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# An Efficient Strategy for the Production of Epoxidized Oils: Natural Deep Eutectic Solvent-Based Enzymatic Epoxidation

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**Abstract** Poor H<sub>2</sub>O<sub>2</sub>-resistance by enzymes is a key bottleneck in the epoxidation process of oil by enzymatic methods. In this study, the stability of three lipases, from *Aspergillus oryzae* lipase (AOL), *Aspergillus fumigatus* lipase B (AflB), and marine *Janibacter* (MAJ1), in the presence of H<sub>2</sub>O<sub>2</sub> was evaluated in different types of natural deep eutectic solvents (NADES). This stability was strengthened significantly in the NADES compared to the buffer. Specifically, AOL retained 84.7% of its initial activity in the presence of choline chloride/sorbitol (1:1 M ratio) and 3 mol L<sup>-1</sup> H<sub>2</sub>O<sub>2</sub> after 24 h incubation at 40°C. In addition, the two-phase epoxidation process was optimized with AOL in ChCl/sorbitol to reach up to 96.8% conversion under the optimized conditions (molar ratio of octanoic acid/H<sub>2</sub>O<sub>2</sub>/C=C-bonds = 0.3:1.5:1,

enzyme loading of 15 U g<sup>-1</sup> of soybean oil, ChCl/sorbitol content of 70.0% of the weight of hydrophilic phase, and reaction temperature of 50°C). Moreover, the lipase dispersed in NADES retained approximately 66% of its initial activity after being used for seven batch cycles. Overall, NADES-based enzymatic epoxidation is a feasible and promising strategy for the synthesis of epoxidized oils.

**Keywords** Epoxidation · Lipase · Natural deep eutectic solvent · Soybean oil · Enzyme catalysis

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**Supporting information** Additional supporting information may be found online in the Supporting Information section at the end of the article.

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## Introduction

Epoxidized oils have gained widespread attention as bio-based and toxicologically less-questionable substitutes for phthalates as plasticizers, which are potentially toxic to human health and the environment (Liu et al., 2016). Chemical methods for the production of epoxidized oils have several disadvantages, such as environmental burdens (e.g., salt wastes that originate from the neutralization of the catalysts or undesirable side-reactions), acute operational risks, and corrosive deterioration of reaction vessels. In recent years, a biocatalytic alternative to chemical synthesis based on lipases has attracted considerable interest from academic researchers (Chen et al., 2017; Liu et al., 2016; Sun et al., 2011). However, to the best of our knowledge, this enzymatic reaction has not yet been applied in industrial production due to the inactivation of enzymes at high concentrations of H<sub>2</sub>O<sub>2</sub>. In the epoxidation process of oils, the substrate H<sub>2</sub>O<sub>2</sub> is detrimental to the structural integrity of enzymes because of its ability to oxidize amino

acids (e.g., Arg, Lys, Met, and His). To address, strategies such as the immobilization and protein engineering of enzymes as well as the use of different types of reaction media have been used to improve the H<sub>2</sub>O<sub>2</sub>-tolerance of enzymes (Khodaverdian et al., 2018; Zhao et al., 2019).

Reaction media play a very important role in efficient epoxidation by a biocatalyst. Aqueous reaction media are not suitable, because perhydrolysis becomes more thermodynamically favorable, leading to hydrolysis of the oils (hence to more complex, low-quality products), and a reduced in situ concentration of peracids, resulting in lower epoxidation rates. Some organic solvents (e.g., toluene, benzene, and chloromethane) have been proven to be excellent media for epoxidation in the presence of lipase, but their potential toxicity is still under criticism (Liu et al., 2016). To circumvent the negative effects of aqueous and organic solvent media, ionic liquids have been evaluated (Sun et al., 2014). Ionic liquids, however, are questionable due to high toxicity and low biodegradability associated with many ionic liquids and high manufacturing costs (due to laborious preparation) (Zhang et al., 2012). To overcome the above-mentioned disadvantages, a new generation of solvents, the so-called deep eutectic solvents, have received interest in recent years (Abbott et al., 2003). Deep eutectic solvents generally refer to a eutectic mixture of two or more, preferably cheap and safe components, which are capable of associating with each other through hydrogen bonding (Zhang et al., 2012). Compared to ionic liquids, deep eutectic solvents have the advantages of easier preparation (therefore to lower production costs), are bio-based, and possess lower toxicity and higher biodegradability. Consequently, deep eutectic solvents have been applied in catalysis, as extraction solvents, in material chemistry and in organic synthesis (Zhang et al., 2012). In 2011, Choi and coworkers reported the concept of “natural deep eutectic solvents” (NADES) (Choi et al., 2011). The components of NADES are natural products such as choline and its derivatives, sugars, alcohols, and amino acids. In recent years, NADES have received considerable attention as environmentally less problematic alternatives to conventional solvents (Dai et al., 2013). In addition, there have been indications that some NADES can also have a beneficial effect on enzyme stability (Zhou et al., 2016). However, little information is available on the application of NADES systems as hosts for enzymatic epoxidation (Ranganathan et al., 2017; Zhou et al., 2017).

