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Ultrasonic pretreatment for lipase-catalyzed synthesis of 4-methoxy cinnamoyl glycerol

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ABSTRACT

4-Methoxy cinnamoyl glycerol (4MCG) is a very promising UV filters material in personal care products. In order to effectively improve the yield of 4MCG, a systematic study on ultrasonic pretreatment enzymatic esterification for 4MCG products was carried out. An ultrasonic frequency of 35 kHz, ultrasonic power of 150 W and ultrasound irradiation time of 1.5 h was determined to guarantee satisfactory degree of esterification and lipase activity. The optimum production was achieved in organic solvent system at 65 °C with 4MCA to glycerol molar ratio of 1:5, enzyme amount of 15 mg/mL, resulting in a monoester yield of above 66% and 55% after 48 h and 24 h of reaction under ultrasonic pretreatment, respectively. The experimental kinetic data were studied. The reactions were modeled by a system of sequential first-order rate expressions, kinetic parameters were estimated from experimental data fit to the model equations. Results show that the monoester yield in the ultrasonic pretreatment process (24 h) were above 1.5-fold as that in mechanical stirring process without essential damaging to lipase activity. The enzymatic method using ultrasonic pretreatment was obviously superior to the mechanical stirring for enzymatic method and chemical method in terms of conversion rate and the monoester yield. These results are of great significance for applying ultrasonic pretreatment method to prepare 4MCG.

1. Introduction

Octyl methoxycinnamate and 2-ethylhexyl-*p*-methoxycinnamate are commonly used as an UV filters in sunscreen and cosmetic formulations to protect skin damage by solar radiation [1,2]. These cinnamates and the related esters formed by combining *p*-methoxy cinnamic acid or ferulic acids with long chain acylglycerides exhibit predominantly lipophilic characteristics [3–6]. However, it would be advantageous to have a UV filter with more hydrophilic character to limit the penetration of a topical formulation into the skin [7]. In order to prepare a sunscreen with hydrophilic character, longer alkyl chain alcohols may be replaced by glycerol in esterification reaction to make it more hydrophilic [6,7].

Glycerol is the main by-product of the conversion of vegetable oils to biodiesel [8–10]. At this point, it may also be an opportune to mention that with the establishment of governments' biodiesel programs worldwide, huge amounts of glycerol surplus are expected to occur in the near future, which will represent an important driving force for the development of new technologies devoted to the transformation of such by-product from biodiesel industrial processing [11].

With a focus on recent developments in the conversion of glycerol into value-added chemicals, the synthesis of 4-methoxy cinnamoyl glycerol (4MCG) was reported by chemical esterification reaction in toluene using *p*-toluenesulfonic acid catalyst [6]. Compared to chemical catalysis, lipase-catalyzed synthesis provides for an environmentally friendlier, more energy efficient and potentially more cost-effective techniques due to low-energy demanding operation and easier downstream processing [12]. Lipase-catalyzed synthesis of 4MCG has been previously reported [7]. Due to the temperature used for most lipase-catalyzed reactions (about 40–70 °C) is quite low, substrates are difficult to dissolve in the reaction system at such low temperature.

The special effects of ultrasound arise from the cavitational collapse which produces extreme conditions and thus induce the formation of chemical species which are not easily attained under conventional conditions, driving a special reactivity [13]. Therefore, ultrasound is a useful tool in the enzymatic heterogeneous systems using immiscible substrates and catalysts [14,15]. Recently, ultrasound irradiation has been used to accelerate the enzymatic reactions such as esterification of phytosterol and different acyl donors, transesterification of methyl benzoate and glycerol in organic solvent, acylation of ascorbic acid with palmitic acid, and synthesis of sugar esters in ionic liquids [16–18]. However, the application of ultrasound to enzymatic reactions is still

