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Selective synthesis of human milk fat-style structured triglycerides from microalgal oil in a microfluidic reactor packed with immobilized lipase

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ABSTRACT:

Human milk fat-style structured triacylglycerols were produced from microalgal oil in a continuous microfluidic reactor packed with immobilized lipase for the first time. A remarkably high conversion efficiency was demonstrated in the microreactor with reaction time being reduced by 8 times, Michaelis constant decreased 10 times, the lipase reuse times increased

2.25-fold compared to those in a batch reactor. In addition, the content of palmitic acid at *sn*-2 position (89.0%) and polyunsaturated fatty acids at *sn*-1, 3 positions (81.3%) are slightly improved compared to the product in a batch reactor. The increase of melting points (1.7 °C) and decrease of crystallizing point (3 °C) implied higher quality product was produced using the microfluidic technology. The main cost can be reduced from $212.3 to $14.6 per batch with the microreactor. Overall, the microfluidic bioconversion technology is promising for modified functional lipids production allowing for cost-effective approach to produce high-value microalgal coproducts.

KEYWORDS: structured triacylglycerols; coproduct; polyunsaturated fatty acid; microalgae oil; microfluidic bioconversion.

1. Introduction

Microalgae has attracted increasing attention compared with other energy crops, as microalgae has specific advantages of rapid growth, high oil yield per unit area, and can be cultivated on land that is not suitable for agricultural cultivation (Dong et al., 2013). However, the cost of microalgal lipid is still very high for the intended fuel applications. Development of algal biorefinery options with a diverse slate of value-added coproducts is required to make algal biofuel commercially possible (Dong et al., 2015). In addition to the ability to accumulate high energy compounds, microalgae can also synthetize high-value compounds such as omega-3 long-chain polyunsaturated fatty acids (LC-PUFAs) (Ferreira et al., 2016). Microalgal oil is well known for healthy benefits thanks to the high content of LC-PUFA, which allows for functional lipid production. Microalgae oil has been used for decades as a safe and sustainable resource for LC-PUFAs supplement. For example, it has been well-known that LC-PUFAs, especially docosapentaenoic acid (DPA) and docosahexaenoic acid (DHA) are vital for human health and nutrition, showing prevention capacity against cardiovascular disease (Bellou et al., 2001). Currently, microalgal PUFAs such as DHA and EPA have been commercialized as nutraceuticals. However, PUFAs are not ideal fuel precursors because they are prone to oxidation and have poor thermal stability (Dong et al., 2015). Co-production of high-value algal PUFAs nutraceuticals adds value to microalgal biofuel production and improves the quality of microalgae-based biodiesel. Re-structuring PUFA-triacylglycerols (PUFA-TAGs) is a promising approach to produce value-added co-products, making microalgal biofuel more competitive and mitigating the risk for stakeholders. To fulfill these targets, proper understanding of fatty acid content and distribution in microalgal TAGs is required (Wang et al., 2014). It is well known that structure is likely to affect the functionalities of TAGs, even for those TAGs with the same fatty

acid composition. For example, FAs bound at *sn*-1, 3 positions are better hydrolyzed by pancreatic lipase while residue monoglyceride with PA at *sn*-2 is helpful for the absorption of Ca^{+}, lipase-modified UPU (U: unsaturated fatty acids, P: palmitic acid) type TAGs are superior to natural TAGs with FA randomly distributed. Thus, UPU-style structured triacylglycerols could be developed to human milk fat analogs (HMFAs), which provide an essential energy for infants and prevent cardiovascular disease for adults (Teichert and Akoh, 2011).

Natural TAGs have a large number of different molecular species, leading to uncertainty in functions (Dai et al., 2014). Hence, it is advisable to produce TAGs with designed structures using natural LC-PUFAs from algae oil as feedstock. Transesterification has been used for modification of microalgae oil. In our previous work, novel structure tricylglycerols (STAGs) with unsaturated fatty acids (UFAs) enriched at sn-1, 3 and low palmitic acid (PA) assembled at *sn*-2 have been synthesized from *Schizochytrium sp*. oil and oleic acid (OA) via a solvent-free acidolysis catalyzed by Lipozyme RM IM (Wang et al., 2014). The majority of the research on the synthesis of desirable structured lipids (SLs) refers to the reaction between the composition of fatty acids and the structure of TAGs.

Traditional method on structured lipids synthesis in a batch reactor was demonstrated in our previous work and the desired production was obtained (Zhao et al., 2015). It is a general method for transesterification which is simple and convenient to operate and widely used for industrial production. However, violent shaking of the mixture in the batch reactor may lead to the crack and collapse of lipase which could obviously reduce activity of lipase (Schwab et al., 2009). In addition, a reaction in a batch reactor is time-consuming, leading to oxidative deterioration of oil and limits the commercialization of products (Dimakou et al., 2007). Thus, to assure the quality of STAGs and improve reaction efficiency, it is critical to develop a

scalable method to reduce cost and maintain the quality of products.

Microreaction technology, which is an interdisciplinary science and engineering area, has been the focus of different fields of research in the past few years. It is well known for its high efficiency, selectivity and security, resulting to a higher product yield in a shorter time than traditional batch reactions (Wang et al., 2014). The continuous microreactor can achieve higher heat and mass transfer rates than operating in a batch system (Marques et al., 2012). These features contribute to conduct reactions with enzymes, which can be immobilized to the microfluidic device. Thus, the microreactor can make full use of the enzyme activity due to repetition utilization; reduce the cost with lower enzyme and energy consumptions (Jeong et al., 2005). For example, the traditional synthesis of ibuprofen takes more than 10 h and the products were isolated in 61-68 % yields. With the microfluidic technology, the needed reaction time was remarkably reduced to 3 min and the yield exceeded 72.9% (Snead and Jamison, 2015). In addition, the technology was used for the esterification of caffeic acid and methanol to sharply reduce reaction time from 9 h to 2.5 h with comparable yield. To date, microfluidic bioconversion technology have not been used for the biotransformation of algae oil to produce UPU-style STAGs. To the best of our knowledge, the synthesis of STAGs with microalgal PUFAs in a packed bed microreactor is investigated for the first time.

The objective of this work was to demonstrate a highly efficient green approach to synthesize STAGs from microalgal PUFAs in a novel miniaturized microfluidic reactor packed with immobilized lipase. In this work, the microalgal PUFAs and tripalmitin was co-fed into a microfluidic reactor packed with Lipozyme TL IM to obtain STAGs. The effects of substrate concentration, substrate molar ratio, reaction temperature, and flow rates on the yield of STAGs as well as the stability of enzyme were investigated. Different reaction conditions and kinetics in

both batch reactor and microreactor were compared.

2. Materials and methods

2.1. Lipases and chemicals

Microalgae oil from *Schizochytrium* sp. was purchased from a branch company of DSM (Royal DSM N. V., Shanghai. China), and tripalmitin was purchased from Sigma Chemical Co. (St. Louis, MO). Immobilized lipase Novozym 435, Lipozyme TL IM and Lipozyme RM IM (*Rhizomucor miehei*, 275 IUN/g, IUN is interesterification units Novo; carrier: phenol formaldehyde) were purchased from Novo Nordisk A/S (Bagsvaerd, Denmark). Thin-layer chromatography (TLC) plates were from Qingdao Haiyang Chemical Co. Ltd. (Qingdao, China), and methanol and *n*-hexane were purchased from Chromatography (Tedia Co., Fairfield, OH, USA).

2.2 Preparation of microalgal free fatty acids (FFAs)

A mixture solution of NaOH (4.8 g) and 95% v/v aqueous ethanol (800 mL) was added into microalgae oil (200 g). The mixture was heated for 6 h at 70 °C until altering to a clear homogeneous solution. Ethanol was evaporated with rotary evaporator at 60 °C and 300 mL of distilled water was added. The solution was acidified to pH = 3–4 with 10% HCl solution. Petroleum ether was used to collect the sample of FFAs from microalgae oil. The petroleum ether layer was removed under the high pressure nitrogen flow to get microalgae FFAs.

