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High-yield synthesis of bioactive ethyl cinnamate by enzymatic esterification of cinnamic acid

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ABSTRACT

In this paper, Lipozyme TLIM-catalyzed synthesis of ethyl cinnamate through esterification of cinnamic acid with ethanol was studied. In order to increase the yield of ethyl cinnamate, several media, including acetone, isooctane, DMSO and solvent-free medium, were investigated in this reaction. The reaction showed a high yield by using isooctane as reaction medium, which was found to be much higher than the yields reported previously. Furthermore, several parameters such as shaking rate, water activity, reaction temperature, substrate molar ratio and enzyme loading had important influences on this reaction. For instance, when temperature increased from 10 to 50 °C, the initial reaction rate increased by 18 times and the yield of ethyl cinnamate increased by 6.2 times. Under the optimum conditions, lipase-catalyzed synthesis of ethyl cinnamate gave a maximum yield of 99%, which was of general interest for developing industrial processes for the preparation of ethyl cinnamate.

1. Introduction

Cinnamic acid esters are widely used as flavor materials in food, perfumery, cosmetic and pharmaceutical industries (Karboune, Safari, Lue, Yeboah, & Kermasha, 2005; Martins et al., 2011). Ethyl cinnamate, as one of the cinnamic acid esters, has already been approved as a flavor by the US Food and Drug Administration (FDA) (Bhatia et al., 2007). Moreover, ethyl cinnamate is also often used in cosmetic products such as face creams, hair sprays, shampoos and toilet soaps (Belsito et al., 2007; Bhatia et al., 2007). Traditionally, ethyl cinnamate could be extracted from natural materials or synthesized by chemical methods (Nguyen & Weizman, 2007; Speed, McIntyre, & Thamattoor, 2004). However, these ways have many drawbacks such as low productivity and tedious purification steps (Martins et al., 2011), use of hazardous chemicals (Lv, Pan, & Li, 2007), and costly equipment (Yadav & Devendran, 2012).

As an promising alternative, enzymatic approach is often preferred because of its high catalytic efficiency, mild reaction conditions, and green and environmentally friendly process (Martins et al., 2013; Sangaletti-Gerhard, Cea, Risco, & Navia, 2015). Recently, Guyot, Bosquette, Pina, and Graille (1997) reported for the first time that it is possible to biosynthesize ethyl cinnamate by Novozym 435-catalyzed esterification of cinnamic acid with ethanol. But the yield of ethyl cinnamate was poor and the reaction time was long. As reported, only 2% of yield was obtained after 7 days of reaction. After that, Sharma, Chauhan, and Kanwar (2011) also investigated the synthesis of ethyl cinnamate by using porcine pancreatic lipase as biocatalyst. More recently, Jakovetic, Jugovic, et al. (2013) have enhanced the yield of ethyl cinnamate to 35.2% and reduced the reaction time to 96 h using Novozym 435 lipase through optimizing the reaction conditions. Also, they studied the effect of bioreactors, including batch and fluidized bed bioreactors, on enzymatic synthesis of ethyl cinnamate (Jakovetic, Lukovic, et al., 2013). However, enzymatic synthesis of ethyl cinnamate did not seem to provide us a competitive way to substitute traditional chemosynthesis until now. Therefore, it is necessary to seek for effective methods to increase the yield of ethyl cinnamate and decrease the reaction time, which could cut costs and meet the demands of enzymatic synthesis of ethyl cinnamate.

In this study, the aim of this work was to develop a competitive strategy for enzymatic high-yield synthesis of ethyl cinnamate. Firstly, ethyl cinnamate was tried to synthesize by Lipozyme TLIM lipase-catalyzed esterification of cinnamic acid with ethanol in different reaction media. Then, a suitable medium was chosen to efficiently enhance the yield of ethyl cinnamate. To optimize the reaction conditions, several parameters such as water activity,

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substrate molar ratio, reaction temperature and enzyme loading were analyzed systematically. Here, we have followed the strategy of keeping all the other experimental conditions constant while only one variable was changed.

2. Materials and methods

2.1. Materials

Lipozyme TLIM (Lipase from *Thermomyces lanuginosus* immobilized on silica gel) was from Novo. Industries (Bagsvaerd, Denmark). Cinnamic acid (99%) was provided by Hubei Yuancheng Pharmaceutical Co., Ltd. (Hubei, China). Ethanol was of analytic grade and purchased from Jinfeng Chemical Company (Tianjin, China). Isooctane was from Kemiou Chemical Company (Tianjin, China). Chromatographic grade methanol was obtained from Xingke Chemical Company (Tianjin, China). All other reagents were of analytic grade and obtained from local sources.