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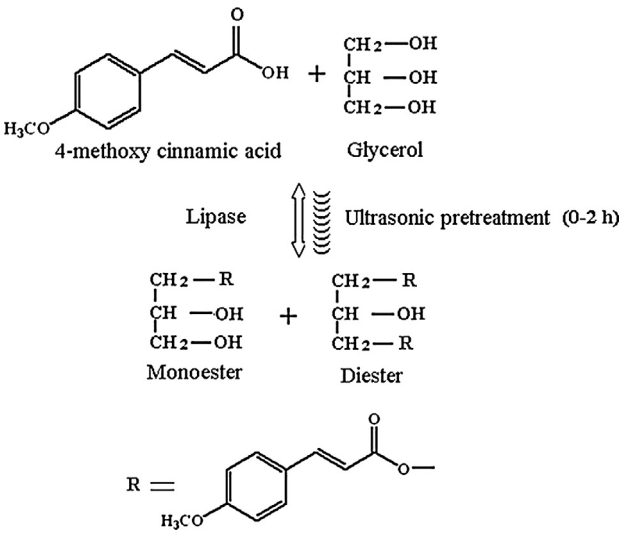

Fig. 1. Ultrasonic pretreatment lipase catalyzed esterification of 4-methoxy cinnamic acid with glycerol.

less extensively studied [19]. The effect of ultrasound on enzymatic reactions could be divided into two aspects. Firstly, ultrasound is used as an enzymatic pre-treatment to reduce particle size and consequent increase in the surface area of substrate and enzyme, which is helpful to reduce mass transfer limitations [20,21]. Secondly, ultrasound is also known to perturb weak interactions and to induce conformational changes in protein structures, thus the substrate's access to the active site is increased [22,23].

In most ultrasound-assistant reactions, ultrasound irradiation was needed throughout the reaction, which was energy-consuming and difficult to scale-up. Therefore, if ultrasound was only used as a pretreatment at the beginning of the reaction, the process would be energy-saving, simple to handle and easy to realize industrialization.

In the present study, we report the lipase-catalyzed synthesis of 4MCG under ultrasonic pretreatment for the first time. The effects of ultrasonic power, ultrasonic frequency, ultrasonic irradiation time, reaction temperature, enzyme amount and substrate molar ratio on the reaction conversion were studied. In order to better understand some transport phenomena, experimental kinetic data of stirring reaction and ultrasound pretreatment reaction was studied. The esterification of 4MCA with glycerol to form the monoester and diester is shown in Fig. 1.

2. Materials and methods

2.1. Materials

C. antarctica lipase B (Novozym 435, immobilized on acrylic resin) was purchased from Sigma–Aldrich (St. Louis, USA). 4MCA (purity >98%) was purchased from Hubei YuanCheng Pharmaceutical Co., Ltd. (Wuhan China). Glycerol (purity >98%) and isooctane were purchased from Sinopharm Chemical Reagent Co., Ltd. (Shanghai China). Methanol (HPLC grade) was purchased from Merk KGaA (64271 Darmstadt, Germany). 4MCA (HPLC grade) was purchased from J&K Scientific Ltd. (Beijing China). All other solvents used for esterification reaction were obtained commercially and were of analytical grade.

2.2. Equipment

Experiments were carried out in a reactor with thermostatic water bath (temperature accuracy of ±0.5 °C), an IKA RW-20 mechanical stirrer and microtip probe (diameter of 13 mm) connected to a multi-frequency phonochemistry generator (Chengdu Jiuzhou Ultrasonic Technology Co., Chengdu China) The ultrasonic unit has an operating frequency of 20–40 kHz and power output of 50–400 W. The progress of the reactions was monitored on an high performance liquid chromatography (HPLC) (Waters, USA) equipped with a Waters Acquity BEH C_{18} column (100 mm × 2.1 mm and 1.7 μm particles, Waters, Milford, MA, USA). The monoester and diester products were separated by Agilent 6890Ngas chromatograph (GC) [6].

2.3. Lipase-catalyzed synthesis of 4MCG by ultrasonic pretreatment

Lipase catalyzed esterification was carried out in 250 mL three-mouth flask by using 10 mmol 4MCA, 30–70 mmol glycerol and 5–30 mg/mL *C. antarctica* lipase B in 150 mL of iso-octane. The solvent had, in advance, been dehydrated with molecular sieves 3 Å (10%, w/v) for at least 24 h. The flask was placed in a water bath with both microtip probe ultrasonic pretreatment (0–2 h) and mechanical stirring or only with mechanical stirring for a certain time. The mechanical stirring rate was kept at 200 rpm. After completion, the reaction mixture was dissolved in methanol and enzyme was removed by filtration. The filtrate was concentrated on rotary evaporator at reduced pressure (0.1 MPa). Concentrated reaction mass was dissolved in methanol for HPLC analysis and conversions were calculated on basis of 4MCA [8].

