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Production of extremely pure diacylglycerol from soybean oil by lipase-catalyzed glycerolysis

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ABSTRACT

Research work was objectively targeted to synthesize highly pure diacylglycerol (DAG) with glycerolysis of soybean oil in a solvent medium of *t*-butanol. Three commercial immobilized lipases (Lipozyme RM IM, Lipozyme TL IM and Novozym 435) were screened, and Novozym 435 was the best out of three candidates. Batch reaction conditions of the enzymatic glycerolysis, the substrate mass ratio, the reaction temperature and the substrate concentration, were studied. The optimal reaction conditions were achieved as 6.23:1 mass ratio of soybean oil to glycerol, 40% (w/v) of substrate concentration in *t*-butanol and reaction temperature of 50 °C. A two-stage molecular distillation was employed for purification of DAG from reaction products. Scale-up was attempted based on the optimized reaction conditions, 98.7% (24 h) for the conversion rate of soybean oil, 48.5% of DAG in the glycerolysis products and 96.1% for the content of DAG in the final products were taken in account as the results.

1. Introduction

In recent years, diacylglycerols (DAG) are widely recognized as functional oils for the prevention of obesity and of other lifestyle-related diseases. The human health concerns of DAG oils are paid central attention and debated along with different metabolic fates versus TAG [1]. It was found that the postprandial triacylglycerols (TAG) levels in serum was decreased and the accumulation of TAG in body fat was being suppressed as DAG oil was in-taken by people [2]. Diacylglycerols, as minor natural component of various edible oils, have been manufactured as health promoting cooking oil in Japan since 1999, and have been approved by United States Food and Safety Administration. The DAG cooking oil was promoted throughout in the United States in 2005 [3]. The oil contains approximately 80% of DAG in weight and TAG match the rest.

Many researchers have focused on the enzymatic production of DAG by esterification, glycerolysis and partial hydrolysis of oils and fats because of the beneficial advantages of employing enzymes such as mild reaction conditions, high regioselectivity and high catalysis efficiency [4]. In lipase-catalyzed esterification, DAG is synthesized through esterification of free fatty acid (FFA) and glycerol with simultaneous removal of water. However, high levels of free fatty acid are needed [5]. A great amount of FFA is generated during the lipase-catalyzed partial hydrolysis and DAG yield is relatively low at the end of DAG production [6]. Comparatively, glycerolysis of natural fats and oils is a prospective process for producing DAG products.

At present, only few reports are available designing the synthesis of DAG by enzymatic glycerolysis as the preliminary goal [7–11]. One significant drawback of enzymatic glycerolysis of oils is the adsorption between the glycerol and the lipases. Kristensen et al. [7] screened seven lipases for their abilities to synthesizing DAG by glycerolysis of rapeseed oil and found that the lipase immobilization carrier was of great importance for catalysis in batch reactions. Glycerol can be easily adsorbed on the carrier to form a layer around the hydrophilic lipase carrier, which limits the contact between the lipases and the hydrophobic oil phase. Since the lipase is immobilized by a hydrophobic carrier – Novozym 435, the catalyzed product mixture contained 60% DAG and 30% TAG residuals. Yeoh et al. [8] studied the influence of silica gel on production of DAG via enzymatic glycerolysis of palm olein. In the absence of silica, no reaction was observed for Lipozyme TL IM and Lipozyme RM IM (immobilized on hydrophilic materials), but a yield of DAG between 45 and 52% was achieved after the addition of silica. Glycerolysis of fats and oils in solvent system alleviate the adsorption between lipase and glycerol. Valério et al. [11] reported the production of MAG and DAG via enzymatic glycerolysis of olive oil in a solvent medium of pressurized n-butane. High extent of conversion of olive oil was achieved in a short reaction time, but MAG took ~70% possession. Further work is required to develop enzymatic glycerolysis process to produce DAG more effectively.

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The research of this paper was aimed to develop an efficient process for producing high-purity DAG via enzymatic glycerolysis reaction of soybean oil in *t*-butanol. Three commercial immobilized lipases (Lipozyme RM IM, Lipozyme TL IM and Novozym 435) were assessed. The influence of conditions that covered substrate mass ratio, substrate concentration and temperature was studied. Short path molecular distillation was applied for purification of DAG.