2.3 Traditional synthesis of SLs in a batch reactor

Initial screening of the suitable tripalmitin and lipase for enzymatic transesterification of

tripalmitin and PUFA to obtain STAGs was performed in a 50 mL conical flask with a ground glass stopper. Tripalmitin and PUFA were added to 3 mL *n*-hexane, then the lipase was added to initiate the reaction. All reactions were performed at temperatures of 50 °C, 60 °C, and 70 °C respectively, in a cube heating equipment (DHG9023A, Minconne instrument manufacture Co. Ltd., Shanghai, China) with a constant shaking speed of 180 rpm in a shaker (SHA-C, Hannuo Instrument Co. Ltd., Shanghai, China). The molar ratios of tripalmitin to FFAs were set to 1:5, 1:7, and 1:9 to achieve the desired molar ratio the enzyme loading was 5, 7 and 9% by weight of total substrates. After 35 h, 150 μL of sample were stored in a freezer which temperature was maintained at -20 °C prior to GC analysis. All experiments were performed in triplicate.

2.4 Production of SLs with a microreactor

The packed-bed reactor with dimensions: width (W) of 8 mm, height (H) of 500 µm, and length (L) of 75 mm, respectively, were built in a stainless steel plate. It has one inlet and one outlet. The design of a packed-bed microreactor was easily assembled, operated and cleaned. Lipozyme RM IM was used as a biocatalyst, and *n*-hexane was employed as the medium. Lipozyme RM IM beads (90 mg) were put in the channel, then the channel was sealed with a glass pane. The stainless steel plate and glass pane can be sealed firmly by glass cement. When the reaction was complete, the stainless steel plate was put in a muffle furnace that was set at 600 °C for 1 hour and the glass cement and the remains were removed. When the temperature of the plate dropped, the microreactor was filled with fresh enzyme for the next reaction. Tripalmitin and PUFAs mixed with 3mL *n*-hexane were fed into the microreactor with flow rates ranging from 2 to 20 μL/min by a syring pump (LSP02-1B, Longer Pump Co. Ltd., Beijing, China). The melting point of tripalmitin is approximately 41 °C; therefore, the reaction

temperatures were set at 45 °C, 50 °C, 55 °C, 60 °C, 65 °C and 70 °C respectively, in a cube heating equipment which had a temperature sensor to control the surrounding temperature of the microreactor to desire temperature. In addition, the substrate ratio is set as 6 gradients as 1:1, 1:3, 1:5, 1:7, 1:9, 1:11. The product of 150 µl was collected for GC analysis. All of the experiments were performed in triplicate.

2.5 Kinetic study of the microreactor and batch reactor

The flow rate dependence of Michaelis constant (K_m) for STAGs was examined at 60 °C to study the kinetics in a continuous-flow microreactor. The tripalmitin concentration ranging from 0.125 mg/mL to 0.5 mg/mL with the fluid flow rates from 2 to 20 μL/min. The process of STAGs synthesis was stable, and the transesterification of STAGs was calculated according to the STAGs concentration difference between the initial state and terminus state (Tran et al., 2013).

The effect of the concentrations of tripalmitin was investigated to determine the kinetics of transesterification in the batch reactor. The concentration of tripalmitin was ranging from 0.125 mg/mL to 1 mg/mL. The initial reaction rates were expressed as the PUFAs incorporation per minute. The relationship of initial reaction rates and the initial substrate concentration was expressed with function and the kinetics constants were determined by the coefficients of the function.

2.6 Purification of STAGs by TLC and preparation of FAMEs for GC analysis

TAGs were isolated by TLC (TLC Silica gel 60, 20 cm × 10 cm) using petroleum ether/diethyl ether/acetic acid (65:35:0.5, v/v/v) as a mobile phase. With triolein as a standard,

the samples of the TAGs were analyzed. The silica gel plate with the sample was air-dried, sprayed with 2, 7-dichlorofluorescein solution which was revealed under ultraviolet light (254 nm). The sample portion was collected with hexane. The isolated TAGs were used for FA compositions analysis (Zhao et al., 2015).

In a 10-mL centrifuge tube, 150 mg oil, 2 mL methanolic potassium hydroxide were sequentially added. The tube was heated in a water bath at 65 °C for 1 hour. Then, 2 mL of *n*-hexane and 4 mL of distilled water were added to extract the FAMEs. C17:0 was used as an internal standard for GC analysis (Wang et al., 2014).

2.7 FA composition analysis with GC and GC-MS

The FAMEs were detected by capillary GC with a DB-5, 30 m × 0.25-mm id, 0.25-μm capillary column (Model 6820N, Agilent) on an Agilent Technologies gas chromatograph equipped with a Hewlett Packard 3396 Series II integrator and 7673 controller, a flame ionization detector, QA/QC NDS Cerity system and split injection (Agilent Technologies Inc., Santa Clara, CA, USA) (Wang et al., 2014). The initial temperature of the oven was 80 °C, ramping to 200 °C at 15 °C/min. After holding for 1min at 200°C, the temperature increased to 250 at 1.5 °C/min. The injector and detector temperature was set 250 °C and 280 °C respectively. The split ratio was 30:1 and the rate of carrier gas was 1mL/min.

GC-MS analysis was performed using an Agilent 6890 gas chromatograph with a 5973 MS detector equipped with 60 m×0.25-mm i.d. 0.25-μm/MS capillary column (DB-WAX). The running condition was the same as the GC. The characterization and identification of FAMEs from microalgae oil was completed in scan mode. The selected ion monitoring mode was from 35 to 320 m/z (Liu et al., 2015). The incorporation of PUFAs was calculated as follows (Wang et

al., 2014):

$$\text{FA}_{(i)} \text{ incorporation at } sn\text{-2 position} = \frac{\text{FA}_{(i)} \text{ at } sn\text{-2 position}}{\sum \text{FA}_{(i)} \text{ at } sn\text{-2 position}} \times 100\% \quad (1)$$

$$\text{FA}_{(i)} \text{ incorporation at } sn-1,\ 3 \text{ positions} = \frac{3 \times \sum \text{FA-FA}_{(i)} \text{ at } sn-2 \text{ position}}{2} \times 100\% \quad (2)$$

2.8 Melting and crystallization analysis

The melting and crystallization of different TAGs were measured by a differential scanning calorimeter (DSC Q2000, TA Instruments, Leatherhead, UK). The indium and octadecane were used for calibration. Samples of 5 to 10 mg were put in an aluminum pan. Also, the empty pan was set as the control. The temperature was set from 25 to 80 °C at 50 °C/min and held at 80 °C for 10 min, then dropped to -65 °C with the rate of 10 °C/min, kept at -65 °C for 10 min, and finally increased to 80 °C with 5 °C/min rate (Lee et al., 2010). The thermographs were analyzed using Thermal Solutions software.

2.9 Statistical analysis

Each preparation was investigated in triplicate in the experiments. The standard deviation of the data was calculated to test the reliability of the results. The analysis was performed with the variance (ANOVA) method. Significance of these data was determined by the Pearson correlation coefficient.

3. Results and discussion

3.1. Traditional synthesis in a batch reactor

As shown in Fig. 1A, PUFAs incorporation reached to 33.4%, 42.6% and 49.8% with the Novozyme 435, Lipozyme TL IM and Lipozyme RM IM, respectively. Positions sn-1, 3 are significantly preferred for PUFA. Compared to the other two enzymes, Lipozyme RM IM displays superior performance in both total PUFA inception (49.8%) and *sn*-1, 3 selectivity (74.7%). The results indicate that Lipozyme RM IM had the highest PUFA incorporation preference and position selectivity.

