Ultrasound-assisted lipase-catalyzed synthesis of ethyl acetate: process optimization and kinetic study

Chengnan Zhang\textsuperscript{a,b}, Xin Liang\textsuperscript{a,b}, Abdullah Abdulaziz Abbod Abdo\textsuperscript{a,b,c}, Benariba Kaddour\textsuperscript{a,b}, Xiuting Li\textsuperscript{b,d}, Chao Teng\textsuperscript{a,b} and Chengyin Wan\textsuperscript{a,b}

\textsuperscript{a}Beijing Advanced Innovation Center for Food Nutrition and Human Health, Beijing Technology and Business University, Beijing, PR China; \textsuperscript{b}School of Food and Health, Beijing Technology and Business University, Beijing, PR China; \textsuperscript{c}Department of Food Science and Technology, IBB University, Ibb, Yemen; \textsuperscript{d}Beijing Engineering and Technology Research Center of Food Additives, Beijing, PR China

\textbf{ABSTRACT}

Synthesis of ethyl acetate (EA) using lipase as a catalyst, as an environmentally-friendly process, has attracted considerable attention. However, the slow rate of enzymatic reaction and higher cost limit its applications. Ultrasound irradiation (UI) is known as an effective tool for accelerating the rate of enzymatic reaction under mild conditions. To attain maximum yield, it is crucial to understand the effect of UI on EA synthesis and the underlying mechanism. Therefore, the present study investigated the effect of UI on lipase-catalyzed ethyl acetate synthesis. The results clearly demonstrated that UI could remarkably accelerate the process of lipase-catalyzed synthesis of ethyl acetate compared with mechanical shaking. The optimal conditions of ultrasound-assisted reaction by using Novozyme 435\textsuperscript{\textregistered} were 6\% enzyme loading, 4:1 molar ratio of ethanol to acetic acid, 150 W & 28 kHz ultrasonic power and frequency, respectively. Bi-bi ping-pong model was employed to give a deep insight into the underlying mechanism of the beneficial effect of UI. It was found that \( V_{\text{max}} \) under UI was elevated by 143\% compared with that under mechanical shaking. Application of UI could decrease \( K_{\text{acetic acid}} \) and \( K_{\text{ethanol}} \) while increase \( K_{\text{ethanol}} \) and \( K_{\text{acetic acid}} \). In conclusion, the present study addresses that UI could remarkably accelerate the process of lipase-catalyzed synthesis of EA compared with mechanical shaking, and kinetic model results explained why ultrasound can significantly speed up lipase catalyzed synthesis of ethyl acetate, which may help to improve the industrial application potential of ethyl acetate biosynthesis.

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\textbf{Introduction}

Ethyl acetate (EA) has been used as a versatile solvent for countless applications [1–3]. Specifically, EA can be utilized as a food additive due to its pear-like and banana-like aromas in food industry [4–6]. The current industrial production of EA involves esterification of acetic acid with ethanol using strong acids as catalysts, dehydrogenation of ethanol and so on, which are typically energy intensive and environmentally-unfriendly processes [7]. In recent years, increased awareness of environmental sustainability has directed the focus on biotechnological production of chemicals. Synthesis of esters using lipase as a catalyst has attracted considerable attention [8,9]. The advantages of using lipase as a catalyst include mild reaction conditions, high substrate specificity and enantioselectivity [10]. However, the relatively low reaction rate of the enzymatic reaction limits the potential application in industry [11].

Ultrasound has been considered as one of the feasible ways to accelerate the process of enzymatic reaction [12]. Accumulative evidence has suggested that the rate of the lipase-catalyzed reaction in the presence of ultrasound irradiation (UI) is significantly increased compared to that in the absence of UI [13,14]. The beneficial effect of ultrasound on the enzymatic reaction is known as cavitation effect, which involves the following two steps: first, violent mechanical vibration of the medium caused by compression and rarefaction cycle of ultrasonic waves generates bubbles or cavities, which subsequently develop and implode [15]. It was reported that the

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intensity and frequency of UI have a great impact on the formation, development and implosion of bubbles [16]. Second, elevated temperature and pressure generated by the collapse of bubbles intensify the mass transfer and homogeneity of substrates to enzyme in the microenvironment, resulting in an increase in the rate and conversion of reaction [17]. It is reported that this phenomenon is largely dependent on the molar ratio of the substrates and enzyme loading [18,19]. Although the effects of ultrasonic treatment on the esterification of several esters have been evaluated, the ultrasound-assisted lipase-catalyzed EA has been scarcely explored.

