Highly Selective Synthesis of Monolaurin via Enzymatic Transesterification under Batch and Continuous Flow Conditions

Fangli Chen, Guiju Zhang*, Changyao Liu, Jieying Zhang, Feifei Zhao, and Baocai Xu*

Abstract: This study aimed to investigate the highly selective production of monolaurin via enzymatic transesterification of methyl laurate and glycerol. It was determined that a binary solvent system (tert-butanol/iso-propanol, 20:80, wt./wt.) was suitable for the enzymatic production of monolaurin, especially in the continuous process. The highest mass fraction of monolaurin in the product mixture (80.8 wt.%) was achieved in a batch mode under the following conditions: a methyl laurate-to-glycerol molar ratio of 1:6, a substrate concentration (methyl laurate in the binary solvent) of 15 wt.%, an enzyme dosage of 6 wt.% of the amount of methyl laurate, and a reaction time of 1.5 h at 50°C. Compared with the results under the batch conditions, a slightly higher yield of monolaurin (82.5 ± 2.5 wt.%) was obtained in a continuous flow system at a flow rate of 0.1 mL/min, while the mass fraction of dilaurin in the product mixture was only 0.7 ± 0.6 wt.%. In addition, the yield of monolaurin remained almost unchanged during the 18 tested days of the continuous experiment.

Key words: monolaurin, enzymatic transesterification, binary solvent system, continuous process, Lipozyme 435

1 Introduction

Monoacylglycerols (MAGs) are widely used as nonionic emulsifiers in food processing, pharmaceutical, and chemical industries due to their excellent emulsifying and stabilizing properties[1]. They can be synthesized by glycerolysis or hydrolysis of oils and fats, direct esterification of glycerol with fatty acids, and transesterification of glycerol with fatty acid methyl esters through either chemical or enzymatic catalysis[2]. At present, the industrial-scale production of MAG is mainly carried out by chemical glycerolysis of fats and oils at high temperatures (220°C - 260°C) employing inorganic alkaline catalysts[3, 4]. In addition to many drawbacks, such as high energy consumption, low product selectivity, and dark-colored byproducts with an undesirable flavor, this conventional chemical glycerolysis process always results in products of low MAG content (usually lower than 60%) [5]. Enzymatic synthesis, which undergoes under moderate conditions (generally below 80°C), has been considered as an alternative to the chemical process for the production of MAG in the past decades[6-7]. The MAG content in the product produced via this biocatalytic process is generally 65% - 80%, which is still not quite desirable[8, 9]. Although this level of MAG content is suitable for most applications, purified MAG (at least 90% MAG content) is required in specific food products such as margarine, shortening, ice cream, and cream filling. Purified MAGs are usually obtained by further molecular distillation, which increases the overall production cost[10].

In past decades, extensive efforts were made to increase the yield of MAG in reaction systems using either chemical or enzyme catalysts such as the selective precipitation of MAG, the rational design of heterogeneous catalysts, and solvation[11]. However, most of these strategies are only suitable for lab-scale synthesis because of the low reaction rates, high cost, or high toxicity. The solvation strategy is efficient and promising for selective MAG catalysis. The introduction of a solvent into the reaction system improves the miscibility and interaction of the reactants, namely, hydrophilic glycerol and hydrophobic fatty derivatives, and thus enhances the reaction.

Various organic solvents, such as tert-butanol, tert-pentanol, acetonitrile, acetone, iso-octane, dioxane, and n-hexane, were employed in the enzymatic production of MAG, and the highest MAG yield was obtained with tertiary...
alcohols, i.e., tert-butanol and tert-pentanol. However, the melting point of tert-butanol is relatively high (25.69 °C), and the temperature difference between the melting point and boiling point of tert-butanol is approximately 57 °C. In实际 production, risks such as solvent crystallization may occur during condensation of the solvent. Solvent crystallization, as well as the high viscosity of tert-butanol, makes the continuous process difficult and limits the possibility for the recycling of the solvent.

Binary solvent mixtures, consisting of tert-butanol and another solvent with a lower melting point and viscosity, can overcome the abovementioned problems. Enzymatic glycerolysis of high oleic sunflower oil, using Novozyme 435 as the biocatalyst, was conducted in a binary solvent mixture of tert-butanol and tert-pentanol (80/20, v/v), and a yield of 75.3% MAG was achieved. Similarly, a tert-butanol/iso-propanol mixture (80/20, v/v) was used as the medium for the enzymatic production of MAG from glycerolysis of soybean oil, and the yield of MAG was reached 72.0%.

