Ultrasonic pretreatment in lipase-catalyzed synthesis of structured lipids with high 1,3-dioleoyl-2-palmitoylglycerol content

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Production of structured lipid, 1,3-dioleoyl-2-palmitoylglycerol (OPO), from tripalmitin (PPP) and oleic acid (OA) using lipases and ultrasonic pretreatment was conducted. Factors influencing both the ultrasonic conditions and enzymatic reaction were investigated. Optimum conditions could be attained with 6 min pretreatment time, 50% ultrasonic power, 3 s/9 s (work/pause) cycle of ultrasonic pulse, 1:8 PPP/OA molar ratio, 12% enzyme dosage and 50 °C temperature of. At the optimum conditions, the OPO yield of 51.8% could be achieved in 4 h. Studies showed that the OPO content increased to 35.9% in 1 h with ultrasonic pretreatment, in comparison to 4 h without ultrasonic pretreatment. Reuse of Lipozyme RM IM for 10 cycles under ultrasonic irradiation did not cause essential damage to its lipase activity. Reaction kinetic model fitted well with the proposed Ping-Pong mechanism. The apparent kinetic constant (V m/K1) of ultrasound pretreatment reaction was 2.52 times higher than the conventional mechanical stirring, indicating that ultrasound pretreatment enhanced the substrates affinity to the enzyme. This study confirmed that ultrasonic pretreatment was more efficient in OPO production than conventional mechanical agitation.

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1. Introduction

Fats and oils are major food constituents. They are also high-energy substances in the human body, and are composed of >95% triglycerides (TAGs). The nutritional value and physicochemical properties of TAGs depend not only on the fatty acid composition, but also on the positional distribution of acyl groups bonded to glycerol [1]. Hence, improvement of the physicochemical properties and nutritive values of TAGs by modification of their fatty acid composition and positional distribution holds promise in nutrition, food and pharmaceutical applications. Structured lipids (SLs), a form of TAGs, have been studied extensively by using chemically catalyzed reactions, enzymatically catalyzed reactions, genetic engineering, or a combination thereof to modify the fatty acid composition, positional distribution in the glycerol backbone, or both [2]. Various SLs have been used to limit the caloric intake of fats and oils, to improve the physicochemical properties of fats, to reduce the absorption of long-chain saturated fatty acids, and to increase the amount of essential fatty acids for specific purposes, such as docosahexaenoic acid and arachidonic acid [3]. 1,3-Dioleoyl-2-palmitoylglycerol (OPO), an important ABA-type SL, is one of the main components of TAGs in human milk [4]. Human milk fat is the richest energy source for infants, supplying essential structural components for the cell membranes of newborns [5]; therefore, numerous investigations have focused on the production of OPO [6].

Enzyme-catalyzed synthesis of SLs has major advantages over chemical synthesis in terms of practical applications such as those involving mild reaction conditions and environmentally friendly processes [7]. Another advantage of enzyme technology is its amenability for use with foodstuffs. Acidolysis is an enzymatic method that is more commonly employed than interesterification for the production of OPO SLs, as its products are easily separable from the final reaction mixtures, consequently decreasing the cost of separation [8]. Acidolysis consists of two steps: the starting TAGs are first hydrolyzed into diacylglycerols and monoacylglycerols, and then the new fatty acids are esterified into the glycerol skeleton to form new TAGs [9]. Acyl migration is a side reaction of esterification that forms byproducts. Acyl migration is affected by many factors such as type of lipase, solubility of the substrate, reaction
temperature, and reaction time [10]. Attempts have been made to reduce acyl migration during enzymatic acidolysis. These include reduction of reaction time, activation of lipase activity, and diminution of mass-transfer limitations [11].