Therefore, in our ongoing efforts to establish NADES-based enzymatic epoxidations, we report on evaluating lipases according to their resistance against H<sub>2</sub>O<sub>2</sub> in the different types of NADES for the preparation of epoxidized soybean oil (ESO).

## Materials and Methods

### Materials

The recombinant *Pichia pastoris* X-33 expression strains, containing pGAPZαA-AOL, AflB, or MAJ1, were stored at −80°C in the laboratory. Soybean oil with an iodine value of 125 g I<sub>2</sub>/100 g (acid value = 0.17 mg KOH g<sup>−1</sup>) was purchased from a local company (ZhiRun Oils & Grains Ltd., Guangzhou, China). Sodium hydroxide (99%), hydrogen peroxide (30%), Wijs reagent, choline chloride (ChCl), sorbitol, xylitol, glycerol, and urea were purchased from Aladdin Chemistry Co., Ltd. (Shanghai, China). All the other reagents were of analytical grade.

### Production of AOL, AflB, and MAJ1 Lipase

The expression strains described above were used to produce *Aspergillus oryzae* lipase (AOL), *Aspergillus fumigatus* lipase B (AflB), and marine *Janibacter* (MAJ1) lipase. The fermentation inocula were prepared by cultivating the cells at 30°C with shaking at 200 rpm for 18–24 h in a 500 mL shaking flask containing 100 mL YPD medium (yeast extract 1% (w/v), peptone 2% (w/v), and glucose 2% (w/v)), and the fermentation was carried out in a 30 L fermenter. After fermentation for 60 h, the supernatant was collected by centrifugation (12,000g, 10 min, 4 °C), and then the recombinant lipase in the supernatant was concentrated and buffer exchanged to Buffer A (pH 8.0, 20 mM Tris–HCl) through a 10 kDa molecular mass membrane (Viva ow 200, Sartorius, Germany).

The lipases were purified in a Q Sepharose Fast Flow column by a Protein Separation and Purification System (GE AKTA Pure, Boston, USA). First, the Q column was eluted with a 20% ethanol solution, the residual protein impurities in the column were washed away, and the column was rinsed with deionized water to an equilibrium state at a flow rate of 4 mL min<sup>−1</sup>. The Q column was flushed with Buffer A until UV absorption, conductivity, and other parameters reached steady state. After that, the crude protease solution was loaded at a flow rate of 2 mL min<sup>−1</sup>. The Q column was then equilibrated with Buffer A, and eluted with 5% Buffer B (pH 8.0, 20 mM Tris–HCl containing 1 M NaCl) to wash away some of the protein impurity at a flow rate of 3 mL min<sup>−1</sup>. Finally, the Q column was eluted with 20% Buffer B at a flow rate of 3 mL min<sup>−1</sup> to obtain the purified lipase. The purified lipase was freeze-dried in a freeze dryer (Christ ALPHA 1–2 LD plus, Osterode, Germany) for subsequent reactions.

### Preparation of NADES

ChCl was mixed with sorbitol, xylitol, or glycerol in a molar ratio of 1:1. These mixtures were heated to 80°C

while being continuously stirred until colorless, homogeneous liquids were obtained.