At present, the enzyme is generally employed in its immobilized form, which would allow the reutilization of the enzymes. In addition, the immobilized enzyme makes continuous enzymatic production possible, which is extremely important for converting laboratory technology into industrial production. Several experiments on the continuous production of MAG have been studied; however, low MAG contents were obtained (14% - 33%) (14-18).

Monolaurin is one of the most important MAGs and is an excellent water-in-oil emulsifier with a low hydrophilic-lipophilic balance (HLB) value (approximately 3.5). Additionally, monolaurin has been used as a preservative in foods and cosmetics due to its desirable antimicrobial activity (18). In the present study, the production of monolaurin via enzymatic transesterification of methyl laurate with glycerol was implemented in the binary solvent system via both batch and continuous process. According to previous research results, tert-butanol was used as the primary solvent, and several solvents were mixed with tert-butanol to achieve a binary solvent system, which made the highly selective synthesis of monolaurin in a continuous process possible.

2 Experimental Procedures

2.1 Materials

Lipozyme 435 (enzyme activity 10,000 PLU/g, Candida Antarctic lipase which is immobilized on a macroporous acrylic polymer resin) was purchased from Novozymes Biotechnology Co., Ltd. (Shanghai, China). Methyl laurate (99%), iso-propanol and tert-pentanol were obtained from TCI Development Co., Ltd. (Shanghai, China). tert-Butanol and iso-octane were obtained from Macklin Biochemistry Technology Co., Ltd. (Shanghai, China). Monolaurin and dilaurin HPLC standards were obtained from TCI Development Co., Ltd. (Shanghai, China). Glycerol (99%) was purchased from J&K Scientific Ltd. (Beijing, China). Acetonitrile and acetone (HPLC grade) were obtained from Thermo Fisher Scientific Co., Ltd. (Shanghai, China). All other chemicals were of analytical grade and used as received.

2.2 Lipase-catalytic synthesis of monolaurin by batch reaction

Methyl laurate (5 g, 23.3 mmol), glycerol (10.7 g, 116.5 mmol) and 25 g binary solvent mixtures (tert-butanol/iso-propanol, 20:80, wt./wt.) were added into a conical flask with a cover. Then, an appropriate amount of lipase (Lipozyme 435) was added and mixed well. The conical flask was placed into an IS-RSV1 thermostat oscillator (Crystal Technology & Industries, Inc., USA) with a 220 r/min reciprocating oscillation at 55 °C. A 0.4 mL sample was taken for HPLC analysis at 0.5 h intervals for 4 h.

Several solvents were used to mix with tert-butanol in addition to iso-propanol. The effects of the type and mass ratio of the binary mixtures on the enzymatic transesterification of methyl laurate with glycerol were examined, and the best binary solvent system was selected accordingly.

The effects of many factors, i.e., reaction time (0 - 4 h), the molar ratio of methyl laurate and glycerol (1:1 - 1:9), reaction temperature (30 °C - 65 °C), enzyme dosage (1 wt.% - 10 wt.% of the amount of methyl laurate), and substrate concentration (methyl laurate accounting for 5 wt.% - 60 wt.% of the binary solvents), on the production of monolaurin were also investigated.

2.3 Preparation of monolaurin by continuous enzymatic reaction

In the continuous flow system, the substrates (methyl laurate and glycerol) were added into the selected binary solvent, and the mixture was stirred by a magnetic stirrer. An appropriate amount of Lipozyme 435 was packed in a jacketed column (0.68 cm inner diameter, 25.0 cm long). The substrate mixture was introduced into the top of the column at a flow rate of 0.1 mL/min with a peristaltic pump. The reaction was maintained at 50 °C by water circulation, and the product was removed from the bottom of the column (Fig. 1). During the reaction, the product solution was periodically sampled and analyzed by HPLC.