2. Materials and methods

2.1. Materials

Lipozyme RM IM (immobilized *Rhizomucor miehei*, enzyme activity 5.9 Batch Acidolysis Units Novo/g), Lipozyme TL IM (immobilized *Thermomyces lanuginose*, enzyme activity 175 Interesterase Units Novo/g) and Novozym 435 (immobilized *Candida antarctica*, enzyme activity 12,000 propyl-laurate units/g) were supplied by Novozymes A/S (Bagsvaerd, Denmark). Isopropyl alcohol (99.8%), n-hexane (97%), *t*-butanol (99%), glycerol (99.5%, included 0.2% water) were provided by Guangzhou Chemical Reagent Factory (Guangzhou, China). Methanolic boron trifluoride solution (12–15% as BF_3) and the standards of 1(3)-monooleoyl-rac-glycerol, 1,3-dioleoyl glycerol, 1,2-dioleoyl glycerol and trioleoyl glycerol for HPLC analysis reference were sourced from Sigma–Aldrich (China). Soybean oil was supplied by Kerry Oils & Grains Ltd. (Shenzhen, China), which represented 0.05% of water, 0.06% of FFA, peroxide value 1.5 mequiv./kg. Triglyceride fatty acid compositions were 10.19% C16:0, 6.34% C18:0, 24.21% C18:1, 54.32% C18:2, 4.01% C18:3 and 0.83% (w/w) of others.

2.2. Enzymatic glycerolysis of soybean oil

The glycerolysis was carried out in a 100 mL conical flask with a solvent medium of *t*-butanol and dissolved glycerol. Soybean oil (triacylglycerols) was charged in the flask, and lipase was followed when the reaction temperature reached the desired level. The reactants were well blended in an orbital shaking water-bath operating at 200 rpm for 36 h. Aliquot fractions (0.1 mL) of reaction mixture were periodically sampled during the reaction process for HPLC analysis. Each glycerolysis experiment was conducted in triplicate and the results were expressed as the average of weight percentage of total acylglycerols.

The conversion of TAG (soybean oil) and the content of DAG (Eq. (1)) in the products of glycerolysis were analyzed against reaction time.

$$DAG\ \ content\ \ (\%) = \frac{DAG\ \ (\%)}{MAG\ \ (\%) + DAG\ \ (\%) + TAG\ \ (\%)} \times 100 \quad (1)$$

2.2.1. Screening of lipases

Three commercial immobilized lipases (Lipozyme RM IM, Lipozyme TL IM and Novozym 435) were screened for the enzymatic glycerolysis of soybean oil. The glycerolysis reactions were carried out via incubating 1 wt% (weight percentage of soybean oil) lipase, with 9.35:1 substrate mass ratio of soybean oil to glycerol and 30% (w/v) of total substrate concentration in *t*-butanol, at 40 °C for 36 h.

2.2.2. Effect of substrate mass ratio on the production of DAG

Mass ratio of soybean oil to glycerol of 18.70:1, 9.35:1, 6.23:1, 4.67:1 and 3.74:1 were studied for their effects on the production of DAG. The total amount of reaction substrate of 9 g (soybean oil and glycerol) was maintained constantly in 30 mL *t*-butanol with a substrate concentration of 30% (w/v). The glycerolysis reactions were undertaken with 1 wt% of lipase at 40 °C for 36 h.

2.2.3. Effect of reaction temperature on the production of DAG

The effect of temperatures (30 °C, 40 °C, 50 °C and 60 °C) on the glycerolysis reactions was evaluated over 36 h with fixed reaction conditions consisting of 1 wt% (weight percentage of soybean oil) lipase, 6.23:1 substrate mass ratio of soybean oil to glycerol and 30% (w/v) of total substrate concentration in *t*-butanol.

2.2.4. Effect of substrate concentration on the production of DAG

The effect of substrate concentration in the solvent medium on the production of DAG was also investigated. 20%, 30%, 40% and 50% (w/v) were chosen for the substrate concentration in *t*-butanol respectively. The total reaction substrate (soybean oil and glycerol with a mass ratio of 6.23:1) was kept constant in different volumes of *t*-butanol as the reaction was carried out with 1 wt% of lipase at 50 °C.

2.3. Evaluation of reusability of lipase

The reusability of the employed immobilized lipase system was evaluated to ascertain the stability of the biocatalyst in the glycerolysis reaction. The lipase was recycled for application in a batch reactor for 15 times. The stability of the lipase was assessed by comparing the data of the DAG contents from batch to batch.

