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Xiumei Wang^a, Daoming Li^b, Weifei Wang^c, Bo Yang^a, Yonghua Wang^{b,*}

^a School of Bioscience and Bioengineering, South China University of Technology, Guangzhou, 510006, China

^b School of Food Science and Engineering, South China University of Technology, Guangzhou, 510640, China

^c School of Chemistry and Chemical Engineering, South China University of Technology, Guangzhou, 510640, China

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ABSTRACT

This study reported that immobilized MAS1 lipase showed high catalytic efficiency in the production of triacylglycerols (TAG) highly enriched with n-3 polyunsaturated fatty acids (PUFA) by glycerolysis of ethyl esters (EE). Immobilized MAS1 lipase was found to have no regiospecificity and be a more suitable catalyst for the glycerolysis of n-3 PUFA-rich EE compared with other enzymes. Higher TAG content (73.9%) and EE conversion (82%) were obtained by immobilized MAS1 lipase than those by Novozym 435 (29.6% and 54.8%, respectively) and Lipozyme RM IM (10% and 49%, respectively). Besides, the effects of temperature, enzyme loading and n-3 PUFA-rich EE/glycerol molar ratio on TAG content were evaluated using response surface methodology. The results showed that temperature, enzyme loading and n-3 PUFA-rich EE/glycerol molar ratio had significant effects on TAG content. The maximum TAG content (76.5%) was achieved under the optimal conditions (enzyme loading of 163.8 U/g substrate, n-3 PUFArich EE/glycerol molar ratio of 4.13:1 at 65 °C). Subsequently, the glycerolysis reaction mixtures were further purified by molecular distillation and highly pure n-3 PUFA-rich TAG (96.2%) with similar fatty acids composition to the substrate (EE) was obtained in the final products. In addition, the obtained final products had low acid value and peroxide value (0.03 mg KOH/g and 3.2 meg/kg, respectively). These results indicated that immobilized MAS1 lipase is a promising catalyst for the synthesis of TAG in industrial application.

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1. Introduction

The health benefits of n-3 polyunsaturated fatty acids (PUFA), especially eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), in the prevention and reduction of some diseases are well recognized in recent decades [1–4]. Besides, many studies have showed that n-3 PUFA-rich triacylglycerols (TAG) are the most desirable form for human nutrition and disease prevention because ethyl esters (EE) are less absorbed and free n-3 PUFA are more easily oxidized [5,6]. Therefore, production of TAG enriched in n-3 PUFA is required in the pharmaceutical and food industry.

Preparation of n-3 PUFA-rich TAG can be carried out using chemical or enzymatic approaches. Compared with chemical methods, enzymatic synthesis of TAG has attracted great attention due to its beneficial advantages such as mild operation conditions, wide fatty acid selectivity, high catalytic efficiency, high substrate specificity and positional selectivity [7,8]. Enzymatic glycerolysis of EE

* Corresponding author. E-mail addresses: 18814123260@163.com, yonghw@scut.edu.cn (Y. Wang).

http://dx.doi.org/10.1016/j.molcatb.2016.09.012 1381-1177/© 2016 Elsevier B.V. All rights reserved. and enzymatic esterification of n-3 PUFA with glycerol are the most commonly used methods for the production of TAG [9-12]. Nevertheless, n-3 PUFA are easy to oxidize, polymerize and isomerize during the reactions [13,14]. Besides, n-3 PUFA-rich EE are the main available form of high grade purity PUFA found in the market. Therefore, lipase-catalyzed glycerolysis of EE is used to synthesize n-3 PUFA-rich TAG in industry.

Although immobilized lipases from various sources [*Thermomyces lanuginose* (TLL), *Candida antarctica* (lipase B, Novozym 435), *Pseudomonas fluorescens* (PFL), *Rhizopus delemar* (RDL), *Rhizomucor miehei* (RML), etc.] were used in the glycerolysis reactions, there were still much monoacylglycerols (MAG) and diacylglycerols (DAG) produced during the synthesis of TAG, resulting in a relatively low TAG yield [11,12,15–18]. Thus, ongoing research is attempting to explore novel lipases for the synthesis of high content of n-3 PUFA-rich TAG through glycerolysis of EE.

In this paper, lipase MAS1 from marine *Streptomyces* sp. strain W007 [19] was immobilized onto XAD1180 resin and its ability to catalyze glycerolysis of EE for the synthesis of n-3 PUFA-rich TAG was investigated. Firstly, effect of temperature on the activity of immobilized MAS1 lipase and regiospecificity of immobilized

MAS1 lipase were investigated. Then, immobilized MAS1 lipase, Novozym 435 and Lipozyme RM IM were used as biocatalysts for the synthesis of n-3 PUFA-rich TAG by glycerolysis of EE in the solvent-free system under vacuum. Subsequently, modeling and optimization of reaction conditions (temperature, enzyme loading and n-3 PUFA-rich EE/glycerol molar ratio) were attempted for the glycerolysis reactions catalyzed by immobilized MAS1 lipase using response surface methodology (RSM). Finally, the glycerolysis reaction mixture was purified using molecular distillation and the properties of the final products were also studied.