Ultrasound irradiation, an environmentally friendly method, has gained popularity and potential for use in applications in organic chemistry and in biotechnology [12,13]. This method has been used to accelerate the rates of numerous chemical reactions [14]. However, its effects on enzymatic reactions have been studied less extensively [15]. Studies on enzymatic reactions subjected to ultrasound irradiation used it mainly for pretreatment and for catalytic reactions. Ultrasound pretreatment for enzymatic reactions aids in reducing the particle size and in increasing the substrate–enzyme interface area through its high cavitation energy. This effect is much more obvious in reactions in organic media using enzyme powders and reduces in mass-transfer limitations [16]. The use of ultrasound throughout the reaction allows substrates to access active sites more easily [17]. Conformations of enzymes change upon exposure to ultrasound. Immersion in an ultrasonic cleaning bath is the most common method used in ultrasound-assisted reactions. However, ultrasound energy cannot be completely transferred to a reaction because of its indirect mode of transmission. Furthermore, it is difficult to realize in large-scale production and is uneconomical in industrial application. Thus, ultrasound pretreatment using microtip probe may be an appropriate method for enzymatic reactions [18]. Many investigators utilized ultrasound to conduct enzymatic interesterification and thus obtained promising results [19,20]. Compared with conventional methods, ultrasound pretreatment could improve the biocatalyst performance [21], shorten the reaction time [22], and increase the product yields [23,24].

In this paper, lipase-catalyzed synthesis of SLs is reported. This approach effectively enriches the OPO content via ultrasound pretreatment in organic media using microtip probe. This work mainly focuses on the reaction parameters that affect lipase-catalyzed synthesis of OPO subjected to ultrasound. A comparison of the OPO content and its effect on lipase reusability was made between ultrasound irradiation and conventional stirring methods under optimal conditions. Kinetic study was also investigated at the optimal experimental conditions to determine the apparent kinetic parameters for reactions under either ultrasound pretreatment or conventional mechanical stirring. This work is a comprehensive study on OPO production and on monitoring of acyl migration during pretreatment ultrasound irradiation.

2. Materials and methods

2.1. Materials

Tripalmitin (>85% purity) and oleic acid (>99% purity) were purchased from Tokyo Chemical Industry Co., Ltd. The immobilized lipases Lipozyme RM IM (immobilized on anion exchange resin) from Rhizomucor miehei, Lipozyme TL IM (immobilized on silica gel) from Thermomyces lanuginosus, and Novozym 435 (immobilized on polyacryl resin) from Candida antarctica were purchased from Novozymes (Bagsvaerd, Denmark). HPLC-grade hexane and acetonitrile were obtained from CNW (Düsseldorf, Germany). All TAG standards (PPP, OPP, 1,2-dipalmitoyl-3-oleoylglycerol (PPO), 1,3-dipalmitoyl-2-oleoylglycerol (POP), 1-palmitoyl-2, 3-dioleoyl-glycerol (POO) and triolein (OOO); Larodan Fine Chemicals, Malmö, Sweden) were dissolved in hexane to a concentration of 5 mg/mL and then stored at −20 °C. Analytical grade n-hexane and other solvents were purchased from Sinopharm Chemical Reagent (Shanghai, China).

2.2. Equipment

Ultrasound pretreatment reactions were carried out in a long-necked round-bottom flask (10 mL) in a thermostated water bath (Pharmacia, Sweden) with a microtip probe (3 mm diameter), and a sonotrode (Sonics Vibracell, VCX 130, 130 W, 20 kHz, USA). The power for the reaction could be adjusted from 20 to 60%. Lipase-catalyzed acidolysis reactions were performed in a thermostated water bath with magnetic stirrer (Aohua Instrument Co., Ltd., Changzhou, China). An Agilent 1200 series HPLC system was used for the separation of TAGs. It was equipped with a G1312A bin pump, a G1379B degasser, a G1329 Aautosampler, and a G1316 Athermostated column compartment (Agilent Corporation, Palo Alto, CA). MS used for analysis was performed on an API 4000 Q-Trap hybrid triple quadrupole/linear ion trap mass spectrometer with an APCI interface (AB SCIEX, Foster City, CA, USA).

2.3. Lipase-catalyzed acidolysis with ultrasonic pretreatment

A typical reaction was carried out by combining 0.1 g of PPP, 0.21 g of commercial OA (1:6 PPP/OA mole ratio), 0.0248 g of lipase (8% by total weight of substrates), and 3 mL of hexane with 4A molecular sieves (50 mg/mL). Hexane was previously dried with molecular sieves (100 g/L) for at least 48 h. The mixtures were placed into a 10 mL round-bottom flask. The flask was firstly placed in a thermostated circulating water bath with microtip probe for ultrasound pretreatment. It was then transferred to a water bath with magnetic agitation at 200 rpm for further reaction. During the reactions, samples were withdrawn from the mixture every hour to analyze for reaction progress. All analyzes were performed in triplicate and the results were reported as the mean standard deviation.