Site-specific lipases that prefer *sn*-1, 3 positions are the most efficient catalyst to synthesize desired STAGs. Immobilized Lipozyme RM IM is an important *sn*-1, 3 specific lipase isolated from *Rhizomucor miehei*. The *R. miehei* lipase has been widely used in transesterification of TAG modification due to the excellent activity and stability. In addition, this lipase shows higher activity toward medium-chain and long-chain fatty acids, especially PUFAs. Additionally, the immobilized lipases are preferred over free enzymes since such formulation simplifies downstream and purification processes (Balcao et al., 1996). Thus, the immobilized lipase Lipozyme RM IM was the optimal lipase to catalyze the transesterification of FFAs and tripalmitin.

Fig. 1B shows the effect of the lipase concentration on the incorporation of the PUFAs. The incorporation of the PUFAs increased linearly until 21 hours. Longer time resulted to marginal increase, and further reaction led to a decline. The optimal lipase amount of 7% led to the highest incorporation of 43.1%. More side effects occurred when equilibrium was reached, resulting to the formation of byproducts (Shu et al., 2010). In addition, when the lipase amount exceeded the optimal amount, the enzyme get to the saturation with the substrate, in which an increase in enzyme amount provided no significant changes on the incorporation (Zou X.G. et al., 2014).

As shown in Fig 1C, a substrate ratio of tripalmitin: FFAs=1:9 resulted in the highest

incorporation at 7 hours. However, when the reaction proceeded to 21 hours, the highest incorporation of 49.8% was obtained with a substrate ratio of 1:7. It is shown that increased substrate ratio (1:9, 1:11) led to slightly reduced incorporation (48.0%, 46.6%). Even though higher substrate concentrations can promote more incorporation of PUFA to TAGs at the initial reaction, the high level molar ratios of substrate may inhibit lipase activity (McEachern et al., 2007) and also complicate the downstream purification. Thus, 1:7 was the optimal substrate ratio.

Fig. 1D shows the effect of temperature on the PUFAs incorporation. The reaction at 70 °C resulted in the highest incorporation of 42.3% after 14 h of reaction. Elevated temperature promoted the acyl migration and reduced the viscosity of substrate; higher temperature, on the other hand, can reduce enzyme activity by damaging the internal structure (Christopher et al., 2014). When the reaction time was prolonged to 21 h, the reaction at 60 °C reached the highest incorporation of 49.8%, while the incorporation at 50 °C kept rising until 28 h. A higher reaction temperature is preferred for enzymatic transesterification due to increased solubility of reactant and enhanced initial reaction rate (Teichert and Akoh, 2011). However, long time reaction at high temperature may result to oxidation of the SLs and increased acyl migration. As discussed in the study of Meng Z (Meng Z et al., 2013), given the higher temperature and longer reaction time, the acyl migration occurred more often, increasing the production of 1, 2-diglyceride. Therefore, 60 °C was chosen as the optimal temperature.

3.2. Continuous flow transesterification in a packed bed microreactor

Fig. 2 shows the effect of the reaction temperature on the transesterification of microalgal PUFAs with tripalmitin using a miniaturized continuous-flow packed bed microreactor with

temperature gradient at different flow rates. An increase in the reaction temperature from 45 to 60 °C led to an increase in the incorporation of the transesterification process. The incorporation of PUFAs was only 26.6% when the transesterification reaction was conducted at 45 °C and 20 μL/min, while the highest incorporation of 38.8% was achieved at 60 °C with 2 μL/min. Further increasing temperature from 60 to 70 °C did not improve the incorporation of PUFAs. High temperature may result in the oxidation of the SLs and increase byproduct formation. Oleic acid (OA), DPA and DHA were also used in this study, OA and DPA are quite alike, but not DHA. In addition, longer chain fatty acids will lead to highly oxidation prone structure and secondary oxidation products (Akanbi et al., 2015), which made the content unstable and decrease with the increasing of temperature. Moreover, Lipozyme RM IM prefers medium chain fatty acid to long chain fatty acids which makes the incorporation trend of DHA different from other fatty acids (Meng et al., 2013). Additionally, the concentration of OA and DPA was too low to show the significant change with the change of reaction conditions. The content of palmitic acid at the *sn*-2 position was stable regardless of reaction temperature, because of the Lipozyme RM IM is sn-1, 3 specificity. The increase of PUFAs and single unsaturated fatty acid incorporation primarily occurred at *sn*-1, 3 positions, with rare transesterification at the *sn*-2 position. The optimal temperature of the microreactor was determined as 60 °C， which is the same as a batch reaction.

Substrate ratio had a significant effect on the incorporation of PUFAs using a continuous flow packed bed microreactor in the evaluated range (1:1~1:11) at different flow rates, respectively (Fig. 3). The highest PUFA incorporations were obtained when the substrate ratio was 1:7 to 1:9 with a similar incorporation yield (about 81.3%). Further increases of the PUFAs ratio reduced the yield to 50.1%. The result implies that excessive FFAs inhibit the enzyme by

acidifying micro aqueous phase surrounding the lipase (Wang et al., 2014).

Fig. 3E and 3F shows the FA content at different positions. The total PA content decreased markedly especially the PA at *sn*-1, 3 positions, indicating PUFAs replaced PA at these positions. Enzymatic transesterification conversion yield increases with the increasing substrate ratio until a substrate ratio of 1:7 and 1:9, while the reaction reached equilibrium when the substrate ratio was 1:7 in a batch reactor. Without the side reaction, the theoretical equilibrium and incorporation of PUFAs can be calculated using the equation (1) (Cvjetko et al., 2012)

$$\mathrm{Inc}_{\max} = \frac{66.67}{1 + 2 / S_r} \tag{3}$$

Where the Inc_{max} is the incorporation of reaction equilibrium and S_r is the mole substrate ratio. In this study, the theoretical incorporation of PUFAs affected by the acyl donor amount was investigated. When the mole substrate ratio was 1, 3, 5, 7, 9 and 11, the incorporation of reaction equilibrium was 22.2%, 44.5%, 47.6%, 51.9%, 54.6% and 56.4%. However, the incorporation decreased when the substrate ratio exceeded 1:7 actually. It is because a large amount of FFAs containing high levels of free or ionized carboxylic acid groups could acidify the enzyme layer which will lead to the decreasing activity of the enzyme (Zou et al., 2014). When the substrate ratio ranged from 1:1 to 1:11 (Fig. 3A), the results are consistent with the maximum incorporation. The substrate ratio of 1:7 was the best condition according to the theoretical value and the actual data.

Table 1 shows the FAs profiles of algae oil, AOSL and tuna oil. Compared to the two materials which are always used for SL synthesis directly, STAGs had 89% PA at the *sn*-2 position which was 4 times higher than that of the others. The incorporation of PUFAs at *sn*-1, 3 positions was similar with the crude algae oil. The DHA incorporation at *sn*-1, 3 of tuna oil was 11.9%, which was far less than the STAGs. The results indicate that the STAGs conform to the

UPU-style and the structure of FAs was the most assessable in digestion tract.

3.3. Kinetic parameters in a batch reactor and a packed bed microreactor

The aim of kinetics study of continuous flow is to calculate the value of kinetic parameters ($K_{m(app)}$) which is relative to the flow rate (Wang et al., 2014). The effect of flow rate on enzyme kinetics in continuous flow reaction systems is usually investigated using the Lilly–Hornby model (Lilly et al., 1966). The kinetic parameters in microreactor can be calculated by the model more specifically. The model adapted from the standard Michaelis–Menten model designed for enzyme kinetics is described by the following equation:

$$f \cdot [A_0] = \frac{C}{Q} + K_{m(app)} \cdot \ln(1 - f) \quad (4)$$

Where f is the substrate which was converted to the product during the reaction, Q is the flow rate, $[A_0]$ is the original concentration of substrate, C is the reaction capacity of the microreactor and $K_{m(app)}$ is the apparent Michaelis constant.

