete conversion from n-3 PUFA to n-3 PUFA-rich TAG was almost achieved. Therefore, immobilized MAS1 is a promising biocatalyst for the synthesis of n-3 PUFA-rich TAG.

As shown in Table 2, the FA composition of the substrate (free n-3 PUFA) mainly consisted of 38.81% of EPA, 6.37% of DPA, and 45.19% of DHA. After 24 h of reaction, the FA composition of n-3 PUFA-rich TAG synthesized by immobilized MAS1 and Novozym 435-catalyzed esterification was similar to that of the substrate. The results showed that immobilized MAS1 may show similar FA-specificity to Novozym 435. Similar results were reported by Cerdán et al. (1998), who found that all the FA were incorporated into TAG in the same proportion in the esterification reaction catalyzed by Novozym 435.

As shown in Fig. 4, DAG and MAG contents in the reaction mixture catalyzed by immobilized MAS1 were very low and not accumulated at any time of reaction. DAG content was lower than TAG content and higher than MAG content throughout the whole reaction. The results indicated that the formation of FA → MAG → DAG → TAG reactions catalyzed by immobilized MAS1 were spontaneous and fast, resulting in a significantly higher TAG content. However, during Novozym 435-catalyzed esterification of glycerol with n-3 PUFA, DAG content was higher than MAG and TAG contents in the first 6 h of reaction time. TAG content increased with decreasing DAG content as reaction time increased. MAG content in the reaction mixture catalyzed by Novozym 435 was almost negligible. DAG content was higher than MAG content during the time course of the reaction. The results indicated that the conversion from MAG to DAG was spontaneous but the conversion from DAG to TAG was not spontaneous. A similar phenomenon was observed by Haraldsson, Gudmundsson, and Almarsson (1995) using Novozym 435. Therefore, DAG accumulation model in the esterification reaction catalyzed by immobilized MAS1 and Novozym 435 was different. The process of the conversion from DAG to TAG was found to be a rate-limiting step in the enzymatic synthesis of n-3 PUFA-rich TAG. Difference in the conversion from DAG to TAG led to different formation rate of TAG by different immobilized lipases. In this work, more than 92% n-3 PUFA was incorporated into TAG by immobilized MAS1.

As shown in Fig. 4c, the highest contents of 1,3-DAG (12.99) and 1,2-DAG (3.74) were obtained by immobilized MAS1 at 3 h where Δ1,3-DAG/Δ1,2-DAG value reached a maximum (3.47). After that, the Δ1,3-DAG/Δ1,2-DAG value, the contents of 1,3-DAG and 1,2-DAG decreased as the reaction proceeded. The decreased Δ1,3-DAG/Δ1,2-DAG value (2.02) at 24 h indicated that a weak acyl transfer occurred during the reaction process. Fig. 4d showed that the contents of 1,3-DAG and 1,2-DAG synthesized by Novozym 435 reached a maximum at 6 h where Δ1,3-DAG/Δ1,2-DAG value was 6.17. The Δ1,3-DAG/Δ1,2-DAG value initially increased and then decreased during the first 6 h. However, the Δ1,3-DAG/Δ1,2-DAG value increased again after 6 h. The maximum of Δ1,3-DAG/Δ1,2-DAG value obtained by immobilized MAS1 was lower than that obtained by Novozym 435. The results provided further evidence that immobilized MAS1 had no regiospecificity in the reaction system investigated. More researches are needed to study the regioselectivity of immobilized MAS1 in different reaction systems.

4. Conclusions

Lipase MAS1 was successfully immobilized onto XAD1180 resin by FT-IR spectra analysis and the best specific activity (24 U/mg) was obtained under the conditions of 75 mg/g (lipase/resin ratio) and initial buffer pH of 8.0. Interestingly, immobilized MAS1 displayed higher thermal stability and catalytic activity than its free form. Highly pure n-3 PUFA-rich TAG was successfully achieved by only one-step using immobilized MAS1-catalyzed esterification of glycerol with n-3 PUFA. The highest esterification degree (99.31%) and TAG content (92.26%) synthesized by immobilized MAS1 were achieved under the optimal conditions (glycerol/n-3 PUFA molar ratio of 1:3, 1500 U of enzyme loading and 65 °C). Comparing to Novozym 435, immobilized MAS1 exhibited better catalytic efficiency due to the different DAG accumulation model. In addition, immobilized MAS1 showed better reusability than its free form during five cycles of batch esterification of n-3 PUFA with glycerol. In conclusion, the results showed that immobilized MAS1 is a promising biocatalyst for the modification of oils and fats. The findings of this study provide useful information for further research involving lipase MAS1.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.foodchem.2016.08.041.

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