2.4 Quantitative analysis by HPLC

The mass fractions of monolaurin, dilaurin and methyl laurate in the reaction system were analyzed by HPLC (Agilent 1200, United States), equipped with a differential refractive index detector (RID) (Agilent G1362A, United States) and an Agilent Eclipse Plus C18 column (4.6 × 250 mm, 5 μm particle size). The conditions for the HPLC anal-
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3 Results and Discussion

3.1 Screening of binary solvents

Due to the high melting point and viscosity of tert-butanol, the partial substitution of tert-butanol by a solvent with a relatively low melting point and viscosity will increase the feasibility of industrial monolaurin synthesis by lipase-catalyzed transesterification. Herein, the production of monolaurin by transesterification in five types of binary solvent systems consisting of tert-butanol mixed with another solvent was studied. The five solvents employed to decrease the melting point and viscosity are as follows: iso-propanol (IP), tert-pentanol (TP), n-hexane (NH), tert-butyl methyl ether (TBME), and iso-octane (IO). For comparison, the corresponding pure single solvents were also used as the reaction medium. For different solvent systems, the mass fractions of monolaurin in the reaction products are summarized in Fig. 2.

When tert-butanol was employed as the single enzymatic reaction medium, a desirable mass fraction of monolaurin (73.6 wt.%) was obtained. For mixed solvents, the mass fraction of monolaurin depended on both solvent types and the mass ratio of tert-butanol and the other solvent. As iso-octane, n-hexane, and tert-butyl methyl ether were employed as cosolvents, the mass fraction of monolaurin in the reaction system decreased as the mass fraction of tert-butanol was reduced. However, when tert-butanol was mixed with tert-pentanol and iso-propanol, the mass fraction of monolaurin in the reaction system increased slightly. As the mass fraction of tert-pentanol increased, the mass fraction of monolaurin in the reaction system increased. At a tert-butanol to tert-pentanol mass ratio of 20:80, a maximum mass fraction of monolaurin of 78.6% was achieved.

Similarly, in the tert-butanol/iso-propanol system, a maximum monolaurin content of 77.5% was also obtained when the tert-butanol to iso-propanol mass ratio was 20:80. Nevertheless, when tert-pentanol or iso-propanol were used individually as the solvent, the mass fraction of monolaurin in the reaction system decreased slightly. Previous literature has shown that functional groups play more critical roles than solvent polarity in enzymatic reactions. All solvent molecules in both tert-butanol/iso-propanol and tert-butanol/tert-pentanol systems have alcoholic hydroxyl groups, which may play a crucial role in the entry of substrates into the active site of the enzyme.

The viscosities of binary solvents and solvents with substrates (methyl laurate and glycerol) were also studied (Table 1). Tert-butanol exhibited a high viscosity of 3.96 mPa·s, and the viscosity was 24.60 mPa·s after substrates.
were added. With the addition of tert-pentanol, the viscosity of the binary solvent decreased slightly to 3.36 mPa·s. When the reactants were added, the decrease in viscosity was negligible compared with the final viscosity of the tert-butanol system. When iso-octane, \(n\)-hexane, and tert-butyl methyl ether were mixed with tert-butanol, the viscosity of both binary solvents and reaction systems decreased dramatically. However, it can be seen from the above results that the production of monolaurin in these binary solvent systems was not satisfactory.

The viscosity of the tert-butanol/iso-propanol binary solvent was lower than that of pure tert-butanol, and in the presence of substrates, the viscosity of the reaction system decreased greatly to 7.22 mPa·s, which is much lower than that of the pure tert-butanol system. In addition, the tert-butanol/iso-propanol system showed good performance in the enzymatic production of monolaurin by transesterification. The boiling point of iso-propanol is 82.4°C, which is the same as the boiling point of tert-butanol. However, the melting point of iso-propanol is \(-88.5\)°C, which is much lower than that of tert-butanol. Therefore, the tert-butanol/iso-propanol binary solvent was chosen for further study.

3.2 Enzymatic synthesis of monolaurin in a batch reaction system

3.2.1 Effect of reaction time

The time course of transesterification of methyl laurate with glycerol in the tert-butanol/iso-propanol(20:80, wt./wt.) system is presented in Fig. 3. In the reaction system, the mass fraction of monolaurin initially increased with time and reached its maximum level (76 wt.% - 77 wt.%) at 1.5 h. It then remained almost constant even if the reaction time was extended to 4 h. The mass fraction of methyl laurate decreased rapidly from 100 wt.% to 22.7 wt.% during the first 1.5 h and then reduced slowly to approximately 21 wt.% after 4 h. This indicated that the maximum conversion of methyl laurate was approximately 79 wt.%. For dilaurin, the mass fraction in the system was lower than 2 wt.% during the whole process. These results indicated that the time required for the reaction to reach equilibrium was approximately 1.5 h.