### Assay on Perhydrolysis Activity and Substrate Specificity of Lipases

The perhydrolysis activity of lipases was tested using the monochlordimedone assay in the presence of NADES (Bernhardt et al., 2005). Here, the formation of peracids was detected indirectly through the formation of hypobromite (via oxidation of bromide by the peracid), which itself reacts with the monochlordimedone. The formed hypobromite was quantified via its characteristic absorption band at 290 nm. The test was performed in 96-well microtiter plates with a reaction volume of 100  $\mu\text{L}$ , with pentanoic acid as the substrate (in 0.1 mol  $\text{L}^{-1}$  pentanoic acid buffer at pH 6.0 containing 90 mmol  $\text{L}^{-1}$  NaBr and 180  $\mu\text{mol L}^{-1}$  monochlordimedone) in the presence of 100 mmol  $\text{L}^{-1}$   $\text{H}_2\text{O}_2$  and constant enzyme (1 mg  $\text{mL}^{-1}$ ), at 40°C. The activity was determined spectrophotometrically and expressed in specific activity units (U), where 1 U represents the amount of enzyme required to produce 1  $\mu\text{mol}$  of monochlordimedone per minute under the reaction conditions.

The substrate selectivity of lipases to carboxylic acid was also evaluated by the method described above. The test was performed in 96-well microtiter plates with a reaction volume of 100  $\mu\text{L}$ , individual carboxylic acids (formic acid, acetic acid, butyric acid, and pentanoic acid or octanoic acid) as the substrates (in 0.1 mol  $\text{L}^{-1}$  carboxylic acid buffer at pH 6.0 containing 90 mmol  $\text{L}^{-1}$  NaBr and 180  $\mu\text{mol L}^{-1}$  monochlordimedone) in the presence of 100 mmol  $\text{L}^{-1}$   $\text{H}_2\text{O}_2$  and constant enzyme (1 mg  $\text{mL}^{-1}$ ), at 40°C.

### Enzymatic Epoxidation of Soybean Oil

The reaction mixture comprised soybean oil and NADES (in the weight ratios of 1.25 to 0.19), hydrogen peroxide ( $\text{H}_2\text{O}_2/\text{C}=\text{C}$ -bonds molar ratio varying from 0.5:1 to 2.5:1), octanoic acid ( $\text{C}_7\text{H}_{15}\text{COOH}/\text{C}=\text{C}$ -bonds molar ratio varying from 0.1:1 to 0.5:1), and the enzyme (5–25 U  $\text{g}^{-1}$  of the weight of the soybean oil). The reaction mixtures were stirred (500 rpm, in a 25 mL conical flask) for 24 h in a thermostatic water bath. After the reaction was stopped, the mixtures were allowed to stand for 5 min then was centrifuged (12,000g, 2 min, 4 °C). The upper oil sample was isolated for subsequent analysis.

The lower layer was recycled. The water (about 30.0 wt. % of the lower layer) present in the lower layer was completely removed under vacuum at 50°C for 20 min and the residue (including NADES and enzyme) was reused to evaluate the reusability of the catalysts. The following

reaction was carried out under the optimized reaction conditions: soybean oil, 3 g; the residue, 4.2 g; the molar ratio of octanoic acid/ $\text{H}_2\text{O}_2/\text{C}=\text{C}$ -bonds, 0.3:1.5:1; and  $\text{H}_2\text{O}_2$  concentration, 2.1 mol  $\text{L}^{-1}$ . The reaction mixture was stirred (500 rpm, in a 25 mL conical flask) for 24 h at 50°C.

The scale-up of the epoxidation reaction was performed under the optimized reaction conditions: soybean oil, 1000 g; ChCl/sorbitol (1:1 M ratio), 1400 g; the molar ratio of octanoic acid/ $\text{H}_2\text{O}_2/\text{C}=\text{C}$ -bonds, 0.3:1.5:1;  $\text{H}_2\text{O}_2$  concentration, 2.1 mol  $\text{L}^{-1}$ ; and enzyme loading of 15 U  $\text{g}^{-1}$  of soybean oil. The reaction mixture was stirred (500 rpm, in a 5000 mL beaker) for 24 h at 50°C.