2.2. Enzymatic esterification

For the typical reaction, 30 mg of Lipozyme TLIM was added to a reaction mixture containing 10 μmol of cinnamic acid and 30 μmol of ethanol in 1 mL of isooctane. The reaction mixture was incubated in a temperature-controlled incubator shaker at 170 rpm at 40 °C for the time appointed in the paper. At various time intervals, 25 μL of the reaction mixture was withdrawn, and then the solvent was evaporated. Subsequently, the above sample was dissolved in 1 mL of methanol and then analyzed by high-performance liquid chromatography (HPLC). All experiments were analyzed in triplicate, and the mean values were calculated.

2.3. HPLC analysis

The samples were monitored via HPLC (ChuangXinTongHeng Science and Technology Co., Ltd., China) with a C-18 column (ZORBAX 300SB-C18 4.6 mm ID × 250 mm (5 μm), Agilent Technologies, Palo Alto, CA) and a UV detector at 280 nm. A 20 μL of the sample was injected, and the methanol was served as eluent with a flow rate at 0.5 mL min^{-1}. The percentages of the products were calculated from areas of their respective peaks. The yield of the reaction was quantified in terms of the mole percentage of esterification based on the ratio of consumed cinnamic acid to the total amount of cinnamic acid before the reaction.

2.4. Setting initial water activity

Initial water activity (a_w) of the reaction system was adjusted by pre-equilibration of the reaction components prior to starting the reaction, and the method is particularly suitable for the reaction, which generate trace amounts of water or even does not generate water. Lipozyme TLIM, cinnamic acid, isooctane and several saturated aqueous salt solutions, including LiCl (a_w = 0.11), K_2CO_3 (a_w = 0.43), NaCl (a_w = 0.75) and KCl (a_w = 0.86) were placed in sealed vessels, separately, and pre-equilibration of the reaction system was achieved after 3 days at room temperature (Zhang, Li, Xie, & Yuwen, 2013). Molecular sieves were used to prepare the nearly anhydrous condition ($a_w \approx 0$).

3. Results and discussion

3.1. Effect of reaction media

Biocatalyst performance in organic solvent is known to be highly sensitive to reaction media (Wescott & Klibanov, 1993, 1997). Initially, Lipozyme TLIM-catalyzed esterification of cinnamic acid was carried out by using acetone as reaction medium. But unfortunately, no ethyl cinnamate was detected in the resulting mixture (Table 1). To synthesize ethyl cinnamate, isooctane was further used as medium to carry out this reaction and the result was shown in Table 1. Surprisingly, the yield of ethyl cinnamate reached 89.2% at 24 h, which indicated that isooctane was a good medium for lipase-catalyzed esterification of cinnamic acid. Previously, several studies on the biosynthesis of ethyl cinnamate in different reaction media have been reported (Guyot et al., 1997; Jakovetic, Jugovic, et al., 2013; Jakovetic, Lukovic, et al., 2013; Sharma et al., 2011). For example, Guyot et al. (1997) synthesized ethyl cinnamate for the first time in a solvent-free system using Novozym 435 lipase through esterification of cinnamic acid with ethanol, but only 2% of yield was achieved after 7 days. Recently, Jakovetic, Jugovic, et al. (2013) have employed isooctane as reaction medium to synthesize ethyl cinnamate from cinnamic acid and ethanol using Novozym 435, and the reaction gave a maximum yield of 35.2% after 96 h. Compared that result with the present study, it was noteworthy that the present yield was much higher than the previous result (Jakovetic, Jugovic, et al., 2013) although the reaction media were the same. We attributed this to the difference in the biocatalyst, i.e. Lipozyme TLIM (Lipase from *T. lanuginosus*) is more suitable than Novozym 435 for this reaction. As known, the lipase from *T. lanuginosus* (formerly *Humicola lagunginosa*) (TLL) is a basophilic and noticeably thermostable enzyme, and it has found applications in many different industrial areas, from biodiesel production to fine chemicals (Fernandez-Lafuente, 2010). Besides, Sharma et al. (2011) also employed DMSO as reaction medium to synthesize ethyl cinnamate by using porcein pancreatic lipase as biocatalyst and the yield reached 54.1% after 27 h. However, it must be pointed out that although DMSO is a potential medium for this reaction, it will also complicate the reaction system and bring some new problems for post-processing because it is difficult to be removed due to its high boiling point and good solvent properties. For comparison purposes, the yields of ethyl cinnamate in various reaction media, including the media studied at present and the media investigated previously, were all listed in Table 1.