3.2.2 Effect of the substrate molar ratio

To investigate the effect of the substrate molar ratio on the production of monolaurin, lipase-catalytic transesterification was carried out at different molar ratios of methyl laurate to glycerol. Figure 4 shows that the mass fraction of monolaurin in the reaction system increased with increasing glycerol concentration, while the substrate molar ratio was in the range of 1:1 to 1:6. A high glycerol concentration was theoretically favored high methyl laurate conversion and monolaurin yield. Optimal monolaurin yield (79.1 wt.%) and methyl laurate conversion (80.3 wt.%) were obtained at a 1:6 molar ratio of methyl laurate to glycerol.

Fig. 3 Effect of time on the production of monolaurin.

Fig. 4 Effect of substrate molar ratio on the production of monolaurin.

However, a further increase in glycerol concentration results in a slight decrease of both the mass fraction of monolaurin and the methyl laurate conversion. This result is consistent with the previous report of enzymatic glycerolysis of castor oil, which was attributed to the instability and heterogeneity of the reaction system resulting from a large amount of glycerol with high polarity and viscosity\(^{20}\). Furthermore, the excess hydrophilic glycerol coated the surface of lipase and hence hindered the enzymatic process\(^{21}\).

3.2.3 Effect of substrate concentration

The effect of substrate concentration on the production of monolaurin is shown in Fig. 5. As the substrate concentration in the whole reaction system increased, the mass fraction of monolaurin first slightly increased and then decreased significantly. The optimal result was obtained at a substrate concentration of 15 wt.%. Under this condition, the highest mass fraction of monolaurin was approximately
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80 wt.%. At low substrate concentrations, the addition of solvent improved the homogeneity and reduced the viscosity of the whole reaction system, which can increase the mass transfer of the enzymatic reaction\(^{13}\). However, further increasing the solvent volume only reduces the substrate concentration, which in turn decreases the reaction rate according to the Michaelis-Menten equation\(^{20}\).

3.2.4 Effect of enzyme dosage

The effect of enzyme dosage in the range of 1 wt.\% - 9 wt.\% on the enzymatic transesterification was determined (Fig. 6). The production of monolaurin increased with increasing enzyme dosage in the range of 1 wt.\% - 6 wt.\%, and the mass fraction of methyl laurate decreased in the same range, indicating that the conversion also increased. This may be due to the increase in the collision frequency of the catalyst with substrates as the catalyst amount increases\(^{22}\). However, above 6 wt.\%, the production of monolaurin and the conversion of methyl laurate remained almost unchanged. This phenomenon may result from the fact that the active sites of the enzyme molecules presented in significant excess would not be exposed to the substrates, as protein aggregation might occur\(^{23, 24}\). From a cost perspective, an enzyme dosage of 6 wt.\% was considered as an appropriate choice for the transesterification reaction.

3.2.5 Effect of reaction temperature

The reaction temperature may affect the mass transfer, enzyme activity, and product composition. The effect of reaction temperature on the production of monolaurin via enzymatic transesterification was determined, and the results are shown in Fig. 7. As the reaction temperature increased from 30°C to 50°C, the mass fraction of monolaurin increased continuously to 80.3 wt.%. However, the monolaurin yield decreased when the reaction temperature was higher than 50°C. The viscosity of the reaction system decreased as the reaction temperature decreased as the reaction temperature increased, which also lowered the mass transfer limit and increased the rate of transesterification. However, too high temperature may reduce the activity of the enzyme, thereby reducing the production of monolaurin\(^{16}\). The optimal working temperature of Lipozyme 435 was determined to be 50°C in the present reaction.

In summary, the optimal reaction conditions for enzymatic monolaurin production under batch mode were as follows: a 1.6 substrate molar ratio (methyl laurate to glycerol), a 15 wt.\% substrate concentration, a 6 wt.\% enzyme dosage, a 50°C reaction temperature and a reaction time of 1.5 h. A maximum monolaurin mass fraction of 80.8 wt.\% was obtained under the abovementioned conditions.