2.4. Reuse of the enzyme

At the end of each round of acidolysis, the immobilized lipase was washed thrice with n-hexane and then separated from the substrate by vacuum filtration. It was then dried for 10 h at 40 °C (in an oven) and reused in a subsequent new reaction.

2.5. Analysis and determination of TAG products by silver ion HPLC atmospheric pressure chemical ionization tandem mass spectrometry (HPLC–APCI–MS/MS)

During synthesis, OPO was obtained. However, TAGs containing oleic acid at the sn-2 position (i.e. POP, POO, and OOO) were also produced because of acyl migration. In this study, Lipozyme RM IM that we used is wildly known as a sn-1,3 specific lipase, which hydrolyzes the ester bonds at sn-1 and sn-3 positions of TAG, but not sn-2 position. Acyl migration from sn-1 and sn-3 to sn-2 position or remigration from sn-2 to sn-1,3 can take place [25]. Fig. 1 shows a proposal for the scheme and mechanism of the current lipase-catalyzed acidolysis reaction. Silver ion HPLC has proven to be an effective means to monitor acyl migration and to control the product quality of the reaction. Thus, HPLC using a Varian ChromSpher 5 Lipids silver ion chromatography column (250 mm × 4.6 mm i.d., 5 µm particle size; Agilent Technologies, Milan, Italy) was performed to separate TAGs. Isocratic elution with hexane/acetonitrile mobile phase (99:4:0.6 v/v) was done at 1.5 mL/min flow rate of, 10 µL injection volume, and 30 °C oven temperature, in accordance with our previous report [26]. MS was carried out in positive APCI mode under the following conditions: curtain gas, 137.9 kPa; nebulizer current, 27.58 kPa; temperature, 450 °C; scan mode, multiple reaction monitoring-enhanced product ion (MRM-EPI); scan rate, 4000 µs; pressure of ion source gas 1, 344.75 kPa; declustering potential, 85 V; collision energies, 100 V. The product ions (m/z 564) in multiple reaction monitoring with product ion (m/z 564) in multiple reaction monitoring were monitored.
35–55 V; collision energy spread, 5 V; collision cell exit potential, 17 V; mass range, 500–1000 m/z. Samples were diluted with hexane and analyzed by silver ion HPLC–APCI–MS/MS.

To evaluate the linearity of the method, each TAG standard was diluted with hexane–acetonitrile solution (99.4:0.6 v/v) at concentrations ranging from 0.01 to 0.2 mg/mL. To construct the calibration curve, triplicate measurements were performed under scan mode of MRM–EPI, and a calibration curve was created by plotting the average peak area and its concentration.

The extent of acyl migration was defined as a percentage of POP, POO, and OOO content of the TAGs (Eq. (1)). The extent of PPP conversion and OPO content were calculated from Eqs. (2) and (3), respectively.

\[
\text{Acyl migration (\%)} = \frac{[A]_{\text{POP+POO+OOO}}}{W} \times 100
\]

\[
\text{PPP conversion (\%)} = \frac{[B]_0 - [B]_t}{[B]_0} \times 100
\]

\[
\text{OPO content (\%)} = \frac{[C]_{\text{OPO}}}{W} \times 100
\]

where \([A]_{\text{POP+POO+OOO}}\) is the total content of POP, POO, and OOO; \([B]_0\) and \([B]_t\) are the amounts of PPP at the beginning and at a given time \(t\) of the reaction. \([C]_{\text{OPO}}\) is the OPO content and \(W\) is the total amount of PPP, PPO, POP, POO, OPO, and OOO.

2.6. Determination of kinetic constants

In order to make models of the reaction rate and compare reaction rate constants, reaction kinetic experiments were performed by a PPP concentration (25 mM) with various OA concentrations (12.5–200 mM) in 3 mL hexane at 50 °C. In all the experiments, a concentration of the Lipozyme RM IM of 10 wt% (based on the total amount of substrates PPP and OA) was used. Initial reaction rates, expressed as mM produced TAGs per hour, were determined from the time course of TAGs concentration by regression analysis and determining the initial slope of the tangent to the curve.