The kinetic characteristics of enzyme reactions provide insights into the underlying mechanism of enzyme–substrate interactions and inhibitory effects of substrates, which can further help to find the most effective strategy to improve the conversion [20,21]. Interestingly, the kinetic parameters under UI were significantly altered compared with those under mechanical shaking. A previous study has shown that the $V_{\text{max}}$ and $k_{\text{m}}$ values of esterification of D-isoascorbyl palmitate under ultrasound was pronouncedly elevated by 2.85 and 1.50 times, respectively, compared with those under mechanical shaking [22]. The Bi-bi ping-pong model is extensively used in estimating parameters of lipase-catalyzed reaction kinetics since it was built for the reaction with two or more substrates and products [20]. A recent study found that ultrasonic assistance significantly altered the kinetic parameters of enzymatic synthesis of isobutyl propionate by using the Bi-bi ping-pong model [23]. However, it still remains unclear how ultrasonic treatment affect the kinetics of EA synthesis.

The present study was therefore conducted to investigate the beneficial effects of UI on lipase-catalyzed synthesis of EA. The effects of power and frequency of UI, enzyme loading as well as the molar ratio of the substrates on the conversion and rate of reaction were studied. The changes in the reaction kinetics in the presence or absence of ultrasound were also explored.

**Materials and methods**

**Materials**

Novozyme 435® (lipase B from *Candida antarctica*, immobilized on microporous polyacrylic resin beads), Lipozyme TL IM® (lipase from *Thermomyces lanuginose*) and Lipozyme RM IM® (lipase from *Rhizomucor miehei*) were supplied by Novozymes (Novo Nordisk, Denmark). Acetic acid, ethanol, n-hexane and acetonitrile were purchased from MREDA (Meridian Medical Technologies, USA). Ethyl acetate (>99%) was obtained from GmbH (Dr. Ehrenstorfer GmbH, Germany).

**Ultrasound equipment**

The experiments were conducted in an ultrasonic instrument DTD5200S (Beijing Hongxianglong Biotechnology Co., Ltd., Beijing, China) equipped with a thermostatic water bath, a microtip probe (diameter of 10 mm) and an ultrasound generator. The power of the ultrasonic equipment could be adjusted ranging from 0 to 300 W, and the frequency could be set at 28, 40, 50, 135 kHz.

**Esterification reaction**

Reactions were conducted by mixing ethanol, acetic acid, n-hexane, deionized water and immobilized lipase in a round-bottom flask. The effect of ultrasound was investigated by placing the flask in ultrasound equipment at certain power and frequency (Figure 1). The aliquot fractions were periodically withdrawn and injected into an Agilent 1260 series high performance liquid chromatography (HPLC) system equipped with a ZORBAX Eclipse Plus C18 column (4.6 mm × 250mm, 5 μm particle size; Agilent, USA) and a UV–visible detector. The conversion of EA was analyzed with an
isocratic elution of acetonitrile:water = 60:40 at 1 mL/min and identified at 210 nm. All the analyses were performed in triplicate and the data were expressed as mean values with standard deviations (±SD).

**Kinetics of esterification reaction**

To determine the kinetic constants of the reaction, the initial rate of reaction was estimated from the time course of EA concentration by calculating the initial slope of the tangent to the curve. The kinetic mechanism of the enzyme-catalyzed esterification of EA was considered based on the Bi-bi ping-pong mechanism as depicted in Equation (1):

$$\text{lipase} + \text{acetic acid} \xrightarrow{K_{\text{acetic acid}}} \text{acyl}$$

$$\text{– lipase} + \text{water} \xrightarrow{K_{\text{water}}} \text{lipase} \xrightarrow{\text{ethyl acetate}}$$

$$\text{– lipase} + \text{ethanol} \xrightarrow{K_{\text{ethanol}}}$$

(1)

where $K_{\text{acetic acid}}$ and $K_{\text{ethanol}}$ are the corresponding Michaelis–Menten parameters, respectively.