2.4. HPLC

Reaction samples were analyzed by HPLC equipped with an auto sampler fitted with a 5 μL loop was used to inject 3 μL of the treated samples (Waters, Milford, MA, USA), and eluted with methanol as the mobile phase at a flow rate of 0.07 mL/min using UV detection at 280 nm. The strong UV absorbance of 4MCA and the glycerol ester products provided a sensitive method of detection. The absorbance of column effluents was monitored at different absorption wavelength using the diode array detector with UV spectra collected under the peaks by scanning from 210 nm to 500 nm. The 4MCA and the esters strongly absorb from 270 nm to 320 nm with maxima near 310 nm. Glycerol does not absorb in this region.

2.5. Products and substrates separation

The concentrated residue from 2.3 was derivatized with BSTF (N, O-bis(trimethylsily)trifluoroacetamide) and pyridine. The derivatized samples were carried out by GC equipped with an auto sampler, and separations were achieved on the HP-5 column, 30 m × 0.32 mm ID × 0.25 μm film thickness. Helium was used as the carrier gas with a linear velocity of 35 cm/s. The oven temperature was programmed from 120 °C to 240 °C at 10 °C/min with an initial 2 min hold and a final 10 min hold. The inlet was heated to 230 °C and set for split injections (split rate 1:10) with a 1 μL injection volume. The detector source was heated to 230 °C and the detector quadrupole was heated to 150 °C. Date were collected and processed via Chemstation software. The Chromatogram of BSTFA derivatives of reaction mixture is shown in Fig. 2.

2.6. Kinetic modeling

In this work, in an attempt to represent the experimental kinetics data obtained from esterification of 4-methoxyl cinnamic acid and glycerol with an immobilized lipase as catalyst in ultrasound pretreatment system and only mechanical stirring system, respectively. Although the reaction rate might be controlled by internal mass transfer in the immobilized enzyme, it was also reported that

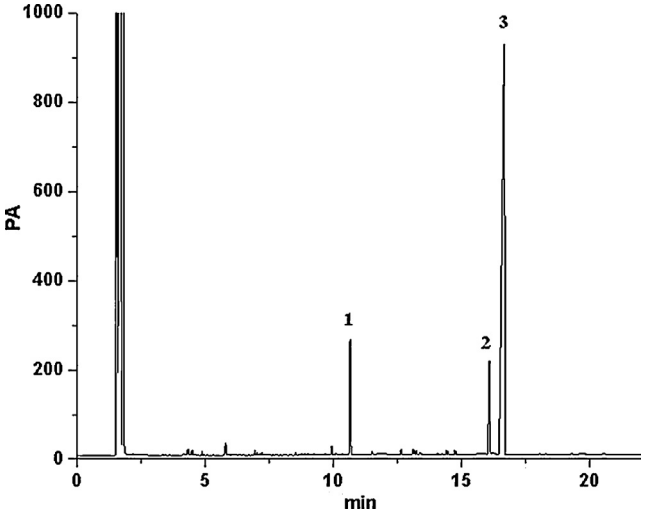

Fig. 2. GC chromatogram of BSTFA derivatives of reaction mixture. (1) 4-methoxy cinnamic acid (2) diester (3) monoester.

the mass transfer limitation could be neglected for porous supports [24]. In the work, only substrates reaction was considered.

3. Result and discussion

3.1. Effect of ultrasonic frequency, power on conversion

Ultrasonic frequency and power are very important influencing factors for ultrasound irradiation reactions [17]. In order to determine the effects of ultrasonic frequency on conversion of 4MCA to 4MCG, the variation of conversion with ultrasonic frequency which is in the range of 20–40 kHz is shown in Fig. 3. It was observed that the conversion increased drastically with the increasing of ultrasonic frequency till 35 kHz after 24 h and 48 h, and the conversion dropped rapidly when ultrasonic frequency exceeded 35 kHz. It is known that the physical effects such as shear forces and cavitation effect decrease with the increase of ultrasonic frequency. It may be due to the fact that the enzyme is partly deactivated at 20 kHz of ultrasonic pretreatment and the deactivation effect decreases with the increasing of ultrasonic frequency [25]. With the ultrasonic frequency increasing above 35 kHz, the deactivation effect could be ignored. Therefore, 35 kHz was used in the following experiments.

