Enzymatic esterification of lauric acid to give monolaurin in a microreactor

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Abstract
Monolaurin is a naturally occurring compound widely utilized in food and cosmetics. In this paper, we present a new method for the synthesis of monolaurin by esterification between lauric acid and glycerol catalyzed by Novozym® 435 using a microreactor. The conversion of lauric acid is 87.04% in 20 min, compared with 70.54% via the batch approach in 5 h. Using an optimized solvent system consisting of t-BuOH/tert-amyl alcohol (1:1, v/v), the selectivity using the microreactor method is enhanced to 90.63% and the space–time yield of the process is 380.91 g/h/L. This newly devised method has the potential for application to other multiphase and enzymatic reactions.

Keywords
enzymatic esterification, microreactor, monolaurin

Introduction
Glycerol monolaurate,1 also known as monolaurin or 1-lauroyl-glycerol (Figure 1), is a naturally occurring fatty acid that is widely utilized in food and cosmetics. It is most commonly used as a surfactant/emulsifier in food and cosmetics. It is also widely used as a dietary supplement, a food ingredient, and a feed additive because of its nutritional function, and is generally recognized as safe (GRAS) by the Food and Drug Administration. Also, monolaurin has antibacterial, antiviral, and other antimicrobial effects in vitro, and can be used as a preservative in the above products.2

Methods to obtain monolaurin include hydrolysis/alcopherolysis/glycerolysis and esterification.3–15 The reactions can be catalyzed either by enzymes or chemical catalysts. The reported chemical catalysts for esterification are p-TSA,14 zeolite Y,13 SBA-15,8 MCM-41,4 and so on.16 The disadvantages of chemical catalysis include harsh reaction conditions (≥120°C), long reaction times (≥6 h), complex post-processing, and low yields (44%–79%). For the industrial synthetic process toward monolaurin, the complicated post-processing is the most urgent problem, because molecular distillation is needed to separate monolaurate from the glyceryl dilaurate and glycerol trilaurate by-products. Therefore, developing a novel preparation with high selectivity toward monolaurin is urgently needed. Generally, lipase-catalyzed esterification is considered to be the most appropriate route for food or pharmaceutical grade monoglycerides.12 However, enzymatic syntheses are mainly focused on producing diacylglycerol via glycerolysis,15 or esterification17 between monoglycerides and fatty acids, and most reports on the enzymatic synthesis of glycerol monolaurin describe low yields (≤40%).3,6 Only one report on the biocatalytic synthesis of monoacyl glycerides gave excellent yield (100%) and selectivity (100%), in which ionic liquids were used as reaction media.11 The enzymatic synthesis of monolaurin is a multiphase reaction, and mass transfer is one important factor affecting the apparent reaction rate of the enzymatic reaction as known. The excellent results described in Lozano et al.’s11 work benefit from a compatible system, created by the water-miscible ionic liquid that enhanced the mass transfer between glycerol (GL), oils, and fats significantly.

One of the most important features of microreactor technology is its highly effective mass-transfer rate. With microreactor technology, the time to reach the chemical equilibrium of an enzymatic transformation can be reduced significantly. The selectivity toward monolaurin in the reaction is affected by multiple factors, such as solvent

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selection, the molar ratio of GL to LA (lauric acid), and the temperature. Combining the high mass-transfer rate of microreactor technology and the high specificity of biocatalysis, the enzymatic synthesis of monolaurin was conducted in a microreactor for both high conversion of LA and high selectivity for monolaurin in a short time.

In order to synthesize glycerol monolaurin using an immobilized enzyme in a microreactor, the experimental conditions needed to be optimized. Some of the parameters such as enzyme screening, the solvent system, the temperature, and the GL/LA molar ratio can be optimized under batch methods and then transferred to a microreactor method. Other factors, which are unique parameters, such as retention time, need to be optimized with microreactors. The optimized batch and flow reactions will be compared based on conversion, selectivity, and space–time yield to demonstrate the advantages of the microreactor method for the synthesis of monolaurin.

**Results and discussion**

The optimization of the key parameters by esterification in batch is presented.

**Enzyme screening**

The catalytic activity of four enzymes, including Novozym® 435, Lipozyme®RM-IM, CalB, and Lipozyme®TL-IM, on the esterification between GL and LA was investigated to identify the best immobilized enzyme for the microreactor technique. The enzyme screening was conducted in batch without a solvent as described in the Supplemental material. The results are summarized in Figure 2. The 90.16% conversion of LA and 63.55% selectivity for monolaurin were obtained in the esterification catalyzed by Novozym® 435. The reaction catalyzed by Lipozyme®RM-IM afforded similar results. Considering the price of these enzymes, Novozym® 435 was selected as the optimum catalyst for further research.