2.4. Purification of DAG by molecular distillation

Organic solvent (*t*-butanol) remained in the products from the glycerolysis of soybean oil were recovered by vacuum distillation using a rotary evaporator. To obtain a highly pure DAG, molecular distillation with a short path falling film distiller (MD-S80, manufactured in Handway Co. Ltd., Guangzhou, P.R. China) was undertaken in two steps. The first step was set to remove the glycerol and residual solvent. An evaporation temperature of 110 °C, a feed flow rate of 3 g/min, a pressure of 10 Pa and a scraper speed of 250 rpm were prepared for the removal process. The second step was to remove MAG generated due to glycerolysis and the conditions were a feed flow rate of 2 g/min, system pressure of 1 Pa, condenser temperature of 40 °C, scraper speed of 250 rpm and an evaporation temperature of 180 °C.

2.5. Scale-up reaction

The scale-up experiments, about 50 folds, of producing highly pure DAG by enzymatic glycerolysis of soybean oil were performed to verify the feasibility of the whole process at a larger scale. 500 g soybean oil was used in the glycerolysis under the optimized batch reaction conditions of substrate mass ratio, reaction temperature, substrate concentration and enzyme load of 1 wt%. The reaction was launched in 200 rpm shaking water bath for 36 h. Samples were collected periodically and consequently for HPLC analysis.

2.6. HPLC analysis of acylglycerols in the reaction mixture

The compositions of the compounds from the glycerolysis reaction and from the molecular distillation were analyzed by the high-performance liquid chromatography (Waters 1525) and refractive index detector (HPLC-RID). Lipids were separated over a Phenomenex Luna column (Phenomenex Corporation, 250 mm × 4.6 mm i.d., 5 μm particle size) at a column temperature of 35 °C. The mobile phase was a mixture of n-hexane and isopropanol (10:1 v:v) with a flow-rate of 1.0 mL/min. A 0.1 mL sample of the reaction mixture was transferred into a centrifuge tube, and 1 mL of n-hexane and isopropyl alcohol (10:1, v/v) was mixed with by vortex. The mixture was centrifuged at 10,000 × *g* for 1 min to separate the glycerol out. 10 μL of supernatant was used for HPLC analysis. Peaks in HPLC were identified by comparison of their retention times with reference standards. Acquisition and processing of data were made using the instrument integrated software.

Analysis was repeated for triplication, and the result was presented as the average of triplicate measurements of the samples. The content of DAG in the final products was determined with Eq. (1).

2.7. Analysis of fatty acid compositions of acylglycerols

The acylglycerols were methylated according to ISO 5509:2000(E) (Animal and vegetable fats and oils – preparation of methyl esters of fatty acids) [12]. The sample was introduced into a 50 mL flask with addition of 4 mL of 0.5 mol/L methanolic sodium hydroxide solution and 5 mL of methanolic boron trifluoride solution (12–15% BF_3), and was boiled under reflux for 10 min. 3 more minutes was given for the boiling. And 2 mL of isooctane was added to the boiling mixture from the top of the condenser. The flask was removed immediately and 20 mL of saturated sodium chloride solution was charged in. The flask was covered and shaken vigorously for at least 15 s. More saturated sodium chloride solution was measured in to make a 50 mL solution in the flask. After the separation of the two phases (the isooctane phase and the saturated sodium chloride solution phase), 1 mL of the upper isooctane layer was pipetted to a 4 mL vial and a small amount of anhydrous sodium sulfate was added for the removal of water marks.

A Hewlett-Packard 7890 gas chromatograph was used to analyze the fatty acid composition of methyl esters obtained from esterification. The products were separated on a FFFAP column (PERMABOND-FFFAP DF-0.25, 25 m × 0.25 mm i.d., Macherey-Nagel, Germany) using nitrogen as the carrier gas. A temperature program was built to keep the samples in a column oven at 150 °C for 2 min. The temperature was increased to 230 °C and held for 8 min for a total running time of 18 min. The split ratio of 20:1 was used. The temperatures of the injector and the flame ionization detector were set at 250 °C and 300 °C, respectively. Standard heptadecanoic acid (17:0) (Sigma–Aldrich) was used for the qualitative determination of the fatty acids.

3. Results and discussion

3.1. Screening of lipases

The effect of three immobilized enzymes on the conversion of TAG from the glycerolysis of soybean oil is represented in Fig. 1. From Fig. 1, the conversion of TAG reached to 92.14%, after glycerolysis of 24 h with the employment of Novozym 435, much higher than those obtained with the other. The effect of enzymes on the DAG content in the mixture of acylglycerols, products of glyc-