Equation (1) represents the formation of EA by esterification of acetic acid and ethanol. Acetic acid firstly binds to the lipase giving out acyl-lipase and water. Then ethanol binds to acyl-lipase, producing ethyl acetate and releasing free enzyme. Ethanol can competitively bind to lipase to form a dead-end product lipase-ethanol, while acetic acid can bind to acyl-lipase to form a dead-end product acyl-lipase-acetic acid. These mechanisms are depicted in Equations (2) and (3).

$$\text{lipase} + \text{ethanol} \xrightarrow{K_{\text{ethanol}}} \text{lipase} \cdot \text{ethanol}$$

$$\text{acyl} \xrightarrow{K_{\text{acetic acid}}} \text{acyl} \cdot \text{acetic acid}$$

(2)

$$\text{– lipase}$$

(3)

where $K_{\text{acetic acid}}$ and $K_{\text{ethanol}}$ were the corresponding Michaelis–Menten inhibition parameters, respectively.

The initial rate equation is given as follows:

$$V_0 = \frac{V_{\text{max}} C_A C_E}{C_A C_E + K_{\text{acetic acid}} C_E \left(1 + \frac{C_S}{K_{\text{water}}}\right) + K_{\text{ethanol}} C_A \left(1 + \frac{C_S}{K_{\text{ethanol}}}\right)}$$

(4)

where $V_0$ is the initial rate of reaction, $V_{\text{max}}$ is the maximum rate of reaction, $C_A$ and $C_E$ are the concentrations of acetic acid and ethanol, respectively. The fitting of the experimental kinetic data was carried out by means of the non-linear multiparametric Equation (4).

**Data analysis**

All the experiments were conducted in triplicate. Data are representative images or mean values with standard deviation (±SD). Statistical analysis was done using the Wilcoxon texts with SPSS software 19.0 version (SPSS Inc., Chicago, IL, USA). Differences were considered statistically significant at $p < 0.05$.

**Results and discussion**

**Effect of ultrasonic treatment on the lipase-catalyzed synthesis of EA**

To examine the effect of ultrasonic treatment, EA was synthesized in the lipase-catalyzed reaction in the presence or absence of UI. As shown in Figure 2, although the final conversion of EA in the absence or presence of UI were similar, this yield was achieved in 100 min under ultrasonic treatment while it took 180 min under mechanical shaking. Similar results were reported by Liu et al. [24] and Bansode et al. [13]: the former found that the hydrolysis reaction rate of triacylglycerides under UI was about 2-fold higher than that under shaking bath process, while the latter found that the maximum time required for isoamyl butyrate conversion was shortened to 3 h in sonication compared with 10 h of stirred reactor. The improvement of reaction efficiency was mainly attributed to
the cavitational effect generated by UI [15]. It is well-known that bubbles are recurrently formed and collapsed under acoustic wave, facilitating the mass transfer of the substrates and resulting in accelerated reaction rate. In addition, the increase in the rate of conversion may partially be ascribed to the conformational changes of lipase induced by UI [25]. The present results suggested that ultrasonic treatment could improve the reaction rate of esterification of EA.

Effect of ultrasonic power on the conversion of EA

To evaluate the impact of ultrasonic power on the rate and conversion of EA synthesis, reactions were conducted under different power ranging from 60 W to 180 W. For the conversion of EA synthesis, an increase in the degree of conversion was observed as the power of ultrasound increased from 60 W to 150 W, and gained optimal conversion at 150 W. However, the final conversion of EA decreased as the power of ultrasound further increased to 180 W (Figure 3). For the rate of reaction, the rate of EA synthesis slightly increased with increase in power from 60 W to 180 W. The results were consistent with those of previous studies, suggesting that an increase in ultrasonic power supply to an extent can lead to an increase in the rate and conversion, while further increase will slow down the progress of reaction [26,27]. Since the magnitude of the cavitation effect depends on the degree of intensity, ultrasound at high power will trigger more effective collapse of bubbles and produce a sufficient shock wave, thus improving mass transfer of substrates [15]. The phenomenon of decrease in the rate and yield of the reaction at higher power were probably ascribed to the extreme temperature and pressure generated by the violent implosion of bubbles, resulting in deactivating the enzyme [28].