As known, solvent play an important role in enzymatic reaction and it affect the catalytic activity of enzyme by changing its three dimensional conformation, which will therefore significantly influence the reaction yield. Laane, Boeren, Vos, and Veeger (1987) once discussed the correlation between log*P* values (essentially partition coefficients that correlated with solvent hydrophobicity) of

Table 1
Lipase-catalyzed esterification of cinnamic acid with ethanol in different reaction systems.

Substrates	Biocatalyst	Reaction medium	Reaction time	Yield
Cinnamic acid and ethanol	Lipozyme TLIM	Acetone	12 h	0%[a]
	Lipozyme TLIM	Isooctane	24 h	89.2%[a]
	Novozym 435	Solvent-free medium	7 days	2% (Guyot et al., 1997)
	Novozym 435	Isooctane	96 h	35.2% (Jakovetic, Jugovic, et al., 2013)
	Porcein pancreatic lipase	DMSO	27 h	54.1% (Sharma et al., 2011)

[a] The reactions were carried out in 1 mL organic solvents with 0.01 mmol cinnamic acid, 0.03 mmol ethanol and 30 mg Lipozyme TLIM, at 40 °C and 170 rpm. Molecular sieves was used to remove the water in solvents.

the solvent and the activity of enzyme. However, partly due to the lack of fundamental insight into the mechanisms of enzyme catalysis in the non-aqueous solvent, the correlation between enzyme activity and the physicochemical properties of solvent is still a critical challenge. Thus, there was relatively little information available to guide us to choose an appropriate reaction medium from the myriad possibilities. Depending on the above observations, further experiments would be carried out by using isooctane as reaction medium.

3.2. Effect of shaking rate

The influence of agitation on lipase-catalyzed synthesis of ethyl cinnamate was evaluated using a shaking incubator at different shaking rate, 50, 170, and 250 rpm. Table 2 listed the yields at 14 h at different shaking rate. As shown, the yield at 50 rpm was 22.1%. An increase in the shaking rate from 50 to 170 rpm would enhance the yield considerably from 22.1% to 37.6%. However, the subsequent increase in shaking rate from 170 to 250 rpm just led to a slight increase of yield from 37.6% to 39.1%, which indicated that the yield was nearly independent of the shaking speed at levels higher than 170 rpm. Therefore, further studies would be performed at a shaking rate of 170 rpm.

3.3. Effect of water activity

Water has multiple roles in regulating lipase activity while also functioning as the hydrolysis of ethyl cinnamate in the reaction. Moreover, water is a product of this reaction, which has clear thermodynamics effects on yields. Thermodynamic water activity (a_w), as an important parameter, is often used to evaluate the effects of water on enzymatic reactions in organic media (Gumel, Annuar, Heidelberg, & Chisti, 2011). Fig. 1 showed the yield of ethyl cinnamate at different time under various initial a_w and the inlet box in Fig. 1 showed the dependence of yield on a_w. As can be seen, a kinetic increase in the yield of ethyl cinnamate with reaction time was observed. Moreover, it could also be seen from the inlet box that lipase-catalyzed esterification of cinnamic acid had a clear a_w dependence. For example, the yield of ethyl cinnamate at 24 h achieved a maximum of 89.2% at $a_w = 0.43$, while reaching lower levels at either lower a_w values (0, 0.11) or higher a_w values (0.75, 0.86). The yield was just 76% and 68% when a_w was set at 0 and 0.86, respectively. These results seemed to be different from several previous observations (Zhang, Bai, & Sun, 2007; Zhang, Lv, & Zhi, 2011; Zhang et al., 2013) in which near-zero low a_w (i.e. a_w is close to 0) was in favor of those lipase-catalyzed esterification. The differences of the effects of a_w on enzyme behavior with these previous reports might be based on the capacity of the enzyme support to capture water. In fact, the moderate optimal a_w could be rationalized by the effects of water on this enzymatic reaction. The presence of moderate water in the reaction system satisfied the requirement of the enzyme for holding an essential water layer to perform its catalytic functions properly (Humeau, Girardin, Rovel, & Miclo, 1998). But in the meantime, water also shifted

Table 2
Effect of shaking rate on lipase-catalyzed esterification of cinnamic acid with ethanol.