Fig. 4A shows the linear relationship between f $[A_0]$ and ln(1 - f), and $K_{m(app)}$ values were calculated as the slopes of the linear plots. As the flow rate ranged from 2 μL/min to 10 μL/min, the $K_{m(app)}$ values were 7.42 mM to 9.67 mM (Table. 2). According to the experimental data, the $K_{m(app)}$ increases as the flow rate increases. The $K_{m(app)}$ values from experimental data indicated that mass transfer appeared to be playing a role in affecting the enzymatic reaction (Matosevic et al., 2011). At the lower flow rate, a lower $K_{m(app)}$ value was obtained, which shows that the acyl donors and enzyme were in sufficient contact with one another at a low flow rate. Therefore, 2 μL/min was the suitable flow rate for a packed bed microreactor.

It is better to understand the mechanism of the reaction when the kinetics of the enzymatic transesterification of STAGs was studied in a batch reactor. Compared to the microreactor, there

is no flow rate in a batch reactor. Hence, the Michaelis–Menten model was the best to be used for determination of the enzymatic kinetics in the batch reaction systems and the model was described by the following equation (Wang et al., 2014):

$$\frac{1}{V}=\frac{1}{V_{max}}+\frac{K_m}{V}\frac{1}{S} \quad (5)$$

Where S is the original substrate concentration, V_{max} is the maximum reaction velocity, and 1/V was plotted against 1/[S] and K_m is the Michaelis constant. Fig. 4B shows the Lineweaver–Burk plots obtained for the batch reactor. The Lineweaver–Burk plots were linear. The kinetic constants and the corresponding correlation coefficient (R^2) of the batch reactor are listed in Table 2. The K_m value was 79.9 mM which is 10.7-fold higher than that of microreactor with a flow rate of 2, 5 and 10 μL/min, respectively. The formation of an enzyme–substrate complex is easier with the immobilized lipase due to porous structure of the micro pipeline (Zou et al., 2012).

The improved kinetic might because better mass transfer can increase the chance of substrate to combine with enzyme, and quickly release of STAG after reaction as well which can improve reaction efficiency in a microreactor. Thus, we intend to explain this process by calculating the mass transfer of the microreactor.

Internal mass transfer of reaction can be determined by Weisz–Prater criterion. Weisz–Prater criterion can be expressed as the following equation and the data in microreactor was used for the calculations (Jia et al., 2012). The calculted results were shown in table 3:

$$C_{WP}=\frac{(r_{obs}\rho_p)R_c^2}{D_eC_{AS}} \quad (6)$$

Where C_{WP} is the internal mass transfer coefficient, ($r_{obs}\rho_p$) is the maximum reaction rate (9.3×10^{-3} mol/(m^3·s)), R_c is the radius of catalyst (1.5×10^{-4} m), De is the effective diffusivity and

can be defined as the following equation:

$$D_e = \left(\frac{\varepsilon}{\tau}\right) DAM \quad (7)$$

Where ε is the particle porosity of immobilized lipase (0.36), τ is the tortuosity factor which is taken as 3 and DAM is scatter factor is taken as 6.6×10^{-7} $m^2 \cdot s^{-1}$ (Yang et al., 2001), C_{AS} is surface concentration of substrate which is the ratio of substrate molar amount and reaction volume (1.2×10^2 mol m^{-3}). With these equations, the C_{WP} in microreactor can be worked out to be 2.5×10^{-5} which is approximately 10-fold higher than that in a batch reactor. The external mass transfer coefficient can be calculated according to the following equation because external mass transfer is similar to the internal mass transfer (Ju et al., 2011) :

$$C_M = \frac{(r_{obs}\rho_p)R_c n}{K_c C_A} \quad (8)$$

Where the C_M is the external mass transfer coefficient, the n is reaction order (2), the K_c is the mass transfer coefficient which can be obtained from Dwidevi-Upadhyay mass transfer correlation (Sert et al., 2013) as 2.2×10^{-5} m/s and the C_A is bulk concentration which is equal to C_{AS}. Hence, the C_M is 1.5×10^{-3} which is also approximately 10-fold higher than that in a batch reactor. Thus, the results support our hypothesis that mass transfer can increase the affinity of the lipase to substrate.

3.4 The analysis of production in both batch and microfluidic reactors

The physical quality of product was studied by the DSC. Fig. 5 shows the crystallization (A) and melting (B) profiles of STAGs produced from a batch reactor and a microreactor, respectively. As shown in Fig 5A, the STAGs produced in a batch reactor have a narrow crystallization range and higher peak compared to STAGs produced in a microreactor. Fig. 5B

shows the STAGs produced in a batch reactor have higher melting point compared to STAGs produced in a microreactor. The STAGs produced in a microreactor have more unsaturated fatty acids and reasonable structure. The melting point of tripalmitin was 41 °C which is higher than body temperature. After the transesterification, the melting point of the STAGs was markedly lower than that of tripalmitin. Compared to the STAGs in the batch reactor, the STAGs in the microreactor had higher melting and lower crystallization points, which indicated that microreactor may improve the selectivity of the lipase. Furthermore, a melting point below a physiological temperature (36.6-37.3 °C) can be emulsified and absorbed quickly (Wang et al., 2014). The plasticity and storage range of the STAGs was improved and the sphere of action was broader after the modification (Rodriguez et al., 2012). In addition, as shown in Fig. S3, the surface of lipase in the microreactor was smoother and has damage compared to that in the batch reactor. These demonstrated the mild and efficient reaction environment in the microreactor.

In addition, the FAs at different positions of triglycerides (Table 4) implied that the developed microreactor has a great advantage for lipase-catalyzed modification of natural lipids. The incorporation of PUFAs at *sn*-1, 3 positions of 74.7% was obtained in the batch with optimal conditions, while the incorporation was 81.3% using a packed bed microreactor. The *sn*-2 position of STAGs from the batch reactor and microreactor were both mainly PA. The OA, DPA and DHA at sn-1, 3 position incorporations were 19.8%, 19.0% and 38.0%, respectively, with the microreactor, while the incorporations were 17.2%, 18.6% and 36.4% with the batch reactor. Additionally, Table 5 shows the FA distribution of STAGs in two reactors under different conditions. The optimal substrate ratio and temperature in the batch reactor and microreactor were the same. However, the results show that the lipase concentration and reaction time in the microreactor were much lower than those in the batch reactor.

3.5 The reusability of lipase

The reusability of the Lipozyme RM IM for the synthesis of STAGs in the microreactor was evaluated at the optimal condition. Fig. 6 shows the activity of the enzyme over 18 runs in the microreactor. Under the optimal conditions, the incorporation of PUFAs at sn-1, 3 positions was maintained at approximately 80% after 18 runs. Further cycles led to the obvious decrease of incorporation. Hence, the results showed that the immobilized lipase could be reused at least 18 times without losing catalytic activity and site-specific selectivity. In contrast, the Lipozyme RM IM only maintained 90% of its original activity after 8 runs in the batch reactors. The results indicate that the microreactor can make better use of enzyme activity and the reactor was more efficient.

As shown in the table 6, every reaction in a microreactor cost 2.5 h and $14.6. With the same time a batch reactor will cost $212.3 to reach the same productivity of microreactor. The experimental data indicate that the use of the miniaturized packed bed microreactor has great potential to reduce cost to produce modify STAGs.