**Solvent screening**

The appropriate solvent choice needs to be determined in order to dissolve LA and GL, and to avoid pump/microchannel blockage and inaccurate flow rates in the microreactor. At the same time, the choice of solvent is also an important factor for high selectivity toward monolaurin over the dilaurate and trilaurate products. The commonly used solvents in esterification, including n-hexane, t-butanol, tert-amyl alcohol, and methyl tert-butyl ether (MTBE), were investigated for their ability to dissolve LA and their influence on the selectivity for monolaurin. The method used for this screening involved using a batch esterification process with the solvent method described in the “Experimental” section. The results showed that all these solvents were able to dissolve LA very well. The influence of the solvents on the selectivity is shown in Figure 3.

The esterification in t-BuOH gave the highest selectivity for monolaurin, but the conversion of LA was low. The esterification in n-hexane and tert-amyl alcohol gave high LA conversion but low selectivity for monolaurin. Hence, a mixed solvent system was tested to study the effect on the monolaurin selectivity. The yield of monolaurin was enhanced a little in the mixed solvent system, even though the esterification gave lower selectivity for monolaurin and a higher conversion of LA, compared to pure t-BuOH. Therefore, the mixed solvent of t-BuOH and tert-amyl alcohol in which the selectivity for monolaurin was 94.06% and the conversion of LA was 67.54% in the batch method was chosen as the medium for the esterification using the microreactor method.

**Temperature optimization**

The optimal temperature for the Novozym® 435 was investigated for the esterification between LA and GL using the batch esterification process. The suitable temperature range for Novozym® 435 is from 30 °C to 60 °C. The temperatures investigated were 46 °C, 50 °C, 54 °C, 58 °C, and 62 °C, and the results are depicted in Figure 4. The conversion of LA and the selectivity for monolaurin reached the highest values (81.04% conversion, 79% selectivity) when the esterification was performed at 58 °C.

**Reaction in flow**

**Flow experimental setup.** Continuous flow reactions were performed using a Vapourtec fixed-bed reactor and an R-Series machine (Scheme 1). t-BuOH was used as the rinsing solution. The fixed-bed reactor was filled with Novozym® 435 (1.32 g). The volume of the reactor was 5.19 mL. The flow rates of the pumps were calculated from this volume and the designed retention time.

**Optimization of the flow esterification**

Influence of the molar ratio of GL to LA on the flow esterification. After the key parameters of reaction had been optimized in batch, the reactions in the microreactor were conducted with a solvent system of t-BuOH/tert-amyl

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**Figure 1.** Esterification between GL and LA.
alcohol (1:1, v/v) at 58°C. First, the effect of the GL/LA molar ratio on the flow esterification was investigated by arranging the molar ratio from 3 to 5. Neither the conversion of LA nor the selectivity for monolaurin changed significantly when the molar ratio was increased from 3 to 5, as shown in Figure 5. Thus, the optimized GL/LA molar ratio was set as 3:1. The flow process was more economical.

Influence of retention time on flow esterification. Furthermore, the influence of the retention time on the flow esterification was investigated. The reactions with retention times of 15, 20, and 25 min were conducted in a microreactor and the results are given in Table 1. The esterification after 20 min of residence time achieved 87.04% conversion of LA and 90.63% selectivity toward monolaurin. Hence, the optimum residence time in flow was 20 min. Also, purification of the reaction was conducted affording 52.80 g monolaurin as a white waxy solid starting from 47.52 g of LA. The yield was 81.12%.

A comparison between the reaction in batch and in a microreactor. In addition, the batch method was optimized in this study in order to compare the results with those using microreactor method. The factors to be optimized with the batch method included treatment of the water produced in the reaction, the enzyme amount, and the reaction time. The esterification reaction between GL and LA is a reversible reaction and continuously produces water (Figure 1). Therefore, the method of handling the generated water can affect the reaction equilibrium significantly, especially in the batch method. To investigate the effect of water on the reaction in batch, three systems, including a closed system, an open system, and a 4 Å molecular sieves system, were designed. The highest conversion of LA and selectivity toward monolaurin was achieved using the open system. For the closed system, accumulation of water will have an adverse effect on the conversion of LA. For the 4 Å molecular sieves system, removing the produced water would be beneficial for the esterification process but also resulted in low selectivity for monolaurin. The conversion of LA was 87.59% and the selectivity for monolaurin was 72.39% in the open system for the batch method. Open system was thus selected for the batch method.