Fig. 5 Effect of substrate concentration on the production of monolaurin.

Fig. 6 Effect of enzyme dosage on the production of monolaurin.

Fig. 7 Effect of temperature on the production of monolaurin.

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3.3 Preparation of monolaurin by a continuous enzymatic reaction

Continuous monolaurin production using Lipozyme 435 was performed under the abovementioned optimal conditions: a 1:6 substrate molar ratio (methyl laurate to glycerol), a 15 wt.% substrate concentration, and a 50°C reaction temperature in a tert-butanol/iso-propanol (20:80, wt./wt.) binary solvent system.

3.3.1 The effect of flow rate on the continuous enzymatic reaction

In the continuous reaction system, the flow rate of the substrate mixture is important for the residence time of the feedstock in the reactor. To some extent, a relatively low flow rate prolongs the residence time, which allows the reaction to proceed to completion, giving a high product yield. The effect of flow rate on continuous monolaurin production was studied, and the results are displayed in Fig. 8. The mass fraction of monolaurin in the product solution reached the highest value of 82.3 wt.% when the flow rate was 0.1 mL/min. The mass fraction of monolaurin no longer increased when the flow rate decreased to 0.08 mL/min. Therefore, the optimal flow rate for the continuous reaction was determined to be 0.1 mL/min.

3.3.2 Enzyme stability under continuous reaction conditions

When the processes involve an immobilized enzyme, the operational stability is a parameter of fundamental importance. Because of the relatively high cost of the enzymes, high enzyme stability would make continuous production more attractive for use in industrial plants. An experiment to ascertain the stability of the enzyme at a flow rate of 0.1 mL/min was performed, and the results are shown in Fig. 9.

Under the optimal conditions determined in the batch reaction mode, the mass fraction of monolaurin under continuous flow conditions was slightly higher than that of the batch reaction. During the 18 tested days, the mass fraction of monolaurin (82.5 ± 2.5 wt.%), dilaurin (0.7 ± 0.6 wt.%) and methyl laurate (16.8 ± 2.5 wt.%) mostly remained constant. These results showed that the catalyst Lipozyme 435 displayed excellent stability in the production of monolaurin.

Although the conversion rate of methyl laurate was not ideal, the desired production of monolaurin was obtained because methyl laurate converted mostly to monolaurin instead of dilaurin. This phenomenon indicated that the transesterification of methyl laurate with glycerol in the chosen binary solvent favored the highly selective production of monolaurin. Moreover, the continuous process and high operational stability of the enzyme make it possible to scale up for the industrial production of monolaurin or other monoacylglycerols.

4 Conclusions

The present research mainly focused on the development of an efficient enzymatic method for the highly selective production of monolaurin from the transesterification of methyl laurate with glycerol. The enzymatic reaction was operated under both batch and continuous mode in a binary solvent system using immobilized lipase (Lipozyme 435) as a biocatalyst. The results showed that the tert-butanol/iso-propanol (20:80, wt./wt.) binary solvent system was suitable for the enzymatic production of monolaurin because of its lower viscosity and melting point but higher reaction efficiency compared with pure tert-butanol.

The optimal reaction conditions for enzymatic monolaurin production under batch mode were determined as
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follows: a 1:6 substrate molar ratio (methyl laurate to glycerol), a 15 wt.% substrate concentration, a 6 wt.% enzyme dosage, a 50°C reaction temperature and a reaction time of 1.5 h. A monolaurin mass fraction of 80.8 wt.% was obtained under these conditions.

Based on the optimal reaction conditions obtained in the batch experiments, the desired monolaurin was produced in a continuous flow system at a flow rate of 0.1 mL/min. The reaction was highly selective towards monolaurin with a yield of approximately 82.5 wt.%. Compared to dilaurin, whose yield was approximately 0.7 wt.%. Furthermore, the catalytic activity of Lipozyme 435 remained almost unchanged during the 18 tested days. The high selectivity of monolaurin production and high operational stability of Lipozyme 435 make it possible to scale up the reaction for the industrial production of monolaurin or other monoacylglycerols via a continuous process.

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