4. Conclusions

A highly efficient technology for converting algal lipids to value-added STAGs was successfully demonstrated in a microfluidic microreactor. The maximum incorporation of total PUFAs was 54.2%, with incorporation at *sn*-1, 3 up to 81.3%. Enzyamtic reusing and recycling experiments had been performed in the microreactor multiple times without losing enzymatic activity, and the microfluidic bioconversion technology can effectively fortify the Lipozyme RM IM-catalyzed synthesis of UPU-style STAGs derived by microalgae oil. Additionally, both the UPU-style STAGs produced via batch and flow reactor showed comparable improved storage

properties. The implementation of this technology could potentially reduce the cost of STAGs.

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Figure Legends:

Fig. 1. The effect of different factors on the incorporation of PUFAs in a batch reactor. Condition: (A) substrate ratio (palmitic acid triglycerides: PUFAs): 1:7, temperature: 60 °C. (B) substrate ratio: 1:3; temperature: 60 °C; lipase amount: 5%, 7%, and 9%; reaction time 35 h. (C) substrate ratio: 1:3, 1:5, and 1:7; temperature: 60 °C; lipase amount: 7%; reaction time 35 h. (D) substrate ratio: 1:7; temperature: 50 °C, 60 °C, and 70 °C; lipase amount: 7%; reaction time 35 h.

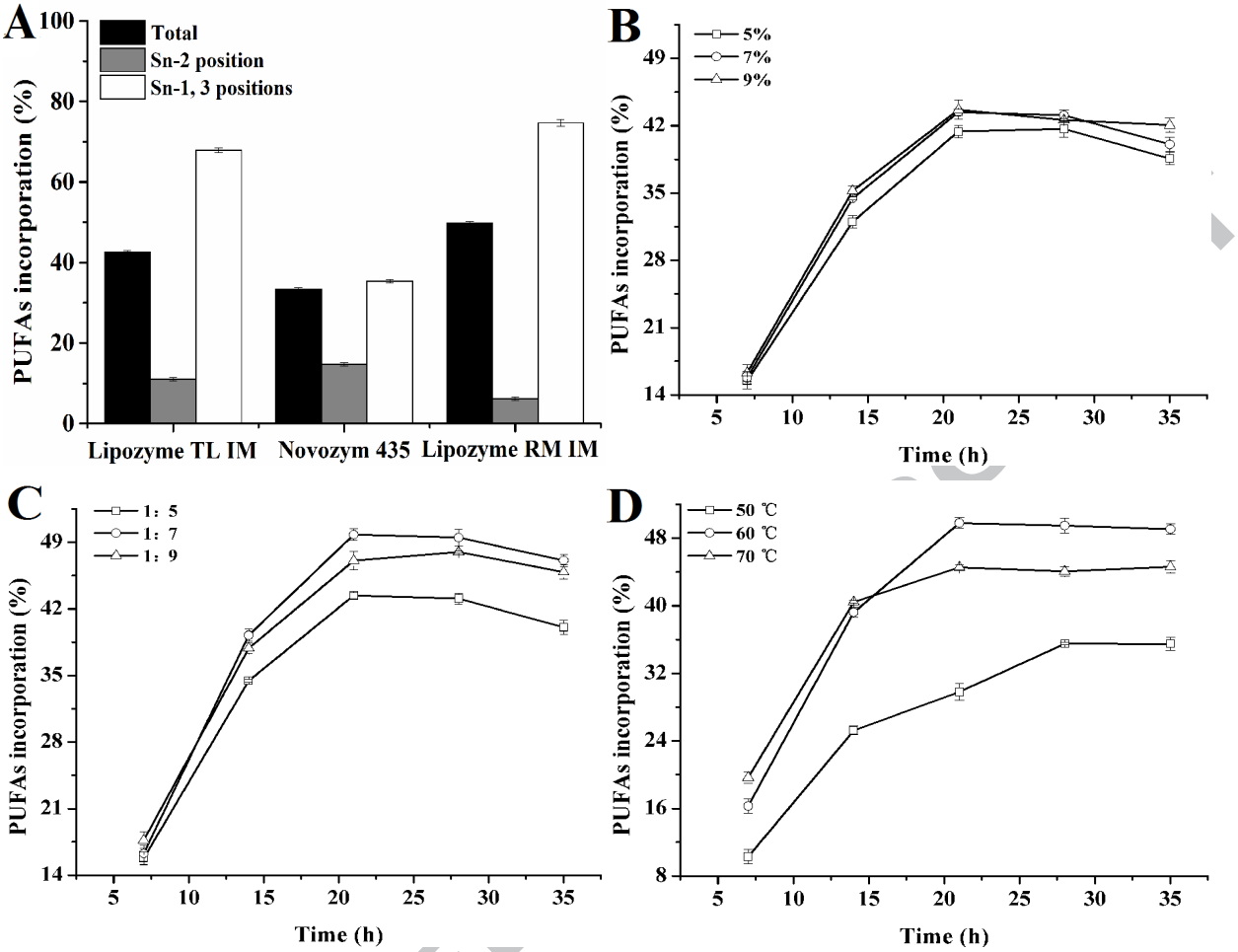
Fig. 2. The effect of temperature on PA and PUFA content in microalgae oil TAGs and SLs after lipase-catalyzed acidolysis. Reaction conditions: The effects of temperature on PUFAs (A), OA (B), DPA (C) and DHA incorporation (D) with substrate ratio of 1:3 at the temperature range from 45 °C to 70 °C and under the flow rate range from 2 μL/min to 20 μL/min. The effect of temperature on PA (E) and PUFAs (F) incorporation at sn-1, 2, 3 positions with substrate ratio of 1:3 at the temperature range from 45 °C to 70 °C and under the flow rate of 2 μL/min.

Fig. 3. The effect of substrate ratio on PA and PUFA content in microalgae oil TAGs and SLs after lipase-catalyzed acidolysis. Reaction conditions: The effects of substrate ratio on PUFAs (A), OA (B), DPA (C) and DHA incorporation (D) with substrate ratio range from 1:1 to1:11 at 60 °C; and under the flow rate range from 2 μL/min to 20 μL/min. The effects of substrate ratio on PA (E) and PUFAs (F) incorporation at sn-1, 2, 3 positions with substrate ratio range from 1:1 to1:11 at 60 °C; and under the flow rate of 2 μL/min.

Fig. 4. Kinetic plots of the lipase-catalyzed synthesis of SLs using different reactors. (A) Lilly–Hornby plots of the continuous flow enzyme microreactor at flow rates of 2 μL/min and 5 μL/min under different palmitic acid triglycerides concentration; (B) Lineweaver–Burk plot of reciprocal initial rates vs. reaction conditions: 7% enzyme, 1:7, 60 °C and 120 rpm.

Fig. 5. Melting (A) and crystallization (B) characterization of STAGs in different reactors.

Fig. 6. Reusability and service life of Lipozyme RM IM in transesterification synthesis of SLs using different reactors. Reaction conditions: (A) Batch reactor, lipase amount: 7%, molar ratio: 1:7, and temperature: 60 °C. (B) Microreactor, flow rate: 2 μL/min, 90 mg lipase, molar ratio: 1:7, and temperature: 60 °C.