The influence of the enzyme amount on the esterification in the batch method was examined by varying the enzyme amount from 1% to 9% (relative to LA + GL) and the optimum enzyme loading was set as 3 wt% (relative to LA + GL) where the conversion of LA was 73.81% and the selectivity for monolaurin was 88.1%.
The reaction time for the batch method was also optimized. The results indicated that the reaction achieved equilibrium after 5 h, which afforded 81.06% of LA conversion and 81.13% of selectivity for monolaurin from 2 g of LA under the optimum conditions in batch.

Finally, the esterification between GL and LA was conducted in batch, with the optimum conditions as follows: GL/LA, 5:1 (mol/mol); reaction temperature, 58 °C; reaction time, 5 h; t-BuOH/tert-amyl alcohol (1:1, v/v) as solvent; and 3 wt% enzyme loading (relative to LA + GL). The reaction starting from 30 g of LA afforded 70.54% of LA conversion and 90.06% selectivity for monolaurin in a 500-mL three-neck bottle. The yield of monolaurin was 63.53%.

The comparison between the reaction in batch and in a microreactor is shown in Table 2. The space–time yield of the reaction in the microreactor was 380.91 g/h/L. With 30 g of starting material, the space–time yield of the reaction in batch was 10.44 and 47.09 g/h/L with and without solvent, respectively.

With the space–time yield of the reaction in the microreactor being one order of magnitude higher than that of the batch method, the high effective mass transfer in the microreactor is obvious. The larger specific surface area in the microreactor enhanced the efficiency of the mass transfer between the enzyme and the reactants. The ratio of the enzyme/starting material in the system was very high. So, the apparent reaction rate was improved significantly. However, the quantity of product produced per unit time per amount of enzyme was similar. This was caused by the small volume of the microreactor. Therefore, keeping the reaction running for a long time to overcome the disadvantage was a common solution. As shown in Figure 6, with the microreactor method, the reactant went through the column reactor and creates a concentration gradient of the product, namely, monolaurate, dilaurate, and trilaurate. The concentration of these products will be the lowest at the entrance and the highest at the exit of the column. In the meantime, the dilaurate and trilaurate concentrations will depend on the monolaurate concentration since these are the products of subsequent reactions. This concentration gradient will favor the reversible esterification reaction toward producing monolaurate at the entrance of the column. With an appropriate retention time selection, monolaurate will leave the column with little conversion to the dilaurate/trilaurate. Thus, optimization of the retention time will improve both the conversion of LA and selectivity for monolaurin. This helps to explain the results of the retention time experiment for the microreactor method. A 15-min retention time did not provide sufficient time for the esterification reaction before the reactants exited the column, thus resulting in lower conversion and selectivity compared to that of the 20 min retention time experiment. The 25 min retention time experiment also resulted in a lower conversion and selectivity compared to that of the 20 min retention time experiment, as a longer retention time leads to more
tendency to reverse the esterification reaction, or toward dilaurate and trilaurate. Hence, the retention time of 20 min was the optimum time for monolaurin.

Conclusion

A new method for the synthesis of monolaurin in a microreactor has been reported for the first time. Commercially available Novozym® 435 was used as the catalyst in the esterification reaction of LA and GL in a medium of t-BuOH/tert-amyl alcohol (1:1, v/v).

With the microreactor method, the esterification was significantly accelerated, and under optimized conditions, 87% conversion of LA was reached and the retention time was reduced to 20 min compared with 5 to 6 h in batch. The reason for the improvement was due to the highly effective mass transfer in the microreactor, which can decrease the mass-transfer limitations of the enzymatic reaction. In the meantime, the selectivity toward monolaurin was also enhanced to 90% by using a solvent system of t-BuOH/tert-amyl alcohol (1:1, v/v) with the microreactor method.

The process in the microreactor could be maintained and kept stable for 12 h, thus allowing a repeatable synthesis of monolaurin. The space–time yield of the process in the microreactor was 380.91 g/h/L, compared with 10.44 ~ 47.09 g/h/L in batch. This showed that this process has good potential in industrial applications for preparing monolaurin. This new method described in this report may serve as an inspiration for applications to other multiphase and enzymatic reactions.