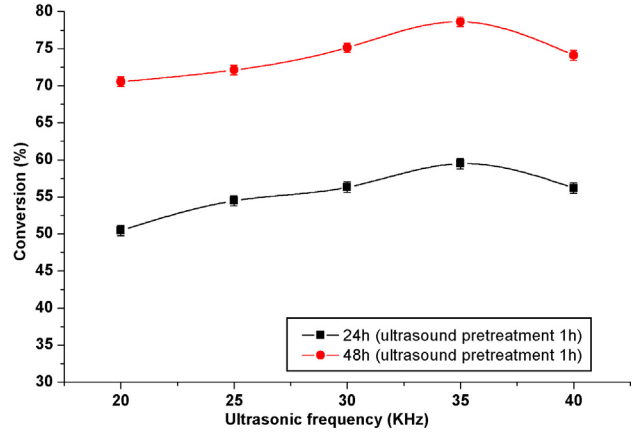

Fig. 3. Effect of ultrasonic frequency on conversion in lipase-catalyzed reaction. Reaction conditions: ultrasonic power of 200 W, glycerol and 4MCA molar ratio of 5:1, reaction temperature of 60 °C, enzyme amount of 12.0 mg/mL.

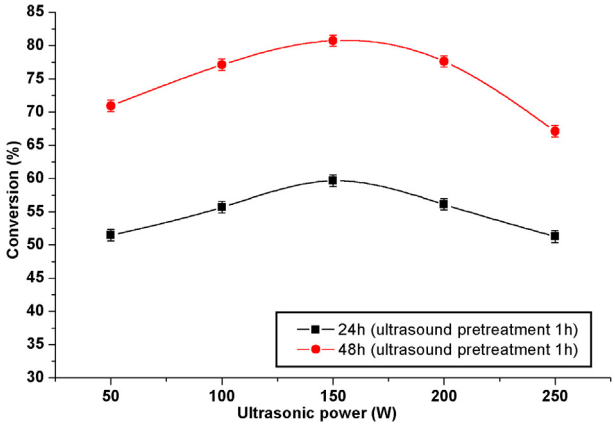

Fig. 4. Effect of ultrasonic power on conversion in lipase-catalyzed reaction. Reaction conditions: ultrasonic frequency of 35 kHz, glycerol and 4MCA molar ratio of 5:1, reaction temperature of 60 °C, enzyme amount of 12.0 mg/mL.

Generally, high intensity ultrasound could have more obvious effect on the mass transfer of the solution than low intensity ultrasound, thus accelerate the enzymatic-catalyzed reaction. However, too high intensity of ultrasound could lead the disruption of enzyme [26]. In the present work, Fig. 4 shows the influence of ultrasonic power on enzymatic esterification of 4MCA with glycerol. It can be observed that the conversion increased sharply with the increasing of ultrasonic power from 50 W to 150 W, and an obvious decrease of conversion was observed when the power exceeded 150 W. These results are consistent with some reports in that increasing ultrasonic power in an appropriate range enhances enzymatic reaction rate [23]. However, too high ultrasonic intensities were reported to reduce or even inactivate the enzyme activity [27]. Therefore, 150 W was selected to study the characteristics of lipase-catalyzed reactions in the following experiments.

3.2. Effect of ultrasonic irradiation time on conversion

The effect of ultrasonic irradiation time on enzymatic esterification of 4MCA with glycerol is shown in Fig. 5. The conversion presented rapid increase with increasing ultrasonic time from 0 h to 1.5 h, but an obvious decrease of conversion was observed when the ultrasonic time exceeded 1.5 h. Some similar reports have been observed in other studies in which ultrasound could help reduce the particle size of substrate and enzyme consequent increase in