### Evaluation of Enzymatic Epoxidation of Soybean Oil

Iodine and oxirane values were determined following titration methods (Monono et al., 2015). The experimentally determined oxirane oxygen content ( $OO_{\text{exp}}$ ) was calculated using Eq. 1.

$$OO_{\text{exp}} = (L \times N \times 1.6) / W \quad (1)$$

where  $L$  is the volume of HBr solution (mL),  $N$  is the normality of the HBr solution, and  $W$  is the mass of the sample (g).

The theoretical oxirane oxygen content ( $OO_{\text{the}}$ ) is defined as the maximum oxirane content in 100 g soybean oil (Zhang et al., 2017), and calculated using Eq. 2.

$$OO_{\text{the}} = A_0 \times IV_0 / 2A_i / [100 + (IV_0 / 2A_i)A_0] \times 100 \quad (2)$$

where  $A_i = 126.9$  (the atomic mass of iodine),  $A_0 = 16.0$  (the atomic mass of oxygen), and  $IV_0$  the initial iodine value of the soybean oil.

The relative conversion to oxirane was calculated using Eq. 3.

$$\text{Relative conversion to oxirane (\%)} = \frac{OO_{\text{exp}}}{OO_{\text{the}}} \quad (3)$$

### FT-IR Analysis of the Final ESO Product

One milligram of the purified sample was mixed with 100 mg KBr using a mortar and pestle. Then, the mixture was pressed into a pellet. Finally, the resultant ESO was analyzed using a Nicolet 8210E FT-IR spectrometer. The wavelength ranged from 400 to 4000  $\text{cm}^{-1}$  during 128 scans, with a resolution of 2  $\text{cm}^{-1}$ . The absorption peaks were identified from the spectra (Gogoi et al., 2015).

### Statistical Analysis

All experiments were performed in triplicate. The results are presented as the means  $\pm$  SD. The differences among

mean values were evaluated in SPSS 19.0 through significant difference tests and variance analysis.

## Results and Discussion

### Influence of Different NADES on Lipase Stability against H<sub>2</sub>O<sub>2</sub>

NADES were employed as reaction media and H<sub>2</sub>O<sub>2</sub> was employed as an oxygen donor in the epoxidation reaction. However, H<sub>2</sub>O<sub>2</sub> is a known inactivator of enzymes. Therefore, the stability of AOL, AflB, and MAJ1 lipases against H<sub>2</sub>O<sub>2</sub> was investigated, and the results are shown in Table 1. Interestingly, much higher resistance of the three lipases against H<sub>2</sub>O<sub>2</sub> was observed in the presence of the three types of NADES (ChCl/sorbitol, ChCl/xylitol, and ChCl/glycerol, 1:1 M ratio) than in the presence of buffer. For example, 3.0 mol L<sup>-1</sup> H<sub>2</sub>O<sub>2</sub> almost completely inactivated the lipases in the buffer within 24 h, whereas under the same conditions, albeit in the presence of ChCl/xylitol (1:1 M ratio), AOL retained 65.6% of its initial activity, AflB retained 33.8%, and MAJ1 retained 33.1%. Particularly in ChCl/sorbitol (1:1 M ratio) media, AOL almost completely retained its initial activity after incubation in the presence of 0.5 mol L<sup>-1</sup> H<sub>2</sub>O<sub>2</sub>, and retained 84.7% of its initial activity after incubation in the presence of 3.0 mol L<sup>-1</sup> H<sub>2</sub>O<sub>2</sub>. The reason for the higher stabilization in NADES is not yet fully understood. Previously, H-bond-donating ionic liquids and polyols,

including sorbitol, were demonstrated to stabilize enzymes (Diego et al., 2004; Kotlewska et al., 2011). Our previous study reported that H-bond donating deep eutectic solvents stabilized the free *Penicillium camemberti* lipase (Zhou et al., 2016). Therefore, the use of some NADES systems as hosts for reaction may be a promising technique due to their stabilization effect. The effect of ChCl/sorbitol (1:1 M ratio) as a reaction medium on AOL-catalyzed lipase was thus investigated in subsequent experiments.

