Journal of Molecular Catalysis B: Enzymatic 72 (2011) 157–162

Contents lists available at ScienceDirect

Journal of Molecular Catalysis B: Enzymatic

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

Optimization of *Candida* sp. 99-125 lipase catalyzed esterification for synthesis of monoglyceride and diglyceride in solvent-free system

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ARTICLE INFO

Article history:
Received 21 November 2010
Received in revised form 11 April 2011
Accepted 30 May 2011
Available online 22 June 2011

Keywords:
Monoglyceride
Diglyceride
Lipase
Esterification
Beta-cyclodextrin

ABSTRACT

Esterification of glycerol and oleic acid catalyzed by lipase *Candida* sp. 99-125 was carried out to synthesize monoglyceride (MAG) and diglyceride (DAG) in solvent-free system. Beta-cyclodextrin as an assistant was mixed with the lipase powder. Six reaction variables, initial water content (0–14 wt% of the substrate mass), the glycerol/oleic acid molar ratio (1:1–6:1), catalyst load (3–15 wt% of the substrate mass), reaction temperature (30–60 °C), agitator speed (130–250 r/min) and beta-cyclodextrin/lipase mass ratio (0–2) were optimized. The optimal conditions to the synthesis of MAG and DAG were different: the optimal glycerol/oleic acid molar ratio, beta-cyclodextrin/lipase mass ratio, catalyst load and reaction temperature were 6:1, 0, 5%, 50 °C for MAG, and 5:1, 1.5, 10%, 40 °C for DAG, respectively. The optimal water content and agitator speed for both MAG and DAG were 10% and 190 r/min, respectively. Under the optimal conditions, 49.6% MAG and 54.3% DAG were obtained after 8 h and 4 h, respectively, and the maximum of 81.4% MAG plus DAG (28.1% MAG and 53.3% DAG) was obtained after 2 h under the DAG optimal condition. Above 90% purity of MAG and DAG can be obtained by silica column separation.

1. Introduction

Monoglycerides (MAG) and diglycerides (DAG) are the most widely used emulsifiers in food and pharmaceutical industries [1]. Furthermore, they have a generally recognized as safe status [2] and some newly uncovered beneficial effects and nutritional properties had been reported, such as the antimicrobial activities of monolaurin, monomyristin, monolinolein, and monolinolenin [3], the weight reducing function of 1,3-diglyceride [4], which contributes to their larger application. Current processes for MAG and DAG production consist of the continuous chemical glycerolysis of fats and oils at high temperatures (220–250 °C) employing inorganic alkaline catalysts under a nitrogen gas atmosphere, and the products are purified through high-vacuum distillation [5]. The major drawbacks of this process include high-energy consumption, low yield, and poor product quality [2].

The replacement of inorganic catalysts by lipases (E.C. 3.1.1.3) in the synthesis of partial glycerides, has advantages of catalysis at lower temperatures which prevents the discoloration and avoids side product formation [6,7], less polluting and energy consuming, moreover, it can produce glycerides with unsaturated fatty acids that is commonly difficult by chemical methods [2]. The three most important processes for the preparation of MAG and DAG catalyzed by lipases are the glycerolysis [8–11], the hydrolysis or alcoholysis of triglycerides [12–14], and the direct esterification of glycerol with fatty acids [6,7,15,16]. S. Ferreira-Dias et al. [17] obtained 43–45% MAG and 20% DAG by two commercial immobilized lipases (Lipozyme IM and Novozym 435) catalyzing the glycerolysis of olive residue oil in n-hexane. Esteban et al. [12] synthesized 2-MAG by enzymatic alcoholysis of fish oils using stirred tank (STR) and packed bed (PBR) reactors, and 63–65% 2-MAG were obtained in the STR operated in discontinuous mode. Duan et al. [18] reported that 40% 1,3-DAG was achieved by Novozym 435-catalyzed esterification in t-butanol system. Watanabe et al. [15] found that esterification catalyzed by Lipozyme RM IM was effectively performed by circulating the reaction mixture between a packed bed column and a water removal vessel, highest 1,3-DAG content of around 70% was obtained.

However, much research focused on the single MAG or DAG, the application of their mixtures is more extensive and varies with the proportion of MAG and DAG. Moreover, commercial lipases reported for the production of DAG and MAG are expensive, such as Lipozyme RM IM and Novozym 435. Previous work demonstrated that a low-cost self-established lipase from *Candida* sp. 99-125 which was produced industrially with a commercial name of LS-20 was effective to catalyze the esterification for fatty esters such as biodiesel and 2-ethylhexyl palmitate production from fatty acids [19–21], but its application to synthesize MAG and DAG was discussed rarely in former research. The preparation of lipase was detailed in previous reports [19–21] and it can be used in the food production through safety test.

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doi:10.1016/j.molcatb.2011.05.014

Although immobilized lipase in many cases can hyperactivate lipases and also can improve it stability, modulate it specificity and allow reutilization, almost no esterification of glycerol and oleic acid catalyzed by immobilized *Candida* sp. 99-125 lipase was noted in our previous study, this maybe due to the high viscosity of the excessive glycerol in the system, the glycerol forms a layer around the immobilized lipase, making it not disperse in the system as well as lipase power, moreover, beta-cyclodextrin as an assistant was mixed with lipase in this paper, it was more exercisable to use the lipase power. So lipase powder from *Candida* sp. 99-125 was employed to catalyze the esterification of glycerol and oleic acid to synthesize MAG and DAG in solvent-free system in this study. And the effects of glycerol/oleic acid molar ratio, initial water content, catalyst load, reaction temperature, agitator speed and beta-cyclodextrin/lipase mass ratio were investigated, then product was purified by silica column separation.