Shaking rate (rpm)	Reaction time (h)	Reaction temperature (°C)	Yield (%)
50			22.1
170	14	40	37.6
250			39.1

The reactions were carried out in 1 mL isooctane with 0.01 mmol cinnamic acid, 0.03 mmol ethanol, and 30 mg Lipozyme TLIM. Molecular sieves was used to remove the water in isooctane.

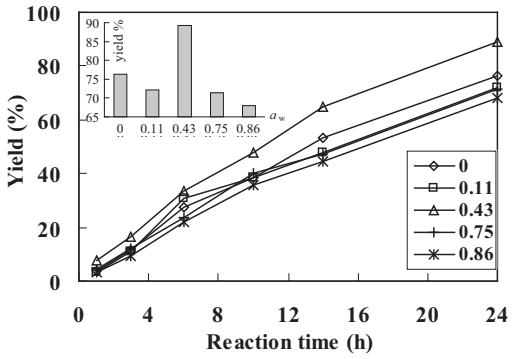

Fig. 1. Effect of water activity (a_w) on lipase-catalyzed esterification of cinnamic acid with ethanol in isooctane. The inlet box showed the yields at 24 h under various a_w. (Reaction conditions: 10 μmol cinnamic acid, 30 μmol ethanol, 30 mg Lipozyme TLIM, 1 mL isooctane, 170 rpm, 40 °C. a_w was set at 0, 0.11, 0.43, 0.75, and 0.86, respectively.)

the reaction thermodynamic equilibrium in favor of the side hydrolysis reaction (Burham, Rasheed, Noor, Badruddin, & Sidek, 2009). The optimal water activity represented the most appropriate water condition from the balance between these conflicts, whereas the lower water activities provided insufficient water for the buildup of the essential water shell for enzyme, and the higher water activities implied excessive water and thereby the increasing competition of water for the acyl-enzyme intermediate (Wehtje & Adlercreutz, 1997). In addition, an over-high water activity probably increased the thickness of water layer around the enzyme molecule, which would cause diffusion-limited problems of substrates entry into or products release from the lipase molecules (Sun et al., 2013). Therefore, the strong competing hydrolysis and the diffusion-limited problems could explain the much lower yield at higher water activities in Fig. 1.

3.4. Effect of substrate molar ratio

The effect of substrate molar ratio on lipase-catalyzed esterification of cinnamic acid was investigated by adding different amounts of ethanol to the reaction system (Fig. 2). As can be seen, the yield of ethyl cinnamate increased from 72.6% to 95.7% when substrate molar ratio varied from 1:1 to 1:6. When further increasing the substrate molar ratio to 1:10, however, the yield was reduced to 86.7% instead of increase. The effect of substrate molar ratio can be in two ways. On one hand, the increase of ethanol will raise the initial reaction rate and the equilibrium conversion; On

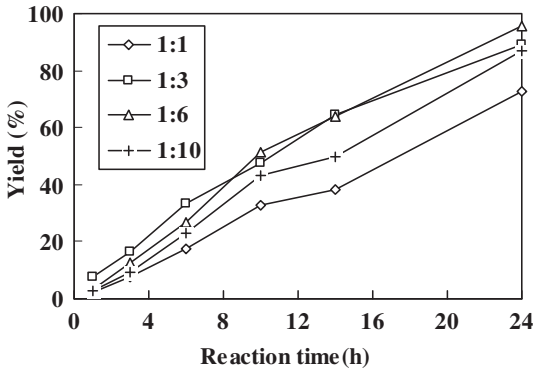

Fig. 2. Effect of substrates molar ratio on lipase-catalyzed esterification of cinnamic acid with ethanol in isooctane. (Reaction conditions: 10 μmol cinnamic acid, 30 mg Lipozyme TLIM, 1 mL isooctane, $a_w = 0.43$, 170 rpm, 40 °C.)

the other hand, it may also exert an effect of substrate inhibition on enzyme activity. Previously, a high substrate molar ratio of cinnamic acid to oleyl alcohol has been reported for biosynthesis of oleyl cinnamate (Lue, Karboune, Yeboah, & Kermasha, 2005). Here, when ethanol was added too much (substrate molar ratio at 1:10), it would inhibit lipase activity and thus cut down the yield of ethyl cinnamate.