### Enzymatic Epoxidation of Soybean Oil by AOL

#### The Substrate Specificity of AOL to Carboxylic Acid

First, the substrate specificity of AOL for different carboxylic acids was investigated (Fig. 1). This study was performed with formic, acetic, butyric, pentanoic, and octanoic acids as substrates. Long-chain saturated carboxylic acids underwent precipitation in the reaction system thereby making them inappropriate candidates. AOL showed the highest preference toward octanoic acid as the active oxygen carrier with a perhydrolysis activity of 13.1 ± 0.5 U mg<sup>-1</sup>.

#### Effect of Temperature

The effect of the reaction temperature (ranging from 30 to 70°C) on the relative conversion of the enzymatic epoxidation reaction was investigated. As shown in Fig. 2a, the conversion increased when the reaction temperature was increased from 30 to 50°C, and the maximum conversion

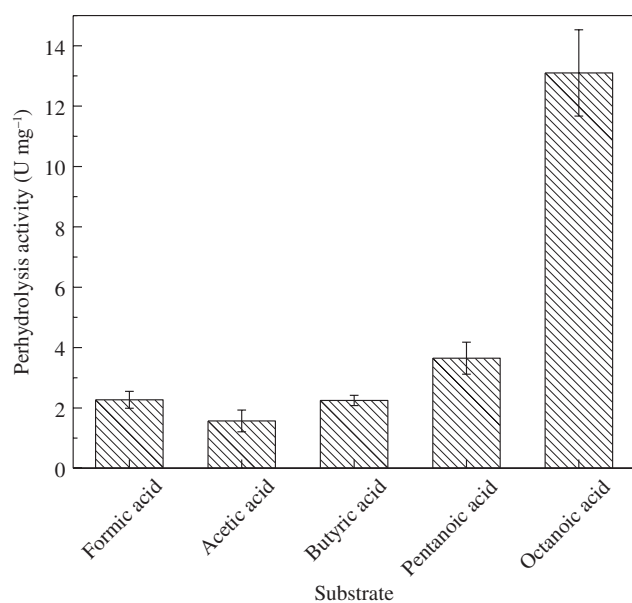
**Table 1** Residual perhydrolysis activity of lipases after incubation with H<sub>2</sub>O<sub>2</sub> in different media<sup>a,b</sup>

Enzymes	Media	Residual enzyme activity after 24 h (%)		
		0.5 mol L <sup>-1</sup> H <sub>2</sub> O <sub>2</sub>	1 mol L <sup>-1</sup> H <sub>2</sub> O <sub>2</sub>	3 mol L <sup>-1</sup> H <sub>2</sub> O <sub>2</sub>
AOL	Buffer (pH = 6, 20 mM phosphate)	53.2 ± 3.13	33.2 ± 1.82	5.1 ± 1.1
	ChCl/sorbitol (1:1 M ratio)	97.3 ± 2.67	90.3 ± 2.13	84.7 ± 1.52
	ChCl/glycerol (1:1 M ratio)	86.6 ± 1.60	76.4 ± 1.22	56.2 ± 2.03
	ChCl/xylitol (1:1 M ratio)	89.2 ± 4.28	78.8 ± 2.95	65.6 ± 1.93
AflB	Buffer (pH = 6, 20 mM phosphate)	46.7 ± 1.31	26.2 ± 0.48	3.3 ± 0.26
	ChCl/sorbitol (1:1 M ratio)	85.3 ± 2.67	79.3 ± 3.22	63.3 ± 1.93
	ChCl/glycerol (1:1 M ratio)	61.6 ± 1.76	46.6 ± 1.94	31.6 ± 0.74
	ChCl/xylitol (1:1 M ratio)	63.8 ± 3.51	49.8 ± 2.71	33.8 ± 1.59
MAJ1	Buffer (pH = 6, 20 mM phosphate)	41.2 ± 2.36	21.2 ± 1.37	4.2 ± 0.49
	ChCl/sorbitol (1:1 M ratio)	81.3 ± 4.68	70.5 ± 2.52	52.3 ± 2.38
	ChCl/glycerol (1:1 M ratio)	69.6 ± 3.83	45.2 ± 2.16	35.6 ± 1.73
	ChCl/xylitol (1:1 M ratio)	63.2 ± 3.06	43.5 ± 3.11	33.1 ± 2.69

<sup>a</sup> General incubation conditions: One milliliter of NADES was supplemented with 75, 150, or 450 μL 30% H<sub>2</sub>O<sub>2</sub> (for 0.5, 1, and 3 mol L<sup>-1</sup> final, respectively) and an appropriate amount of buffer (pH = 6, 20 mmol L<sup>-1</sup> phosphate) to a total volume of 1.5 mL, the enzyme loading of 15 U mL<sup>-1</sup>, and the mixture was incubated at room temperature for 24 h.