3. Results and discussion

3.1. Quantitation of TAGs by silver ion HPLC–APCI–MS/MS

Various analytical methods have been used to determine and quantitate the targeted products, TAGs, and their positional distributions. These methods include thin-layer chromatography [27], lipase hydrolysis [28], chemical degradation using a Grignard reagent [29], and \(^{13}\)C NMR spectroscopy [30]. However, these methods merely determine the positional distributions of the fatty acids in the TAGs and are incapable of identifying the TAG species.

Silver ion HPLC is an efficient means of separation and quantitation of positional isomers of fatty acids and TAGs. It principle is based on the number, geometrical configuration, and position of double bonds in the molecule [31]. Thus, positional isomeric pairs such as PPO, POP, POO, and OPO in the reaction product can be effectively separated. Fig. 2 shows good separation of TAGs from a standard lipid mixture by silver ion HPLC. Linear responses of the TAGs were obtained in the concentration range of 0.01–0.2 mg/mL. Amounts of PPP, PPO, POP, POO, OPO, and OOO were quantified by using the corresponding calibration curves.

3.2. Effect of reaction time and lipase in ultrasonic pretreatment on OPO content

As shown in Fig. 3, the OPO content increased with reaction time and then remained essentially constant or even decreased after 4 h. Lipzyme RM IM resulted in the maximum OPO content in a relatively short reaction time compared with those obtained with Lipozyme TL IM and Novozym 435. Enzyme structure can become flexible under the condition of ultrasound irradiation, and thus, the enzyme may shift into its active configuration. However, different enzymes may have different stereo configurations, they can display different effects even in the same enzyme-catalyzed reaction under the same adopted parameters of ultrasonication [32]. Therefore, according to our experiment results, Lipozyme RM IM was selected as a catalyst to analyze the effect of ultrasonic pretreatment on OPO content in the subsequent experiments.

3.3. Effect of ultrasonic power in ultrasonic pretreatment on OPO content and extent of acyl migration

Ultrasonic power is one of the most critical factors influencing OPO content and acyl migration. To obtain the maximum OPO content and minimum acyl migration, the ultrasonic power was set in the range of 20–60%. Ultrasonic pretreatment enhances the interfacial area [33]. However, high-intensity ultrasound could lead to disruption of the enzyme’s catalytic activity [34]. Fig. 4 shows the effect of ultrasonic power on OPO content and extent of acyl migration during the catalytic reaction. The results show that increasing the ultrasonic power to an appropriate range could enhance the OPO content.

Fig. 1. Reaction scheme of the sn-1,3-specific lipase-catalyzed acidolysis of PPP with oleic acid. P, palmitic acid; O, oleic acid.

Fig. 2. Silver ion HPLC–APCI–MS/MS separation the six standard TAG mixtures (PPP, PPO, POP, POO, OPO and OOO).
content and enzymatic reaction rate, consistent with a previous report [35]. An obvious decrease in OPO content was observed when the ultrasonic power was increased to 60%. The main explanation for this trend can be that ultrasound pretreatment caused a decrease in the particle size of the catalyst and consequently increased the catalytic surface area. Ultrasound energy helped reduce mass-transfer limitations, but further increasing the power during pretreatment resulted in enzyme inactivation, in agreement with other studies [36]. Therefore, 50% ultrasonic power was selected for further experiments according to the maximum yield of OPO and minimum extent of acyl migration.