Effect of ultrasonic frequency on the conversion of EA

Ultrasonic waves are typically defined as the frequency above 20 kHz which cannot be detected by human hearing. When ultrasonic waves pass through the reaction medium, the periodic compression and rarefaction cause mechanical vibration of the liquid, leading to formation of voids or bubbles, which subsequently grow and further implode [15]. The frequency of UI has a significant effect on the duration of compression and rarefaction cycle which will affect the size and shape of bubbles, ultimately impact the cavitational effect [29].

The effects of ultrasonic frequency on the rate and conversion of EA synthesis were explored. For the rate of synthesis, the initial rate of reaction decreased as the frequency increased from 28 kHz to 135 kHz. For the degree of conversion, the final EA yield under 28 kHz was greater than those under 50 kHz and 135 kHz (Figure 4). The present study indicated that an increase in ultrasonic frequency will hamper the efficiency of enzyme-catalyzed EA synthesis. Similar results were also observed in ultrasound-assisted production of troxerutin ester [30] and methyl caffeate [14]. It was reported that the expansion of alternative compression and rarefaction cycle was reduced under higher frequency, and such shorter expansion time...
resulted in the formation of small, intense and stable bubbles which were ineffectively imploded and generated a weak cavitation effect in the reaction [31]. Taken together, we selected 28 kHz as the appropriate frequency for the subsequent experiments.

**Effect of enzyme source on the conversion of EA**

The structure of enzymes under UI tends to be flexible, which will lead to changes in the catalytic performance [15]. It was reported that lipases produced from *Burkholderia cepacia* and *Pseudomonas fluorescens*, which have been immobilized onto Novozyme 435® and Lipozyme TL IM®, respectively, were subjected to conformational changes under lower-intensity ultrasonic treatment, including increase in random coil fraction and decrease in \( \alpha \)-helix and \( \beta \)-sheets structures [32]. Shan and Gupta found that the secondary structure of UI pre-treated lipase was slightly changed while the tertiary structure of that was perturbed [33].

To examine the effects of different enzyme sources on the ultrasound-assisted enzymatic reaction, EA was synthesized with three commercial immobilized enzymes: Novozyme 435®, Lipozyme TL IM®, and Lipozyme RM IM®, respectively (Figure 5). The EA yield with Novozyme 435® as catalyst was higher than those with Lipozyme TL IM® and Lipozyme RM IM® as catalysts, indicating that the Novozyme 435® is an appropriate immobilized enzyme for the synthesis of EA under UI. It is speculated that the catalytic performance of the immobilized enzyme is altered due to the conformational changes induced by ultrasonic treatment, which is different from that of free lipase [15]. Such structural changes deserve further study.

**Effect of enzyme loading on the conversion of EA**

Apart from the slow reaction rate, another drawback of using enzymes as catalysts is the high cost of enzymes [34]. Although numerous strategies have been attempted to reduce the cost of enzyme, the most direct way is to optimize the enzyme loading. To gain the optimal enzyme concentrations, EA was synthesized by using immobilized enzyme at 1–7% (w/v) with ultrasonic power of 150 W and ultrasonic frequency of 28 kHz. The yield of EA gradually increased as the lipase loading increased from 1% to 6%. In contrast, an obvious decrease in conversion of EA was detected when the lipase loading was increased to 7% (Figure 6). A similar result was reported by Zhao et al. [35], who found that the degree of polymerization was improved with 15% lipase loading, while lowered with 20% lipase loading. The possible explanation for this phenomenon was steric hindrance induced by excessive enzyme supplementation. It was reported that crowding of enzyme tends to cause aggregation thus limiting the diffusion of substrate and reducing the mass transfer at the surface of the enzyme [15]. Another possible explanation was that the large amount of the carrier brought by the immobilized enzyme will scatter the ultrasonic wave and therefore lead to energy loss [15].
Effect of molar ratio of substrates on the conversion of EA

Although the molar ratio of ethanol to acetic acid of 1:1 is stoichiometric requirement of EA synthesis, an excess of ethanol was always pursued to drive the equilibrium towards EA formation in view of esterification being a reversible reaction [1]. In the present study, the molar ratio of ethanol to acetic acid ranging from 1:1 to 5:1 was investigated so as to maximize the conversion of EA under UI. For the yield of EA, it can be seen that the formation of EA increased as the molar ratio of ethanol to acetic acid increased from 1:1 to 4:1, whereas decreased when the molar ratio was 5:1 (Figure 7). The results are in agreement with those previously reported by Khan et al. [36], in which conversion of cetyl oleate with 2:1 cetyl alcohol:oleic acid molar ratio was significantly higher than that with 4:1 molar ratio. The decrease in the conversion of EA synthesis could be due to the excessive ethanol accumulated in the vicinity of the enzyme, reaching a concentration level sufficient to cause enzyme inactivation [37].