Experimental

Materials and methods

Novozym® 435, Lipozyme RM-IM, Lipozyme CalB, and Lipozyme TL-IM were purchased from Novozymes (Beijing, China). Monolaurin (Analytical Reagent (AR), 99%) was purchased from Sigma-Aldrich. LA (AR, 98%), tert-butanol (AR, 99.5%), tert-amyl alcohol (AR, 98%), and methanol (AR, 99.8%) were purchased from Macklin Shanghai, China. n-hexane, ethyl acetate, acetone, and ethanol were purchased from Sinopharm Chemical Reagent Co., Ltd. GL (98%) was obtained as a gift from Lemonchem Co., Ltd.

Using the standard curve methods, the quantitative determination of LA and monolaurin was performed via gas chromatography (GC, Agilent 7890A) equipped with a flame ionization detector. The calibration curves for LA and monolaurin were obtained by using LA (AR, 98%) and monolaurin (AR, 99%) as standards. The actual content was calculated by the equation “mass × purity” (mass: the number shown on the scale). The procedural details for generating the calibration curves are given in the Supporting Information. The reactant mixture (200 µL for LA determination and 50 µL for monolaurin determination) was added into a 20 mL volumetric flask and immediately made up to the mark with 1:1 hexane/ethyl acetate solvent mixture. The above solution (1 µL) was injected into an Agilent 7890A GC system (HP-5 Column, 30 m × 0.320 mm × 0.25 µm), with helium as the carrier gas.

Table 2. Comparison between the reaction in batch and in a microreactor.

| Entry | Reaction | Enzyme loading (wt%) | GL/LA | Retention time (min) | Conversion (%) | Selectivity (%) | GC yield (%) | Space–time yield (g/h/L) | Product quantity/time/enzyme (g/h/g) |
|-------|----------|-----------------------|-------|----------------------|---------------|---------------|--------------|-------------------------|--------------------------------------|
| 1     | Batch in solvent | 3b | 5:1 | 300 | 70.54 | 90.06 | 63.53 | 10.44c | 1.76 |
| 2     | 2.5d | 3:1 | 300 | 73.83 | 88.1 | 65.04 | 10.69c | 2.99 |
| 3     | Microreactor | 2.53e | 3:1 | 20 | 87.04 | 90.63 | 78.88 | 380.91f | 1.50 |
| 4     | 3e | 5:1 | 20 | 88.45 | 90.13 | 79.72 | 384.97h | 1.51 |

GL: glycerol; LA: lauric acid; GC: gas chromatography.

Temperature: 58 °C. Solvent: t-BuOH/tert-amyl alcohol (1:1, v/v).

*Product quantity/time/enzyme, quantity of product produced per unit time per amount of enzyme.

**2.97 g Novozym® 435.

***1.32 g Novozym® 435.

****1.32 g Novozym® 435, running for 12 h.

*****1.32 g Novozym® 435.

******1.32 g Novozym® 435, running for 10 h.

Figure 6. Illustration of the production of glyceryl laurate esters in the microreactor.

| Conversion | Selectivity | Yield |
|------------|-------------|-------|
| 30%        | 40%         | 50%   |
| 40%        | 50%         | 60%   |
| 50%        | 60%         | 70%   |
| 60%        | 70%         | 80%   |
| 70%        | 80%         | 90%   |

Figure 6. Illustration of the production of glyceryl laurate esters in the microreactor.

GL/LA molar ratio

Table 2.

Enhanced results for the reaction in batch and microreactor.
carrier gas at a flow rate of 2 mL/min. The detector and injector temperatures were set at 330 °C. First, the column temperature was set at 80 °C and kept for 1 min, then increased to 320 °C with a rate of 20 °C/min and kept for 2 min. The concentrations of LA and monolaurin in the samples could be calculated via the standard curve. From GC results, the yield of monolaurin could be calculated from equation (1). The “yield” is also called “GC yield” on assuming no product loss during the purification. The “yield” shown in all tables and schemes is the “GC yield”

\[
\text{Monolaurin calculated from GC in moles} \times \frac{\text{produced monolaurin in theory}}{100\%} \quad (1)
\]

Furthermore, the conversion rate of LA can be calculated from equation (2). The remaining LA was determined by GC using the calibration curve method

\[
\text{Conversion rate of lauric acid} = \frac{\text{lauric acid used in moles} - \text{remaining lauric acid in moles}}{\text{lauric acid used in moles}} \times 100\% \quad (2)
\]