### 3.4. Effect of ultrasonic time in ultrasonic pretreatment on OPO content and extent of acyl migration

Many studies reported that ultrasound-assisted treatment leads to energy dissipation during the entire enzymatic reaction, resulting in difficulties in increasing production [37]. However, if ultrasound irradiation is used merely as a direct pretreatment method before the enzymatic reaction, it is much more efficient and feasible for use in industrial applications. The effect of ultrasonic pretreatment time on the enzymatic acidolysis of PPP with OA is shown in Fig. 5. The OPO content rapidly increased with the increase in ultrasonic pretreatment time from 0 to 12 min. However, degree of conversion decreased when the ultrasonic pretreatment time exceeded 12 min. The main reason for this decrease is the action of ultrasound irradiation in reducing the particle size of the substrate and in reducing mass-transfer resistance. This action is significant in heterogeneous reactions in organic solvents. According to Shah and Gupta [38], ultrasound irradiation of lipases in organic solvents enhanced enzyme activity, their results from circular dichroism (CD) spectra show that ultrasound irradiation led to slight perturbation of the lipase tertiary structure. Similarly, scanning electron microscope (SEM) demonstrated significant morphological changes in the enzyme preparation as a result of ultrasonicitation. However, further treatment induces enzyme inactivation [39]. Upon considering the OPO content, extent of acyl migration, reaction efficiency, and energy consumption of the process, we selected 6 min as the pretreatment time for ultrasound-assisted reaction in the succeeding experiments.

### 3.5. Effect of ultrasonic pulse mode in ultrasonic pretreatment on OPO content and extent of acyl migration

Ultrasonic pulse is defined here as the working time and intermittent time during ultrasonic irradiation. A previous study pointed out that continuous operation without pulse damages the transducers that produce ultrasonic waves [40]. Moreover, the catalytic property of the enzyme is deactivated with extended irradiation time. Thus, ultrasonic pulse was performed at 3 s/9 s, 3 s/6 s, 3 s/3 s, 6 s/3 s, 9 s/3 s work/pause cycles in this study. Fig. 6 shows the effect of the ultrasonic pulse on OPO content and extent of acyl migration. The mode of the pulse had an irregular influence on OPO content and extent of acyl migration. Modes with shorter working time were sufficient to stimulate enzyme activity. Longer working time led to greater extent of acyl migration, although it had no significant influence on the OPO content. Thus, a 3 s/9 s work/pause cycle was chosen as the pulse mode for subsequent experiments.

### 3.6. Effect of substrate molar ratio in ultrasonic pretreatment on OPO content and extent of acyl migration

The rate of enzymatic reaction depends on the concentrations of enzyme and substrate; thus, variation of the molar ratio of the substrate may have an effect on the reaction. For this reason, the
substrate molar ratio (PPP/OA ratio) was changed from 1:2 to 1:10 upon increase of the OA concentration in this experiment. All other parameters were held constant. As shown in Fig. 7, the OPO content slightly increased with the increase in PPP/OA from 1:2 to 1:8; however, further increasing the substrate ratio did not result in an increase in OPO content. This result may be due to the reduced ability of enzyme active sites to accommodate more substrates in the presence of excess substrate. Upon considering the
OPO content and economy of the reaction, we deemed 1:8 as the optimum substrate molar ratio.

3.7. Effect of enzyme dosage in ultrasonic pretreatment on OPO content and extent of acyl migration

The effect of Lipozyme RM IM dosage on OPO production under ultrasound irradiation was investigated. The enzyme dosage was varied from 4% to 20% according to the weight of the substrates, with the mole ratios of the reactants held constant. As shown in Fig. 8, the OPO yield increased with the increase in enzyme dosage from 4% to 12%. This may due to the available of extra enzyme active sites for catalytic reaction. However, at higher enzyme dosages (16–20%), the OPO yield decreased sharply while acyl migration increased rapidly after reaction times of 4 and 2 h for reactions with 16–20% enzyme respectively. These results could be explained by the facilitated contact between the enzyme and substrate caused by the increased enzyme dosage leading to TAG hydrolysis and thus acyl migration. Taking into consideration the OPO content and cost of the enzyme, we found that 12% was the ideal dosage of Lipozyme RM IM.

3.8. Effect of reaction temperature in ultrasonic pretreatment on OPO content and extent of acyl migration

In theory, an increase in temperature provides a reaction with sufficient energy to surmount its energy barrier. However, higher temperatures lead to the disruption of the enzyme tertiary structure, thereby easily deactivating the enzyme [41].