Kinetic study

A series of experiments were conducted by varying the concentration of acetic acid ranging from 0.10 to 0.74 mol/L, with keeping the concentration of ethanol at 0.9 or 1.2 mol/L with or without UI to estimate the parameters of enzyme kinetics. Figure 8 depicts plots of reciprocal initial rates of esterification versus reciprocal acetic acid concentrations. An increase in the initial rate of conversion was observed when the acetic acid concentration increased from 0.1 to 0.4 mol/L (Figure 8 (I)). As the concentration of acetic acid further increased above 0.4 mol/L, a significant decrease in the initial reaction rate was observed, indicating that there is an inhibitory effect at higher concentrations of acetic acid. It could be perceived from Figure 8 (II) that the slope and the minimum value of curve (A) decreased compared with those of curve (B), suggesting that UI treatment could increase the rate of reaction. When the fixed ethanol concentration increased to 1.2 mol/L, a similar trend was observed (Figure 8 (III)).

Figure 8 (III and IV) depict the comparative analysis of the results obtained with or without UI at 0.9 or 1.2 mol/L fixed ethanol concentration studies. The reaction rates under mechanical shaking or UI at 1.2 mol/L ethanol concentration were lower compared with those at 0.9 mol/L ethanol concentration, suggesting that the rate of EA synthesis was inhibited by ethanol. The present results clearly demonstrated that not only the substrate acetic acid, but also the substrate ethanol had an inhibitory action on the reaction.

It is generally recognized that esterification catalyzed by lipase follows the Bi-bi ping-pong mechanism [38,39], which includes the following steps: firstly, the enzyme reacts with acetic acid to form an acyl-enzyme complex and release water; secondly, the acyl-enzyme complex interacts with ethanol to yield ester and release enzyme [23]. It was reported that ethanol may competitively bind to enzyme to inhibit the formation of acyl-enzyme complex [39,40]. The interaction of an acid with the acyl-enzyme complex could lower the yield of esters by forming a dead-end acid-enzyme-acyl complex [23]. In this regard, modified formulation modeled from the Ping-Pong Bi-Bi mechanism with substrates inhibition was employed to evaluate the kinetic constants. Table 1 shows the kinetic parameters calculated by using Equation (4). The $V_{\text{max}}$ under UI was increased by 143% compared with that under mechanical agitation, suggesting that ultrasound could elevate the rate of reaction. It was speculated that the wave shock generated by the cavitation effect of ultrasound could increase the mass transfer and decrease the activation energy, resulting in improving the interaction of substrate and enzyme and the formation of intermediate complex [16]. Such effect was evidenced by a decrease in $K_{\text{acetic acid}}$ and increase of $K_{\text{ethanol}}$ under UI compared with those under mechanical agitation, implicating that the enzyme was prone to form an acyl-enzyme complex rather than a dead-end ethanol-enzyme complex. In addition, the higher
value of $K_i$ acetic acid and lower value of $K_i$ ethanol under Ul showed that the inhibitory effect of acetic acid was elevated, while that of ethanol was reduced [36]. This was possibly attributed to the greater affinity of lipase toward acetic acid under Ul, which may lead to accumulation of acetic acid at the microenvironment of enzyme thereafter exert inhibitory effect on the enzyme [41].

Conclusions

The present study showed that ultrasound irradiation could significantly shorten the time of lipase-catalyzed synthesis of ethyl acetate compared with mechanical shaking. The optimal conditions of ultrasound-assisted reaction by using Novozyme 435® were 6% enzyme loading, 4:1 molar ratio of ethanol to acetic acid, 150 W & 28 kHz ultrasonic power and frequency, respectively. The kinetic study indicated that ultrasound accelerated the rate of reaction and this was mostly mediated by enhancing the affinity of acetic acid toward the enzyme and alleviating the inhibitory action of ethanol.

Disclosure statement

No potential conflict of interest was reported by the authors.

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Data availability statement

The data supporting the findings reported in this study are available from the corresponding author upon reasonable request.

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