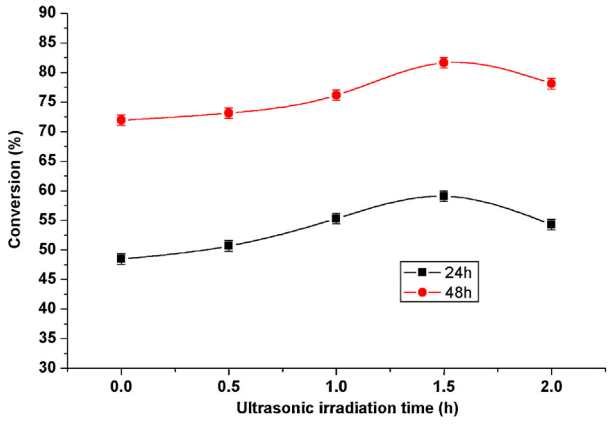

Fig. 5. Effect of ultrasonic irradiation time on conversion in lipase-catalyzed reaction. Reaction conditions: ultrasonic frequency of 35 kHz, ultrasonic power of 150 W, 4MCA and glycerol molar ratio of 1:5, reaction temperature of 60 °C, enzyme amount of 12.0 mg/mL.

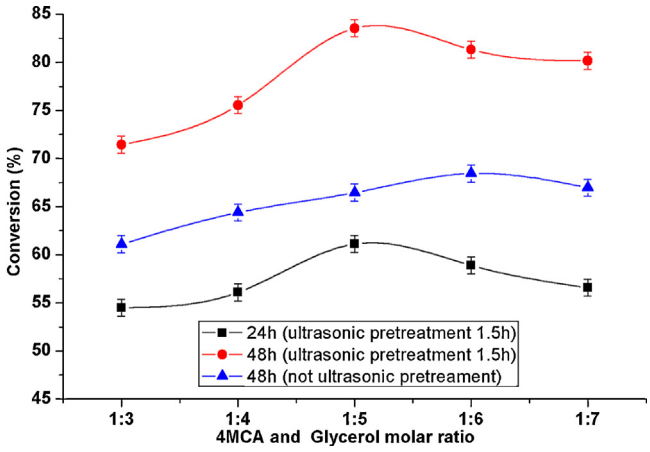

Fig. 6. Effect of substrate molar ratio on conversion in lipase-catalyzed reaction. Reaction conditions: ultrasonic frequency of 35 kHz, ultrasonic power of 150 W, reaction temperature of 60 °C, enzyme amount of 12.0 mg/mL.

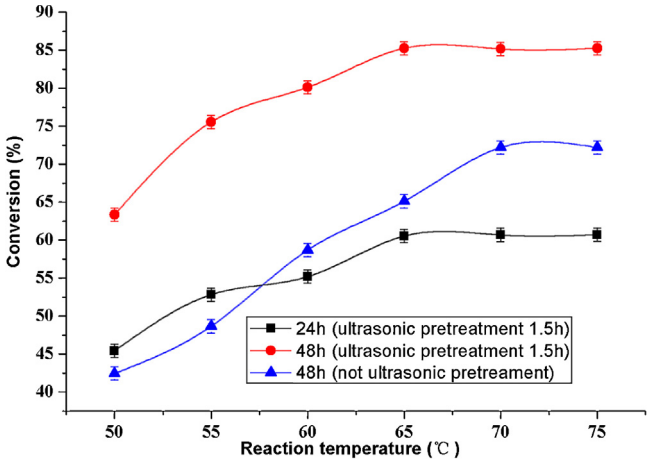

Fig. 7. Effect of reaction temperature on conversion in lipase-catalyzed reaction. Reaction conditions: ultrasonic frequency of 35 kHz, ultrasonic power of 150 W, enzyme amount of 15.0 mg/mL, the molar ratios were 5:1 and 6:1 for ultrasonic reaction and stirring reaction respectively.

the enzyme surface area are useful to reduce mass transfer limitations. This is especially relevant when using enzyme powders to catalyze reactions in organic solvent [21]. However, too long ultrasonic time with high intensity might produce lots of heat thus reduce the enzyme activity in some extent. Considering the conversion, energy consumption of the process, the ultrasonic irradiation time was determined to be 1.5 h in the subsequent experiment.