2. Materials and methods

2.1. Materials

The pPICZ α A-MAS1-X33 expression strain was stored at -80 °C in our laboratory. Novozym 435 and Lipozyme RM IM were obtained from Novo Nordisk (Denmark). DHA/EPA-rich EE were kindly provided by Sinomega Biotech Engineering Co., Ltd. (Zhejiang, China). Standards of monooleoylglycerol, dioleoylglycerol (15% of 1,2-dioleoylglycerol and 85% of 1,3-dioleoylglycerol), trioleoylglycerol, and 37-component fatty acid methyl esters (FAME) mix (C4-C24) were sourced from Sigma-Aldrich. *n*-Hexane, 2-propanol and formic acid of chromatographic grade were purchased from Kermel Chemical Reagent Co., Ltd. (Tianjin, China). The Amberlite XAD1180 resin was acquired from Rohm and Haas Company (USA). All other chemicals were of analytical grade unless otherwise stated.

2.2. Production of crude lipase MAS1

The pPICZ α A-MAS1-X33 expression strain was used to produce lipase MAS1 and fermentation was carried out in a 30-L fermentor according to the method described by Lan et al. [20]. After fermentation, the supernatant was collected by centrifugation. Then, the supernatant containing lipase MAS1 was concentrated and used for the subsequent immobilization. The hydrolytic activity of crude lipase MAS1 was 953.3 U/mL and its specific activity was 202 U/mg according to the method described previously [21].

2.3. Preparation of immobilized MAS1 lipase

The resin XAD1180 (20 g) was added to a 2000 mL-conical flask containing lipase MAS1 solution (75 mg protein/g resin) and an equal volume of sodium phosphate buffer (0.02 M, pH 8.0). Then, the mixture was stirred at a temperature of 30 °C and a speed of 200 rpm for 8 h. Subsequently, the obtained immobilized MAS1 lipase was washed with sodium phosphate buffer (0.02 M, pH 8.0) repeatedly until no protein was detected in the eluate. Finally, the obtained immobilized MAS1 lipase was dried under vacuum at 40 °C for 8 h and stored in closed vials at 4 °C before use. The esterification activity of immobilized MAS1 lipase was 2002 \pm 6.1 U/g according to Novozymes Standard Method [22].

2.4. Effect of temperature on the activity of immobilized MAS1 lipase

The effect of temperature on the hydrolytic activity of immobilized MAS1 lipase was evaluated at temperatures varying from 50 to 90 °C at pH 7.0. The hydrolytic activity was determined according to the method described previously [21].

2.5. Regiospecificity of immobilized MAS1 lipase

The hydrolysis reactions of triolein catalyzed by immobilized MAS1 lipase were performed according to the method of Li et al. [23] with a slight modification. The hydrolysis reactions were carried out in tightly colsed, screw-capped glass vials (10 mL) containing 1 g triolein, enzyme amount of 30 U/g oil and 0.2 g sodium phosphate buffer (0.1 M, pH 7.0). Then, the sample vials were incubated at 65 °C with a speed of 200 rpm for 10 min. The experiments about the hydrolysis of triolein catalyzed by lipase AYS were carried out as a control at a temperature of 40 °C. Samples were withdrawn periodically for high-performance liquid chromatography (HPLC) analysis. The regiospecificity of the lipases was assessed by the 1,2-DAG/1,3-DAG value. The hydrolytic activities of immobilized MAS1 lipase and lipase AYS were measured as described in Section 2.4.

2.6. Glycerolysis of n-3 PUFA-rich EE

The glycerolysis reactions were carried out in a glass vessel containing 10 g substrate. The reactions catalyzed by different amounts of immobilized lipases were incubated at various temperatures with a speed of 200 rpm under vacuum for 24 h. Samples were withdrawn at selected times for HPLC analysis.

2.7. Experimental design for glycerolysis of n-3 PUFA-rich EE using RSM

A three-level-three-factor RSM based on Box-Behnken design was employed to optimize the glycerolysis reactions catalyzed by immobilized MAS1 lipase. Temperature (A), enzyme loading (B) and substrate molar ratio (C) were three independent variables selected for the optimization of the glycerolysis reactions. TAG content (Y) was chosen as the response of the experiments. The actual and coded levels of three independent variables are shown in Table S1. Seventeen experiments containing five replications at the center points were carried out to determine the pure error. All experiments were performed in a randomized order to minimize the effects of unexplained variability in the observed response due to extraneous factors. The range of each factor level was determined according to the results of preliminary experiments using a single-factor test.

2.8. Purification of n-3 PUFA-rich TAG by molecular distillation

In order to obtain n-3 PUFA-rich TAG with high purity, the glycerolysis reaction mixture obtained under the optimized conditions was further purified using molecular distillation with a short path falling film distiller (MD-S80, Hanwei Co., Ltd., Guangzhou, China). The conditions of the purification process were as follows: a feeding temperature of 60 °C, an evaporating temperature of 175 °C, a condenser temperature of 35 °C, a pressure of 3.0 Pa, a feed flow rate of 2 g/min, and a scraper speed of 250 rpm. After molecular distillation, a large amount of TAG with a little DAG were acquired in the residues (final products) and EE with MAG from the reaction mixture were collected in the distillates.

2.9. Analysis of the composition of the reaction mixture by HPLC

The composition of the reaction mixture was separated and quantified using a normal-phase HPLC equipped with a refractive index detector and a Phenomenex Luna column (250 mm × 4.6 mm i.d., 5 μ m particle size, Phenomenex Corporation). The detailed analysis was carried out according to the method described by Wang et al. [24] with a slight modification. The mobile phase was a mixture of *n*-hexane, isopropanol with formic acid (21:1:0.003, by volume) and its flow rate was 1 mL/min. Peaks in HPLC were identified by comparison of their retention times with those known standards. Retention times were 3.10 (TAG), 3.34 (EE), 4.63 (1,3-DAG), 5.86 [1,2(2,3)-DAG], 29.51 [1(3)-MAG], 37.93 (2-MAG).

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