In this work, the effect of temperature (45–65 °C) on the products of reaction with Lipozyme RM IM was examined. Fig. 9 illustrates that reaction temperatures of 45–50 °C resulted in an increase of the OPO content. A decrease in OPO content was observed when the temperature was further increased above 55 °C. Moreover, when temperatures were increased from 55 to 65 °C, there were obvious decreases of OPO produced throughout the reaction period. This decrease is especially significant for the reaction occurred at 65 °C, indicating the deactivation of the enzyme. On the other hand, the acyl migration shows opposite trends to those of the OPO content. The increase of temperature affects the bubble formation and collapse caused by ultrasound cavitation, the effect of ultrasound appeared to be weak at higher temperature [42]. Based on current results, we selected 50 °C as the optimum reaction temperature for subsequent experiments.

Fig. 8. Effect of enzyme dosage on OPO content and extent of acyl migration. Reaction conditions: 50% ultrasonic power, 3 s/9 s ultrasonic pulse mode, 6 min pretreatment time, 1:8 PPP/OA molar ratio and 3 mL of hexane with molecular sieves 4A (50 mg/mL), 55 °C.

Fig. 9. Effect of temperature on OPO content and extent of acyl migration. Reaction conditions: 50% ultrasonic power, 3 s/9 s ultrasonic pulse mode, 6 min pretreatment time, 1:8 PPP/OA molar ratio, 12% enzyme dosage and 3 mL of hexane with molecular sieves 4A (50 mg/mL).
3.9. Reusability of Lipozyme RM IM in ultrasound pretreatment

The recovery and reusability of immobilized enzymes are among the most important factors that determine their suitability in terms of economic and environmental aspects for industrial applications. Therefore, enzyme reusability experiments were carried out in the presence and absence of ultrasound pretreatment. As shown in Fig. 10, PPP conversion was still retained 73.5% when immobilized Lipozyme RM IM was reused for 10 cycles. Additionally, PPP conversion was slightly higher with ultrasound pretreatment than that with conventional stirring only. This indicates that ultrasound technology could improve operational stability. Thus, ultrasound pretreatment is a promising method for large-scale production of OPO using immobilized lipase.

3.10. Comparison between ultrasound pretreatment and conventional method

In this study, we compared the presence of ultrasound pretreatment method and the conventional absence method at the optimal conditions established in a previous study (Sections 3.2–3.8). It is observed that the production of OPO is consistently higher by at least 10% when ultrasound pretreatment is used throughout the experimental period of 7 h (Fig. 11), while the acyl migration showed no significant differences between these two methods. Ultrasonic pretreatment resulted in a 51.8% yield of OPO within 4 h, producing 35.9% OPO within 1 h. In contrast, the conventional stirring method requires 4 h to produce similar OPO within 1 h. This marked difference is due to the effect of ultrasonic irradiation. Cavitation energy and sonic pressure caused by microjets aid the rapid movement of the substrate to the active site of enzyme, leading to formation and collision of microbubbles involved in the reaction. They also help to maximize the surface area of the enzyme for the esterification reaction.

3.11. Mathematical modeling of the acidolysis reaction kinetics

Enzyme kinetics for acidolysis reaction have been usually expressed by the Ping-Pong Bi Bi mechanism, which is applicable to lipase reactions [43]. The mechanism proposed is shown in Fig. 12. According to the Ping-Pong Bi Bi mechanism, the acidolysis reaction involves sequential execution of the hydrolysis and reesterification reactions by binding of a glyceride molecule (E₁) and rupture of the ester bond to form the mixed acyl complex (E₁A₁) between the released fatty acid (A₁) and lipase. Then the following step involves reesterification between the new fatty acid (A₂) and the ester bond to form a new ester–enzyme complex (E₁A₁A₂). At last, the ester–enzyme complex dissociates into the free enzyme (E) and formation of the final product (P).

The rate equation for the Ping-Pong Bi Bi mechanism can be expressed as follows (Eqs. (4)):

$$v_0 = \frac{V_m[A_0][E_0]}{K_m[E_0] + K_m[A_0] + [A_0][E_0]}$$

Fig. 10. Reusability of Lipozyme RM IM for OPO production.