3.3. Effect of substrate molar ratio on conversion

The effect of molar ratio of 4MCA to glycerol on the conversion under ultrasound irradiation and mechanical stirring was evaluated in the range from 1:3 to 1:7. As shown in Fig. 6, there is a critical point of 4MCA/glycerol ratio, below which the conversion increases with the increasing of 4MCA/glycerol ratio and above which conversion shows little change with the increase in 4MCA/glycerol ratio. At the critical point, the 4MCA is probably already required by glycerol, so that further addition of glycerol even leads to the decrease of conversion. Due to the relative activity of the enzyme may be dependent on the interaction between the substrate and the active site of the native enzyme, thus an increase in glycerol concentration may change the catalytic environment of lipase to some extent [28]. On the other hand, ultrasonic wave provided excellent mixing of 4MCA and glycerol, because it could break down the liquid and form the cavitation bubbles resulting in the rising of mass transfer rate and acoustic streaming mixing [30]. Judging from the experimental results, the proposed critical points are near 4MCA/glycerol molar ratio of 1:6 in stirring condition and 1:5 in ultrasonic condition, which means that the substrate requirement in ultrasonic condition is litter than that not ultrasonic condition. These results could be attributed to the dispersion effect of ultrasound that might produce more contact area between 4MCA and glycerol, thus accelerate mass transfer. Therefore, the substrates molar ratios of 1:5 and 1:6 were chosen for ultrasonic reaction and stirring reaction in the following experiments, respectively.

3.4. Effect of enzyme amount on conversion

The influence of the enzyme amount was evaluated using varying amounts of *C. antarctica* lipase B from 5 mg/mL to 30 mg/mL. It was observed that the conversion of 4MCA with glycerol increases with the increasing of lipase amount. A good synthesis method should consider the esterification rate and economical interest of the reaction, in other words, using less amount of lipase to obtain satisfactory production of 4MCG. Using minimal amount of *C. antarctica* lipase B such as 5 mg/mL would be economically attractive, but conversion only reached 32.4%, 42.4% and 53.4% after 48 h (no ultrasonic pretreatment), 24 h (ultrasonic pretreatment) and 48 h (no ultrasonic pretreatment), respectively. Increasing of lipase amount led to better production of 4MCG. Under the ultrasonic pretreatment reaction condition, the conversion was much higher with 15 mg/mL enzyme amount and resulted in 56.5% and 80.8% conversion after 24 h and 48 h of reaction, respectively, and the conversion was not obvious change when the enzyme amount beyond 15 mg/mL. Under mechanical stirring reaction condition, the conversion was much higher with 15 mg/mL enzyme amount and resulted in a 68.2% conversion after 48 h of reaction. It is also observed that there was almost no obvious difference in conversion when the enzyme amount beyond 15 mg/mL. Thus, the enzyme amount of 15 mg/mL was selected for ultrasonic reaction and stirring reaction in the further experiments.

3.5. Effect of reaction temperature on conversion

The effect of reaction temperature on esterification of 4MCA with glycerol in lipase-catalyzed reaction was investigated at different temperatures from 50 °C to 75 °C. As shown in Fig. 7, under mechanical stirring, the conversion after 48 h increased with the rising of the temperature from 50 °C to 70 °C, little change in conversion was observed with temperature further increased above 70 °C. But under ultrasound irradiation, the optimum temperature was observed at 65 °C during the first 24 h and 48 h. It might be due to the fact that the cavitation efficiency from ultrasound irradiation could be increased with the increasing of solution temperature in a certain range [29]. However, the extent of cavitational effect is dampened at higher operating temperature due to the cavitation may also lead to the localized increase in temperature at the phase boundary of mixing reaction [31]. At 65 °C, more than 85% of enzyme activity remained in ultrasonic reaction; by contrast, only 70% lipase activity was retained in the stirring reaction. The results revealed that optimum temperature and inactivation temperature of the lipase was 5 °C lower under ultrasound irradiation than that in mechanical stirring. Therefore, the optimal reaction temperatures were 65 °C and 70 °C for ultrasonic reaction and stirring reaction, respectively.

3.6. Reuse of lipase

The reusability of lipase is very important for its practical application. At the end of each reaction batch, the lipase was washed

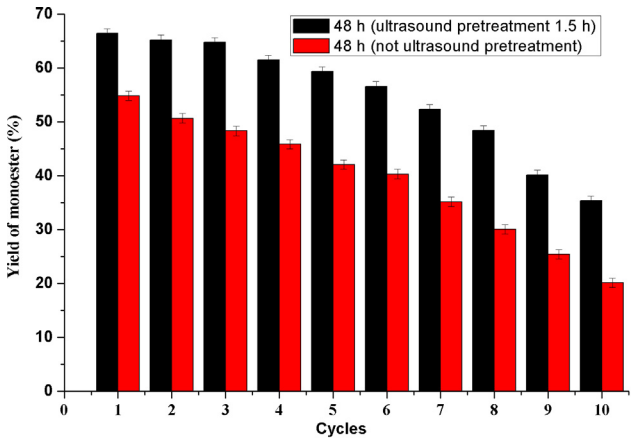

Fig. 8. Reuse of *C. antarctica* lipase B for yield of monoester.