3.5. Effect of reaction temperature

Temperature not only affects the stability and the activity of the enzyme but also the reaction equilibrium, as well as the properties of reaction media such as viscosity (Li, Chen, & Tan, 2011; Sun et al., 2013). Fig. 3 showed the effect of temperature on Lipozyme TLIM-catalyzed esterification of cinnamic acid in the range of 10–50 °C. As indicated, the initial reaction rate increased markedly with the increase of reaction temperature. Although the yield of ethyl cinnamate was low at 10 °C, it was increased with reaction temperature. For instance, after 24 h of reaction, the yield was only 16% when the reaction was conducted at 10 °C, while the yield reached 99% when the reaction was conducted at 50 °C. These results suggested that temperature played an important role in such a reaction system, which was different from several other reactions (Zhang et al., 2007, 2011). Nevertheless, it should be point out that too high temperature would cause the loss of enzyme activity. Here, it was interesting that the yield of 99% obtained through Lipozyme TLIM-catalyzing esterification of cinnamic acid at 50 °C for 24 h was much higher than the recently reported yield of 35.2% (Jakovetic, Jugovic, et al., 2013) obtained through Novozym 435-catalyzing esterification of cinnamic acid at 55 °C for 96 h.

3.6. Effect of enzyme loading

The effects of enzyme loading on Lipozyme TLIM-catalyzed esterification of cinnamic acid were studied in the range of 10–40 mg/mL solvent (Fig. 4). As can be seen, lower amount of enzyme loading led to lower yield of ethyl cinnamate. Fortunately, the yield at 24 h increased rapidly from 19.7% to 89.2% with the increase in enzyme loading from 10 to 30 mg/mL solvent. Thereafter, a further increase of lipase to 40 mg/mL solvent brought about 97.3% of yield. In short, these main variables, including shaking rate, water activity, reaction temperature, substrate molar ratio and enzyme loading, determined the yield in this process, which has been thought as a thermodynamically controlled process by Kasche (1986).

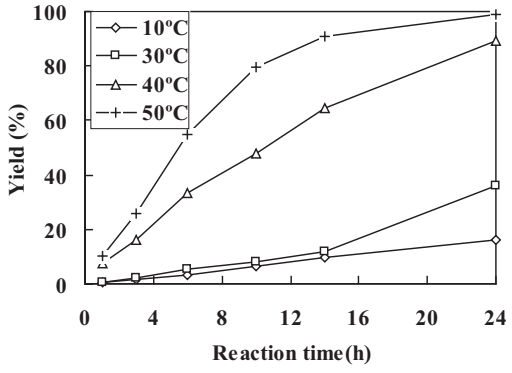

Fig. 3. Effect of reaction temperature on lipase-catalyzed esterification of cinnamic acid with ethanol in isooctane. (Reaction conditions: 10 μmol cinnamic acid, 30 μmol ethanol, 30 mg Lipozyme TLIM, 1 mL isooctane, a_w = 0.43, 170 rpm.)

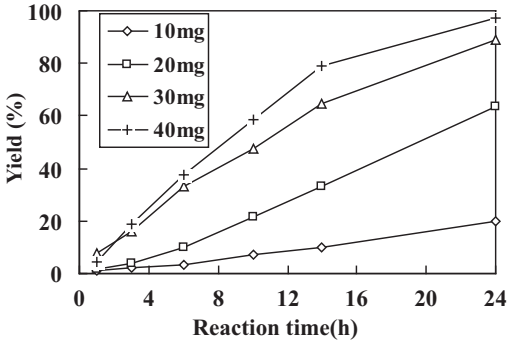

Fig. 4. Effect of enzyme loading on lipase-catalyzed esterification of cinnamic acid with ethanol in isooctane. (Reaction conditions: 10 μmol cinnamic acid, 30 μmol ethanol, 1 mL isooctane, a_w = 0.43, 170 rpm, 40 °C.)

4. Conclusion

In the present work, Lipozyme TLIM-catalyzed synthesis of ethyl cinnamate through esterification of cinnamic acid with ethanol was studied. The result showed a high yield of ethyl cinnamate in isooctane, which was much higher than the results reported previously. Through optimizing the reaction conditions, the highest yield reached 99% at 24 h under a_w = 0.43, 50 °C, 30 mg/mL Lipozyme TLIM, and substrate molar ratio of 1:3. The results are of general interest for developing industrial processes for the preparation of ethyl cinnamate that is useful for food additives, cosmetic formulations, and the synthesis of other cinnamic acid derivatives. In addition, other lipases should also be tried to use in this reaction by improving their properties via immobilization using more adequate immobilization protocols.

Conflict of interest

The authors have no conflict of interest to declare.

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