Fig. 11. A comparison between ultrasound pretreatment and conventional stirring method on the production of OPO. Ultrasound reaction conditions: 50% ultrasonic power, 3 s/9 s ultrasonic pulse mode, 6 min pretreatment time, 1:8 PPP/OA molar ratio, 12% enzyme dosage and 3 mL of hexane with molecular sieves 4A (50 mg/mL), 50 °C. Conventional stirring reaction conditions: 1:8 PPP/OA molar ratio, 12% enzyme dosage and 3 mL of hexane with molecular sieves 4A (50 mg/mL), 50 °C.

Fig. 12. Schematic illustration of Ping-Pong Bi Bi mechanism of lipase-catalyzed acidolysis of PPP with oleic acid. E, enzyme; W, water; Es, PPP; A, fatty acid; P, final product.
where \( V_0 \) is the initial reaction rate, \( V_m \) is the maximum reaction rate, \([A_0]\) is the initial concentration of OA and \([E_0]\) is the initial concentration of PPP. \( K_m \) and \( K_E \) are the Michaelis–Menten constants of OA and PPP. Eqs. (4) can be simplified and rearranged to the Michaelis–Menten equation (Eq. (5)):

\[
\frac{V_0}{V_m K_m [A_0]} - \frac{V_m [A_0]}{K_m [A_0] + [E_0]} = \frac{V_m}{V_m K_1} - \frac{1}{V_m}
\]

where \( K_1 = \frac{K_E}{K_m + K_E} \), \( K_2 = \frac{K_m K_E}{K_m + K_E} \), and \( V'_{m_0} = V_m K_1 \)

\[
\frac{1}{V_0} = \frac{1}{V_m} + \frac{1}{V_m}
\]

The Lineweaver–Burk plot or double reciprocal plot is a common way of illustrating kinetic data, Michaelis–Menten equation (Eq. (5)) can be transferred to the Lineweaver–Burk form (Eq. (6)). The PPP concentration \([E_0]\) was kept at 25 mM with various OA concentrations (12.5–200 mM). Initial rates were calculated using the linear slope of the TAG products concentration–time curve. As can be seen in Fig. 13, a good linearity showed that there is no OA inhibition occurred. Thus, Eqs. (4) and (5) that we can be used in this study. According to Fig. 13 and Eq. (6), the ultrasonic pretreatment apparent \( V_m \), Michaelis constant \( (K_2) \) and apparent kinetic constant \( V_m/K_2 \) were 0.04975 mMs⁻¹, 58.3533 mM⁻¹, 0.0008499 h⁻¹, while the conventional stirring were 0.01478 mMs⁻¹, 43.8444 mM⁻¹, 0.0003370 h⁻¹. According to Kuo [44], the value of apparent kinetic constant \( V_m/K_2 \) is often recognized as a suitable evaluation parameter of enzyme performance. The value of ultrasonic pretreatment was 2.52 times higher than that of conventional stirring. Higher value of \( V_m/K_2 \) indicated higher catalytic efficiency. Moreover, from Eq. (5), the Michaelis–Menten constant of PPP \( (K_m) \) in ultrasonic pretreatment was less than conventional stirring, which indicated ultrasonic pretreatment strengthened the affinity of PPP and the enzyme. Thus, the application of ultrasound is useful to overcome the drawback of enzyme catalyzed reaction.

4. Conclusions

In the present study, we demonstrated that ultrasonic pretreatment using microtip probe is a more efficient method than conventional stirring in lipase-catalyzed synthesis of SLs with high OPO content. Optimal reaction conditions for the pretreatment were 50 °C, 50% ultrasonic power, 3 s/9 s of ultrasonic pulse, 6 min pretreatment time, PPP/OA molar ratio of 1:8, and 12% dosage of Lipzyme RM IM. At the selected optimal conditions, reaction under ultrasonic irradiation generated a higher yield of OPO in a relatively short reaction time compared with conventional stirring. Immobilized Lipzyme RM IM was stable under ultrasound, as PPP conversion was still retained 73.5% when reused for 10 cycles. The enzyme kinetics study for acidolysis reaction under ultrasonic pretreatment and conventional stirring fit the Ping-Pong Bi Bi mechanism, the apparent kinetic constants showed that ultrasonic pretreatment can improve the enzyme performance and the affinity between the substrate and enzyme. Use of this environmentally benign, energy-saving, and efficient method in large-scale production can be explored in the near future.

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