with isooctane in order to remove any substrate or product. Then, the lipase was consecutively reused after each reaction cycle. The effect of repeated lipase use on the yield of monoester after 48 h under ultrasonic reaction and mechanical stirring reaction is shown in Fig. 8. The ultrasonic reaction conditions were ultrasonic frequency of 35 kHz, ultrasonic power of 150 W, glycerol and 4MCA molar ratio of 5:1, glycerol concentration of 250 μmol/mL, enzyme amount of 15 mg/mL and reaction temperature of 65 °C, the stirring reaction conditions were glycerol and 4MCA molar ratio of 6:1, 4MCA concentration of 250 μmol/mL, enzyme amount of 15 mg/mL and reaction temperature of 70 °C. As shown in Fig. 8, after the 7 reuses, the yield of monoester decreased from 66.2% to 53.3% under ultrasonic condition, and the conversion decreased from 56.6% to 35.2% under stirring condition. This result confirmed that ultrasonic pretreatment could enhance the conversion of lipase-catalyzed 4MCA esterification without essential damaging to the lipase activity.

3.7. Kinetics of 4MCG formation

At first, blank reaction tests were carried out, without the presence of enzyme or without irradiation power supply to the reaction medium. Results corroborated the fact that the contents of monoester and diester were negligible, hence demonstrating that the esterification reaction at the condition studied may not be considered as auto-catalytic.

Fig. 9A and B presents the plots of concentration versus time for the esterification of cinnamic acid and glycerol under stirring reaction and ultrasonic reaction. As seen from Fig. 9, the monoester was produced rapidly in the first 30 h and 24 h before the appearance of the diester product in the two different reaction systems, respectively. After 48 h of reaction time the 4MCA has decreased to 32.1% and 14.9% of the starting concentration, production of the monoester has increased to 50.2% and 66.4%, and production of the diester has increased to 17.7% and 18.7%, respectively. In all cases the yields of monoesters exceeded those of the diesters which indicated that the reaction rates for the formation of the monoesters was greater than the rates for the diesters. The result is similar to kinetics of cinnamoyl glycerol formation in chemical catalytic reaction system reported by Holser [6].

The kinetics of the sequential esterification reactions were modeled by a series of first-order irreversible rate expressions [7]. Experimental data were fit to the model equations by a least squares technique to obtain values of the rate constants. These results are listed in Table 1. The rates of monoester formation ($K1$) were respectively 4 times and 4.5 times of the diester formation rates ($K2$) for the mechanical stirring reaction and ultrasound