<sup>b</sup> Error bars represent SD.



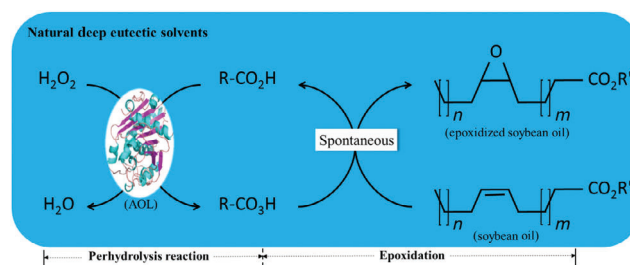


**Fig. 1** The substrate selectivity of AOL toward carboxylic acid (formic, acetic, butyric, pentanoic, and octanoic acids). The test was performed in 96-well microtiter plates with a reaction volume of 100  $\mu\text{L}$ , with carboxylic acid as the substrate (in 0.1 mol L<sup>-1</sup> carboxylic acid buffer at pH 6.0 containing 90 mmol L<sup>-1</sup> NaBr and 180  $\mu\text{mol L}^{-1}$  MCD) in the presence of 100 mmol L<sup>-1</sup> H<sub>2</sub>O<sub>2</sub> and constant enzyme (1 mg mL<sup>-1</sup>), at 40°C

(83.5%) was obtained at 50°C. However, there was a decrease in the conversion when the reaction temperature was increased from 50 to 70°C, which may be attributable to decreasing intrinsic stability of AOL at higher temperatures. Therefore, a reaction temperature of 50°C was selected in subsequent experiments. Apparently, this temperature provided the highest conversion for the two-step reactions involving perhydrolysis and epoxidation (Scheme 1).

#### Effect of the H<sub>2</sub>O<sub>2</sub>/C=C-Bonds Molar Ratio

Fig. 2b shows the effect of hydrogen peroxide addition on the relative conversion of the enzymatic epoxidation reaction. The conversion increased when the H<sub>2</sub>O<sub>2</sub>/C=C-bonds molar ratio was increased from 0.5:1 to 1.5:1. The maximum conversion (94.1%) was observed at a H<sub>2</sub>O<sub>2</sub>/C=C-bonds molar ratio of 1.5:1. In addition, the reaction rate of the AOL-catalyzed perhydrolysis was also affected by the concentration of the H<sub>2</sub>O<sub>2</sub> substrate. Above a molar ratio of H<sub>2</sub>O<sub>2</sub>/C=C-bonds of 1.5:1, the initial reaction rate and the final conversion decreased, which was probably due to the inactivation of AOL by excess H<sub>2</sub>O<sub>2</sub> and/or peracids. Therefore, the H<sub>2</sub>O<sub>2</sub>/C=C-bond molar ratio was fixed at 1.5:1 for further experiments.



**Scheme 1** The reaction process of natural deep eutectic solvent (NADES)-based enzymatic epoxidation. The percarboxylic acid was generated in situ from enzymatic reaction (by *Aspergillus oryzae* lipase, AOL) of carboxylic acid with H<sub>2</sub>O<sub>2</sub>, and then in Prileshajev reaction forms the epoxide and the corresponding carboxylic acid

In a previous study, the relative conversion of alkene to oxirane reached 77.0% (Sun et al., 2014). This reaction was catalyzed by the immobilized lipase B from *Candida antarctica* and the molar ratio was 1.5:1 of H<sub>2</sub>O<sub>2</sub>/C=C-bonds, using ionic liquid [Bmim]PF<sub>6</sub> as a reaction medium. However, in this study, the same molar ratio resulted in a higher conversion (94.1%, Fig. 2b). The lower conversion of the cited study may be due to a decreased activity of the enzyme caused by the high concentration of hydrogen peroxide in ionic liquid. Alternatively, in the NADES system, enzymes showed excellent activity as well as stability at high concentrations of hydrogen peroxide (Table 1). Therefore, this characteristic behavior of the NADES systems makes them an excellent choice for enzymatically producing epoxidized oils with high yield.