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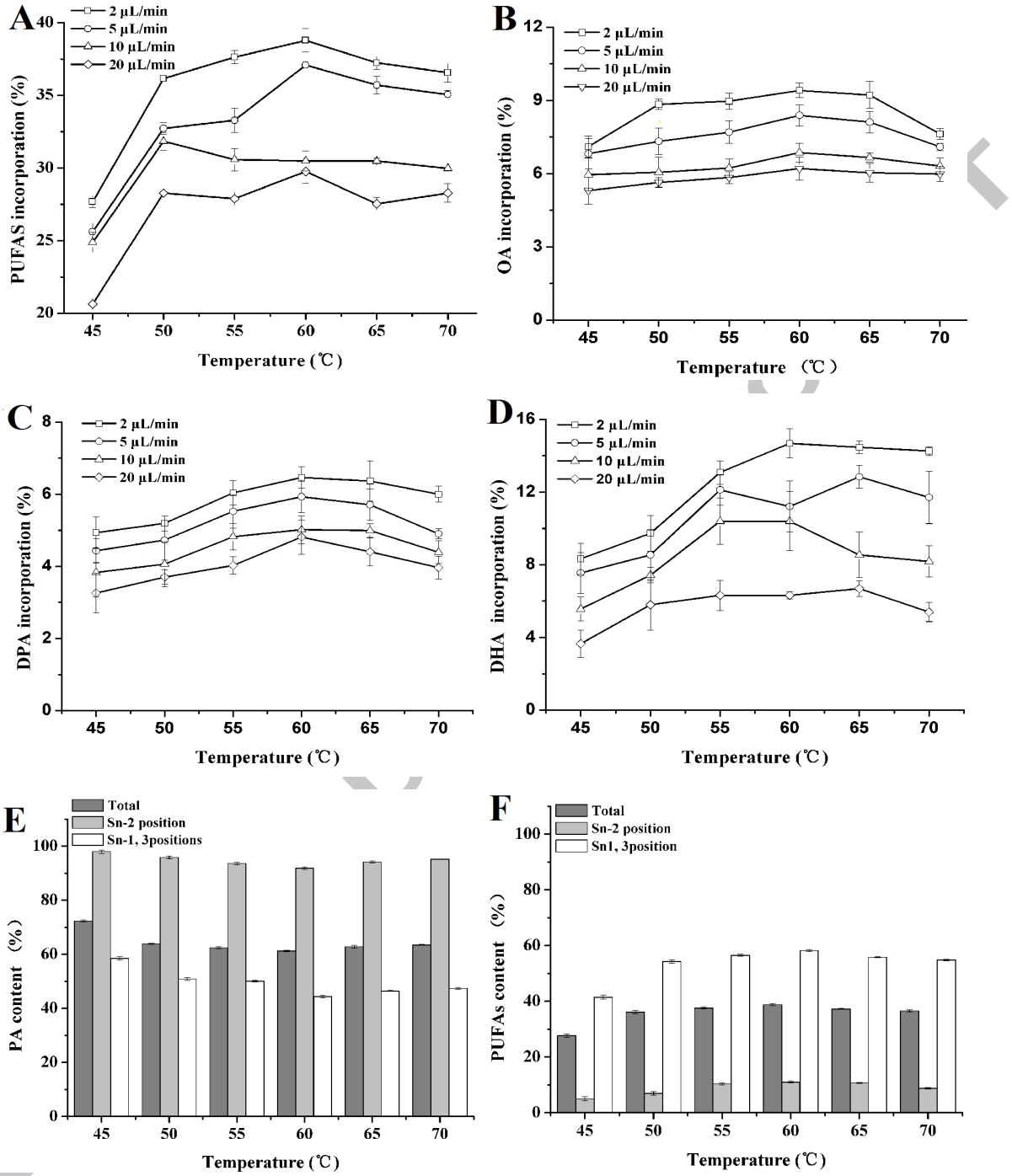

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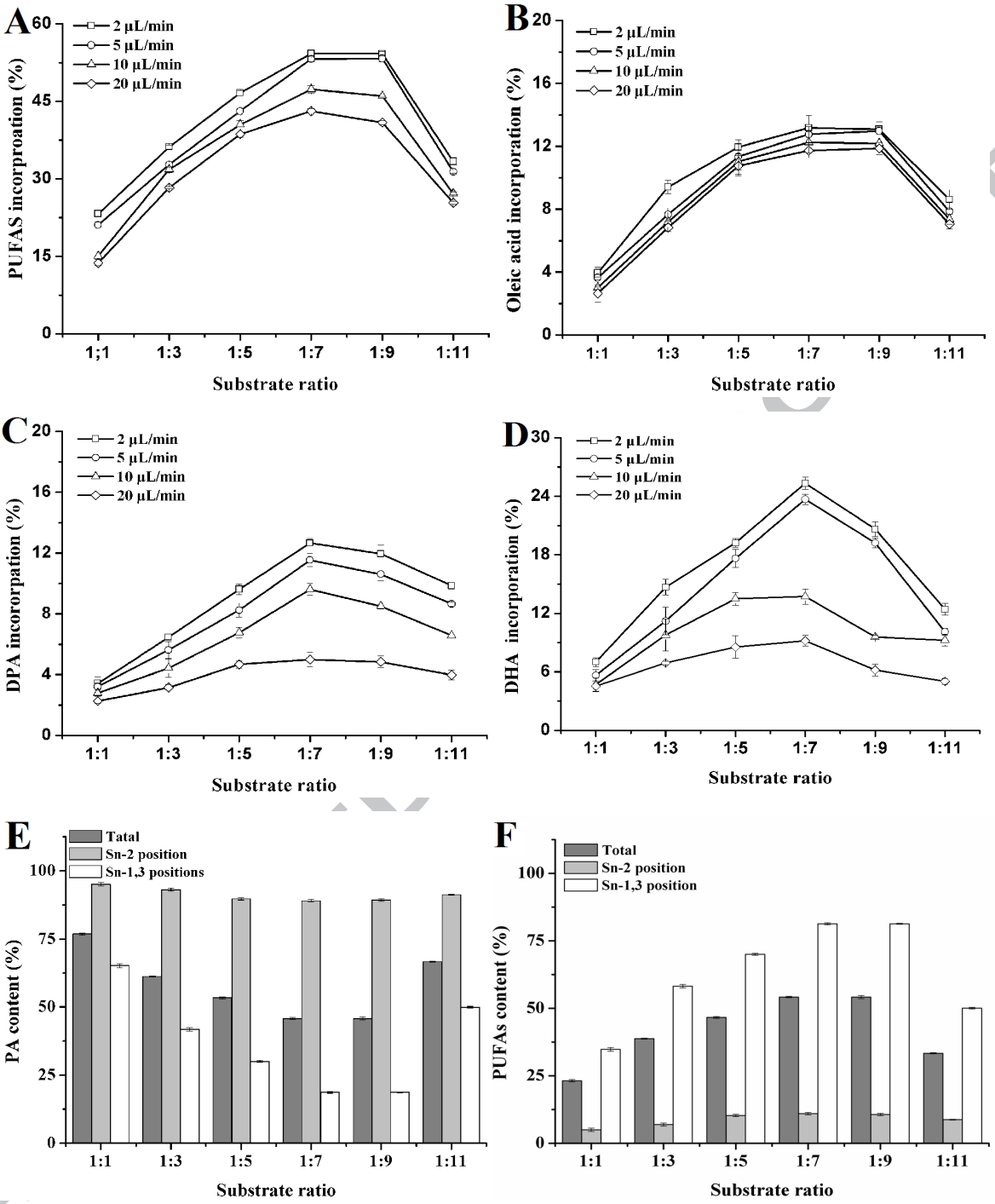