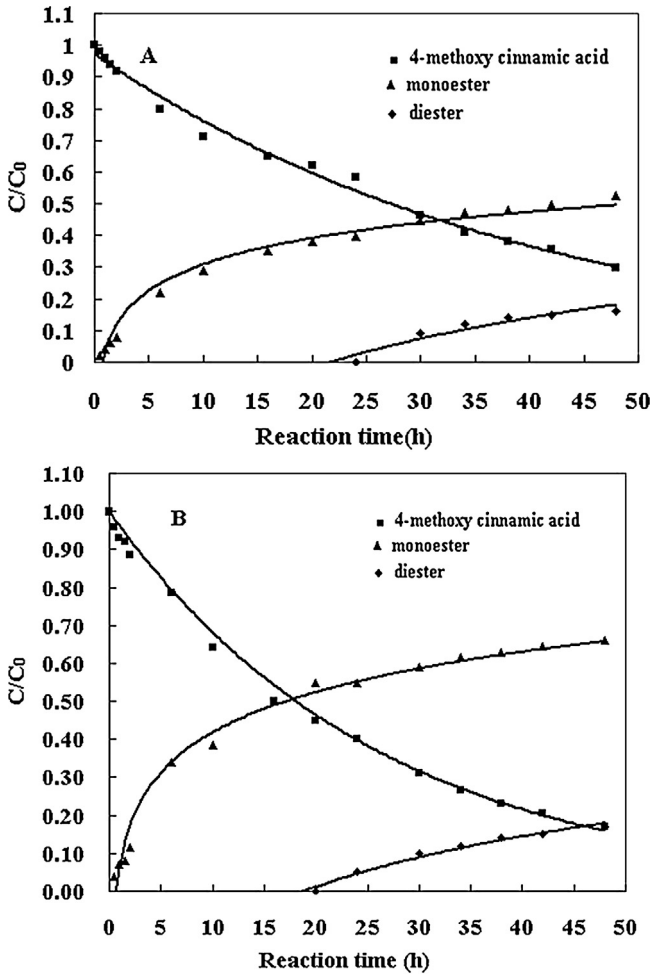

Fig. 9. Experimental data and kinetic modeling results for the reaction of 4-methoxy cinnamic acid and glycerol (A) mechanical stirring, (B) ultrasound pretreatment and mechanical stirring.

pretreatment reaction studied. The differences in the reaction rates observed between the ultrasound pretreatment and stirring reaction systems are attributed to the cavitation efficiency from ultrasound.

3.8. Comparison of different esterification methods on formation of 4MCG

A comparative study was carried out on the production of 4MCG using different esterification methods. Table 2 summarizes the data on conversion in esterification of 4MCA with glycerol using chemical method, mechanical stirring for enzymatic method and ultrasonic pretreatment for enzymatic method. Holser et al. [6] studied the esteriffication of 4MCA with glycerol using *p*-toluenesulfonic acid catalyst in toluene, where they got 35.8% of monoester. Patil et al. [7] found the enzymatic method using mechanical stirring was superior to the chemical method in terms of conversion yields. Consequently under the optimized condition, the conversion of ultrasonic pretreatment for enzymatic

Table 1
Rate constants of monoester and diester in different esterification methods.

Esterification methods	$K1$ (h^{-1})	$K2$ (h^{-1})
Enzymatic method (mechanical stirring)	0.1185	0.0304
Enzymatic method (ultrasonic pretreatment)	0.1461	0.0307

Note: $K1$ is rate constants of monoester. $K2$ is rate constants of diesters.

Table 2
Comparison of different esterification methods on conversion and mono and diesters yield.

Esterification methods	Time (h)	Conversion (%)	Monoester (%)	Diester (%)
Chemical method[a]	2	20.0	20.0	–
	8	52.5	35.8	16.7
Enzymatic method (mechanical stirring)[b]	24	34.3	34.3	nd
	48	73.1	56.6	16.5
Enzymatic method (ultrasonic pretreatment)[c]	24	60.2	55.4	4.8
	48	85.3	66.2	19.1

nd: not detected.
[a] Ref. [6].
[b] Ref. [7].
[c] Reaction conditions: ultrasonic frequency of 35 kHz, ultrasonic power of 150 W, ultrasonic pretreatment time of 1.5 h, the glycerol and 4MCA molar ratio of 5:1, enzyme amount of 15 mg/mL, reaction temperature of 65 °C.

esterification was significant higher than that of mechanical stirring for enzymatic method and chemical method. The overall esterification rate and monoester yield in the ultrasonic pretreatment process was above 1.5 times as that in the mechanical stirring process after 24 h of reaction. The results suggest that the ultrasonic pretreatment could be used to accelerate the esterification of 4MCA with glycerol with relatively high conversion.

4. Conclusion

A systematic study on ultrasonic pretreatment enzymatic esterification for 4MCG products is reported. The results showed a promising perspective of the technique to overcome mass transfer limitations arising from the use of 4MCA as the substrate. Both the optimum temperature and inactivation temperature of lipase under ultrasound irradiation was 5 °C lower than that of mechanical stirring condition. Less substrate molar ratio was required under ultrasonic pretreatment than no ultrasonic pretreatment possibly due to stimulated emulsification in the former case.

The experimental kinetic data and parameters show that enzymatic method using ultrasonic pretreatment was obviously superior to the mechanical stirring for enzymatic method and chemical method in terms of conversion yields. The overall esterification reaction rate in the ultrasonic pretreatment process was above 1.5-fold as that in mechanical stirring process after 24 h reaction, and the process did not essentially damage to lipase activity. This mild, convenient and improved protocol for the ultrasound-promoted preparation of 4MCG can potentially be explored for cinnamoyl glycerol synthesis.

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