#### Effect of the Octanoic Acid/C=C-Bonds Molar Ratio

The effect of the octanoic acid/C=C-bonds molar ratio on the relative conversion of the enzymatic epoxidation reaction is shown in Fig. 2c. The conversion increased from 58.8% to 94.9% when the octanoic acid/C=C-bonds molar ratio was increased from 0.1:1 to 0.3:1. The octanoic acid/C=C-bonds molar ratio of 0.4:1 produced the highest initial reaction rate, but the conversion (approximately 95.2%) at reaction equilibrium (after 12 h) was similar to that observed with the octanoic acid/C=C-bonds molar ratio of 0.3:1. There was a slight decrease in the conversion when the octanoic acid/C=C-bonds molar ratio was further increased to 0.5:1. The conversion was similar or lower at higher octanoic acid/C=C-bonds molar ratios (0.4:1 and 0.5:1) than that at the molar ratio of 0.3:1, which may be due to the denaturation of the lipase at a higher acid value. Additionally, excessive amounts of octanoic acid will lead to time-consuming and energy-consuming removal of residual acids. Therefore, an octanoic acid/C=C-bonds molar ratio of 0.3:1 was used for further experiments.

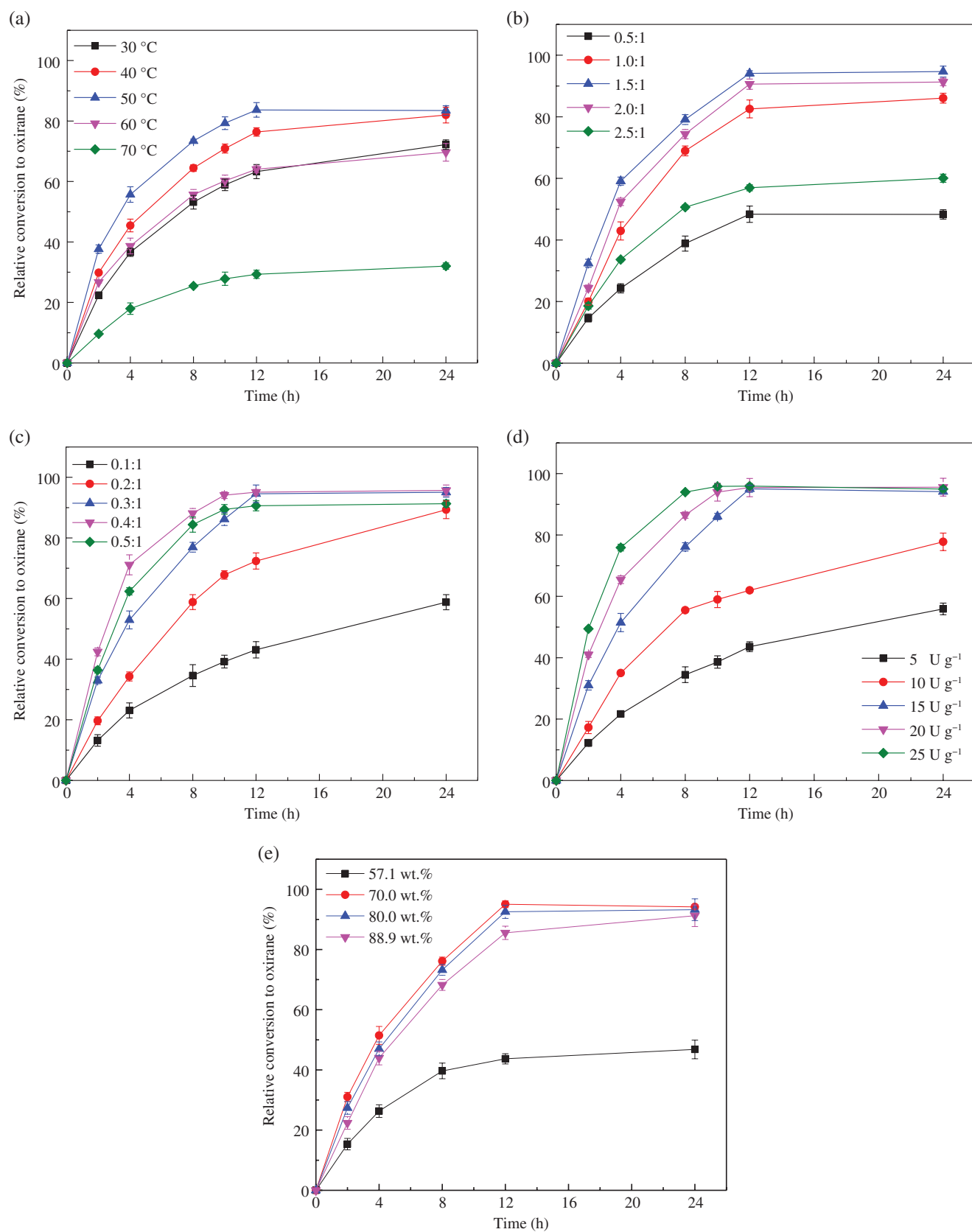


Fig. 2 Legend on next page.



### Effect of Enzyme Loading

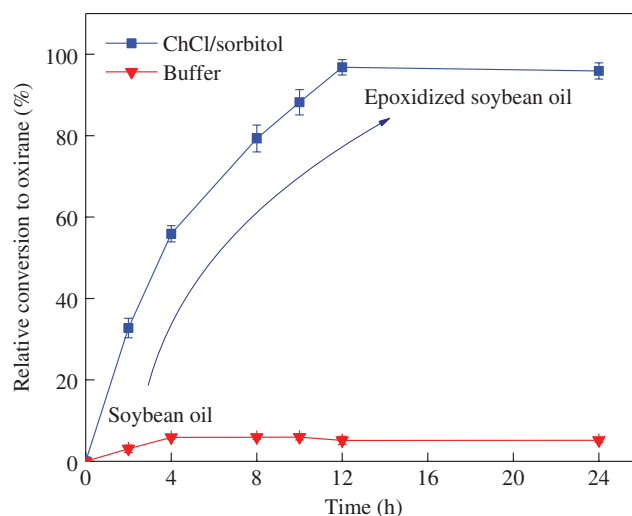
As shown in Fig. 2d, the conversion of the enzymatic epoxidation reaction after 12 h significantly increased from 43.6% to 95.1%, with increasing enzyme loading from 5 to 15 U g<sup>-1</sup> of soybean oil. Above 15 U g<sup>-1</sup>, the