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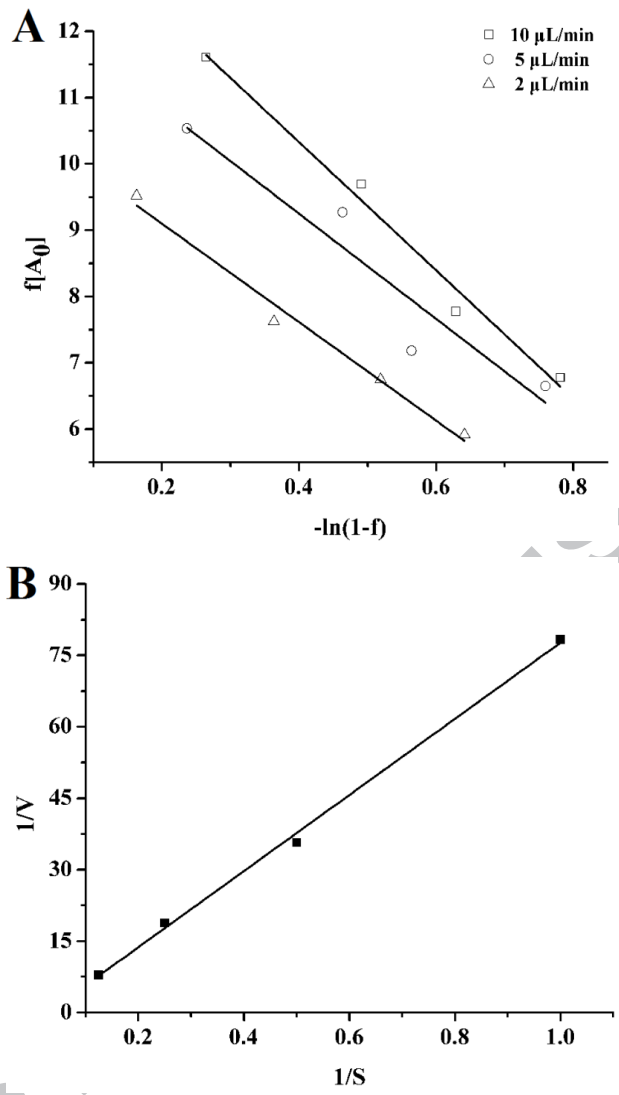

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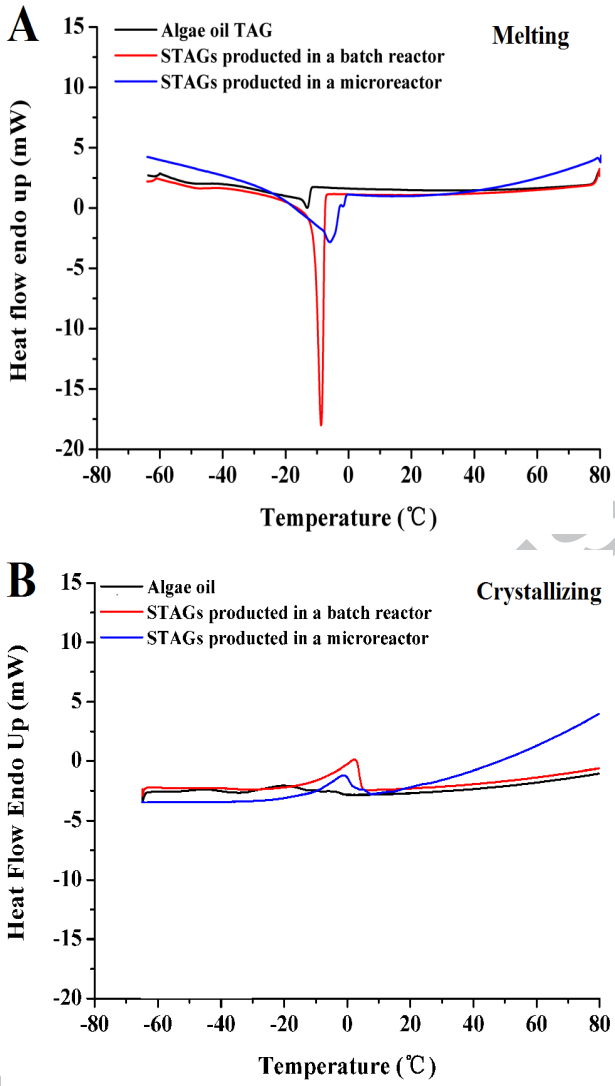

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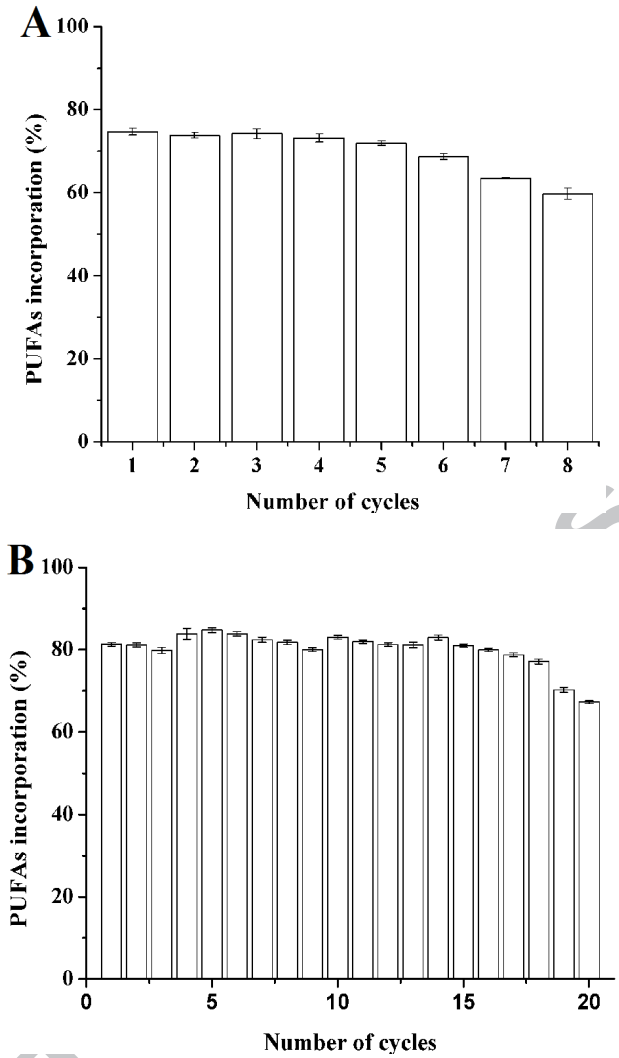

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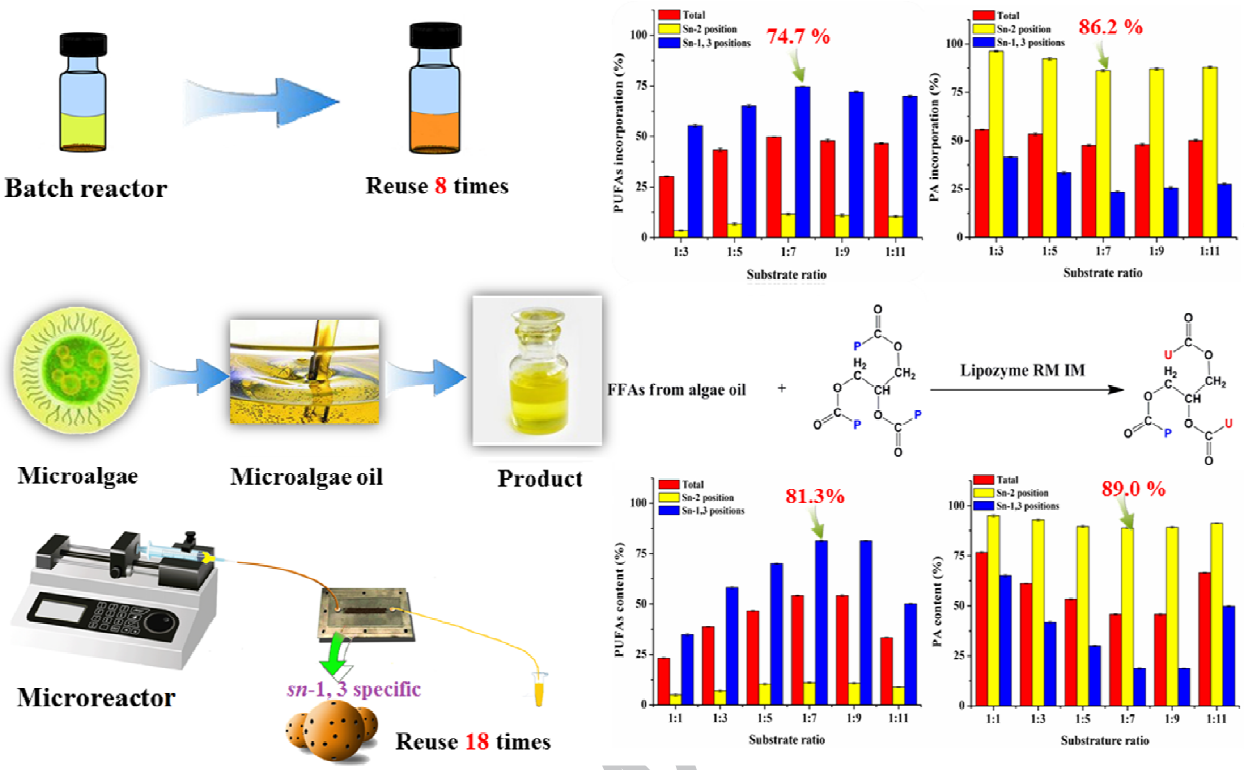

Table 1. Relative fatty acid compositions of different oils.