2. Materials and methods

2.1. Materials

Glycerol anhydrous (99.9%), oleic acid (analytical grade), beta-cyclodextrin (analytical grade), silica gel (300–400 mesh) were purchased from Beijing Chemicals Factory, Beijing, China. Lipase powder from *Candida* sp. 99-125 namely LS-20 was purchased from Beijing CTA New Century Biotechnology Co., Ltd, Beijing, China. *Candida* sp. 99-125 was screened by our lab [19–21], and 99-125 is a deposit number of a cell bank. All other reagents were obtained commercially and were of analytical grade.

2.2. Esterification reaction

The esterification was carried out in a 250 ml three-necked round-bottomed flask. The substrates were pre-mixed by agitation with an impeller at the desired temperature. Cyclodextrin (CD) has been reported to improve the functional and stability properties of enzymes [22–25]. So beta-cyclodextrin was mixed with lipase as an assistant in the process, powdered enzyme–cyclodextrin conjugates were added gradually to the reaction mixture with agitation, water was added at last. The reaction was stopped until the concentration of products had no raise over time.

Initial water content, glycerol/oleic acid molar ratio, catalyst load (enzyme–cyclodextrin conjugates), reaction temperature, agitator speed and beta-cyclodextrin/lipase mass ratio were changed to study their effects on the esterification, only one parameter was varied at a time in the optimization studies. Aliquots of 10 μl were periodically withdrawn, and analyzed by thin layer chromatography coupled with a flame ionization detector (TLC-FID). All the experiments were replicated at least three times and the results presented were the mean values for the replicated data.

2.3. Separation of product by silica column

After the reaction, acylglycerols and free fatty acid were extracted with n-hexane. The resultant solution was evaporated to remove the solvent under reduced pressure, preparing for the separation on silica column.

A silica column, 300 mm in height and 30 mm in diameter, was used to as the separation device. 5 g reactant was applied to silica column in a loading solvent of petroleum ether. A minimal volume (3 ml) was used to load the sample, and the loading solvent was then pulled through under gravity. Reactants containing TAG, FFA, DAG and MAG were then sequentially eluted from the column using the solvents of follows: (I) 200 ml petroleum ether:ethyl acetate:acetic acid (90:10:1, v/v); (II) 200 ml petroleum ether:ethyl acetate:acetic acid (80:20:1, v/v); (III) 200 ml petroleum ether:ethyl acetate:acetic acid (70:30:0.7, v/v); (IV) 100 ml petroleum ether:ethyl acetate:acetic acid (50:50:1, v/v); (V) 100 ml ethyl acetate; (VI) 150 ml methanol. Eluents were collected by an automatic fraction collector, and then the components of each tube were analyzed by TLC-FID. The eluents containing the same component combined, and the solvent evaporated, giving the final products.

2.4. Analysis by TLC-FID

Samples were analyzed by thin layer chromatography coupled with a flame ionization detector (Iatroscan MK-6s, Iatron Laboratories, Japan). Aliquots were dissolved in 0.5 ml of n-hexane, and 1 μl of diluted sample was spotted onto silica-coated chromarod quartz rods by a semiautomatic sample spotter. Samples were developed with the developing system of methylbenzene/chloroform/acetic acid mixture (70:30:2, v/v). The rods were dried for 3–5 min at 60 °C in an oven prior to analysis. Data handling was performed on a computer equipped with SES I-Chromstar1 6.0 software. The area percentages of TAG, DAG (1,3- and 1,2-isomers separately), MAG, and free fatty acids (FFA) were used for the calculation of product concentration. All the reactions in this work were conducted in duplicates. The means of duplicated determinations were used for result evaluation.

3. Results and discussion

3.1. Optimization of reaction variables

3.1.1. Effect of initial water content

It is well known that water content is one of the key factors that affect the activity of an enzyme in a non-aqueous medium. In this study, esterifications were performed at glycerol/oleic acid molar ratio 4:1, catalyst load (relative to the weight of total substrates) 10%, beta-cyclodextrin/lipase mass ratio 1.5:1, reaction temperature 40 °C, agitator speed 190 r/min, the effect of water was investigated by varying initial addition content of water in the range of 0–14% (w/w) of the substrate mass (Fig. 1). Almost no reaction was observed at 0% initial water content. The production of MAG and DAG increased when the initial water content increased. As indicated, the esterification at 10% initial water content is fastest with high reaction degree, in which MAG concentration amounts up to 35.2% after 8 h, DAG concentration amounts up to 29.5% after 6 h, and the concentration of MAG plus DAG achieves maximum of 61% around 8 h. However, 14% initial water reduced the concentration of MAG and DAG on the contrary. The result validated the water is necessary to maintain enzyme activity, once the water content reached the proper catalytic amount, the rate of the esterification reaction decreased along with a further increase in water content, which seemed to be due to inhibition of the esterification because one of the reaction products of the esterification between glycerol and oleic acid is water, thus prompted the esterification to the reverse direction. The results were similar to the synthesis of ethyl oleate catalyzed by 15 biocatalysts (native and immobilized lipases) in the solvent-free system reported by Foresti [26]. He also found that esterification performed in media with added water percentages of 10% led to much higher esterification rate than systems with the addition of little water. Although some authors agree on the need of very small amounts of water (0.2–0.3%) to successfully employ lipases in esterification reactions in organic/solvent-free media [27,28], the *Candida* sp. 99-125 employed in this study need much more water to maintain activity, this result was in consistent with former studies [21,29]. So the initial water content was fixed at 10% of the substrate mass in the following reactions.