Finally, the selectivity was calculated from equation (3). The selectivity for monolaurin was defined as the ratio of the formed monolaurin (in moles) with respect to the converted starting material (in moles)

\[
\text{Selectivity for monolaurin} = \frac{\text{GC yield}}{\text{conversion rate of lauric acid}} \times 100\% \quad (3)
\]

**Synthetic procedures**

**Batch esterification process in solvent.** GL (1.38 g) and LA (1.00 g) were added to the appropriate solvent (6 mL, 0.397 g/mL) in the 15 mL batch and were kept at the selected temperature using a thermostatic water bath, followed by the addition of lipase Novozym® 435. The catalyst amount (relative to LA + GL) was modified during the research. The reactions were carried out for the appropriate period of time (5–6 h) in an oil bath under continuous stirring conditions. The stirring rate was 350 r/min. For kinetic research, 200 µL aliquots were withdrawn every 1 h and analyzed by GC. For optimization, the reaction was quenched by adding 0.5 mL of acetone/ethanol (1:1) into the reaction mixture and 200 µL aliquots were withdrawn and analyzed by GC.

**The purification of final product.** The purification was conducted after the reaction under optimized reaction conditions (15 min, 58 °C, GL/LA 3:1) kept running for 24 h starting with 47.52 g LA. First, saturated sodium bicarbonate and ethylacetate (EtOAc) were added into the reaction mixture to remove the remained GL and extract the esters. Vacuum rotating distillation was done after workup, affording the final product as a white waxy solid (52.80 g).

\[
\text{Volume} = 0.3421 \times \text{scale} \quad (4)
\]

**Calculation of the yield and space–time yield.** The space–time yield was calculated as follows

\[
\text{Space – time yield in batch} = \frac{\text{mass of starting material (g) \times yield}}{\text{reaction time (h) \times volume of reactor (L)}} \times 274.401 \times 200.322 \quad (5)
\]

\[
\text{unit: g/(h·L)}
\]

\[
\text{Space – time yield in microreactor} = \frac{\text{rate of pump B (mL/min) \times concentration of solute B (g/mL) \times GC yield \times 274.401 \times 60}}{\text{volume of reactor (L)}} \quad (6)
\]

\[
\text{unit: g/(h·L)}
\]

The product quantity per time per enzyme, and the quantity of product produced per unit time per amount of enzyme is calculated as

\[
\text{The quantity of product produced per unit time per amount of enzyme (see Supplemental materials)} = \frac{\text{space – time yield \times volume of reactor (L)}}{\text{amount of enzyme (g)}} \quad (7)
\]

**The monolaurin produced in the study.** The monolaurin produced in the study was confirmed to be 1-lauroyl-glycerol.
by $^1$H NMR and $^{13}$C NMR methods as shown in the Supporting Information. It was in good agreement with the reported data.²⁰ Also, a comparison between GC of 1-lauroyl-rac-glycerol standard and the reaction mixture is shown in the Supporting Information.

**Calculation of the residence time.** The residence time was calculated from equations (8) and (9)

**Table 3. Esterification in the microreactor.**

| Entry | Pump A (mL/min) | Pump B (mL/min) | Time (min) | Enzyme (%) | Notes |
|-------|-----------------|-----------------|------------|------------|-------|
| 1     | 0.169           | 0.176           | 15         | 1.92       | Volume of reactor: $0.3421 \times \text{scale} = 5.19 \text{mL}$ |
| 2     | 0.127           | 0.132           | 20         | 2.53       | Mass of enzyme = 1.32g |
| 3     | 0.102           | 0.106           | 25         | 2.71       | |

GL: glycerol; LA: lauric acid.

The number 0.3421 was the manufacturer setting, which is labeled on the tub. Scale is the bed length, which indicates where the catalyst was filled to, and is shown in the Supplemental material.

**Declaration of conflicting interests**
The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

**Funding**
The author(s) disclosed receipt of the following financial support for the research, authorship, and/or publication of this article: This research was financially supported by National Key Research and Development Project of China (2019YFD1101200) and the Jiangsu Synergetic Innovation Center for Advanced Bio-Manufacture (XTE1852).

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**Supplemental material**
Procedures for determining the calibration curve for lauric acid (LA) and monolaurin, $^1$H NMR and $^{13}$C NMR of monolaurin are supplied as Supporting Information.
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