Fatty acids	Content (%)								
	AO [a]			AOSL [b]			Tuna oil [c]		
	Total	*sn*-2	*sn*-1,3	Total	*sn*-2	*sn*-1,3	Total	*sn*-2	*sn*-1,3
C8:0	ND	ND	ND	ND	ND	ND	45.2	12.8	61.4
C14:0	5.5	7.5	4.5	3.0 ± 0.3	1.4 ± 0.5	0.5 ± 0.6	3.8	8.0	1.6
C16:0	19.1	8.3	24.5	45.8 ± 0.3	89.0 ± 0.9	18.2 ± 0.7	12.4	22.0	7.6
C16:1	ND	ND	ND	ND	ND	ND	4.3	7.5	2.7
C18:0	1.2	1.1	0.8	1.3 ± 0.7	ND	ND	2.0	1.5	2.3
C18:1	16.2	38.7	9.6	12.8 ± 0.9	1.5 ± 0.3	19.8 ± 0.6	7.2	8.8	6.4
C18:2n-6	4.1	1.9	5.2	1.0 ± 0.2	0.4 ± 0.1	4.6 ± 0.5	ND	ND	ND
C20:1	ND	ND	ND	ND	ND	ND	1.2	1.4	1.1
C22:5n-6	16.1	11.5	18.5	10.9 ± 0.4	2.2 ± 0.2	19.0 ± 0.7	4.7	7.8	3.2
C22:6n-3	38.6	34.0	40.9	25.3 ± 1.0	2.5 ± 0.6	38.0 ± 0.8	16.2	24.9	11.9

ND: not detected;

[a] Fatty acid compositions of microalgae oil from *Schizochytrium sp*;

[b] Fatty acid compositions of microalgae oil interesterified SLs product using optimum conditions;

[c] Adapted from Hita et al, 2007.

Table 2. Kinetic constants for the lipase-catalyzed synthesis of STAGs using a continuous flow packed bed microreactor at 60°C.

Reactor	Flow rate (μL min^{-1})	$K_{m(app)}$ (mM)	R^2
Packed bed microreactor [a]	2	7.4	0.978
Packed bed microreactor [b]	5	7.9	0.859
Packed bed microreactor [c]	10	9.7	0.979
Batch reactor [d]	-	79.9	0.996

[a] Molar ratio of microalgae oil to palmitic acid triglycerides = 1:7, lipase amount: 90mg, flow rate: 2μL min^{-1};

[b] Molar ratio of microalgae oil to palmitic acid triglycerides = 1:7, lipase amount: 90mg, flow rate: 5μL min^{-1};

[c] Molar ratio of microalgae oil to palmitic acid triglycerides = 1:7, lipase amount: 90mg, flow rate: 10μL min^{-1};

[d] Molar ratio of microalgae oil to palmitic acid triglycerides = 1:12, lipase amount: 7% of the mass of the substrate.

Table 3. Mass transfer coefficient of both batch and microfluidic reactors

Reactor	$r_{obs}\rho_p$ (mol $(m^3 \cdot s)^{-1}$)	R_c (m)	De (mol $(m^2 \cdot s)^{-1}$)	CAS/ CA (mol m^{-3})	K_c (m s^{-1})	C_{WP}	C_M
Packed bed microreactor[a]	9.3×10^{-3}	1.5×10^{-4}	7.9×10^{-8}	1.2×10^{2}	2.2×10^{-5}	2.3×10^{-5}	1.5×10^{-3}
Batch reactor[b]	1.5×10^{-3}	1.5×10^{-4}	7.9×10^{-8}	2.2×10^{2}	2.2×10^{-5}	3.6×10^{-6}	1.4×10^{-4}

[a] Molar ratio of microalgae oil to palmitic acid triglycerides = 1:7, lipase amount: 90mg, flow rate: 2μL min^{-1};

[b] Molar ratio of microalgae oil to palmitic acid triglycerides = 1:12, lipase amount: 7% of the mass of the substrate.

Table 4. The comparison of production structure in two reactors.

Fatty acids	Content in a batch reactor (%) [a]			Content in a mircoreactor (%) [b]		
	Total	*sn*-2	*sn*-1,3	Total	*sn*-2	*sn*-1,3
C8:0	ND	ND	ND	ND	ND	ND
C14:0	2.3 ± 0.9	2.8 ± 0.5	3.3 ± 0.7	3.0 ± 0.3	1.4 ± 0.5	0.5 ± 0.6
C16:0	47.2 ± 0.5	86.2 ± 0.6	17.2 ± 0.6	45.8 ± 0.3	89.0 ± 0.9	18.2 ± 0.7
C18:0	0.7 ± 0.5	ND	1.8 ± 0.9	1.3 ± 0.7	ND	ND
C18:1	12.0 ± 0.7	4.2 ± 0.2	17.2 ± 0.9	12.8 ± 0.9	1.5 ± 0.3	19.8 ± 0.6
C18:2n-6	2.7 ± 0.8	0.2 ± 0.3	5.6 ± 0.3	1.0 ± 0.2	0.4 ± 0.1	4.6 ± 0.5
C22:5n-6	10.3 ± 0.3	2.4 ± 0.6	18.6 ± 0.2	10.9 ± 0.4	2.2 ± 0.2	19.0 ± 0.7
C22:6n-3	24.8 ± 0.3	4.2 ± 0.8	36.4 ± 0.7	25.3 ±1.0	2.5 ± 0.6	38.0 ± 0.8

[a] Molar ratio of microalgae oil to palmitic acid triglycerides = 1:7, lipase amount: 7% of the mass of the substrate, temperature: 60 °C, react time: 21h;

[b] Molar ratio of microalgae oil to palmitic acid triglycerides = 1:7, lipase amount: 90mg, temperature: 60 °C, react time: 2.5h.

Table 5. Main economic costs of the reaction in two reactors

Reactor	Parameters	Lipase	Tripalmitin	Algae oil	Total [a]
Batch reactor	Weight (g)	0.78	3.36	9.80	--
	Price ($)	1.00	13.96	2.77	212.31
Microreactor	Weight (g)	0.09	3.00	8.75	--
	Price ($)	0.11	12.46	2.48	14.58

[a] React for 2.5h and keep the production field of 72.09 % with the space-time productivity in both reactors respectively.

Highlights

1. UPU-style STAGs was produced in a packed bed microreactor with algae oil as feedstock.

2. Incorporation of PUFAs is 9 % higher than that in a batch reactor.

3. Reaction time was reduced by 87.5 % compared to that needed in a batch reactor.

4. The Michaelis constant decreased ten times compared to that needed in a batch reactor.

5. The lipase reuse times increased 2.25-fold compared with that in a batch reactor.