conversion remained almost constant with enzyme loading increasing to 25 U g<sup>-1</sup> of soybean oil. These results indicate that high enzyme loadings increased the reaction rate and shortened the time required to reach the reaction equilibrium. Therefore and also considering economic factors, an enzyme loading of 15 U g<sup>-1</sup> of soybean oil was used in the subsequent experiments.

### Effect of the ChCl/Sorbitol Content

In addition, the effect of the ChCl/sorbitol (1:1 M ratio) content was investigated as shown in Fig. 2e. When the content of ChCl/sorbitol was decreased from 4.2 g (70.0 wt.%, in hydrophilic phase) to 2.4 g (57.1 wt.%), the conversion after 12 h was decreased from 95.1% to 56.2%. This may be due to damaged hydrogen-bond networks between ChCl and sorbitol in the NADES that were weakened by excessive water content (Hammond et al., 2017), as seen in Fig. S1 (Supporting Information), thereby resulting in a lower conversion. The conversion was similar or lower when the content of ChCl/sorbitol (1:1 M ratio) was further increased to 7.2 g (80.0 wt.%) and 16.2 g (88.9 wt.%). Therefore, the ChCl/sorbitol (1:1 M ratio) content was fixed to 70.0 wt.% of hydrophilic phase (consisting mainly of hydrogen peroxide solution and NADES).

### Scaled-up Production of ESO

The scale-up of the reaction system (3–1000 g scale for soybean oil) was performed under the optimized reaction conditions. As shown in Fig. 3, the conversion was 96.8% after 12 h of scale-up reaction using ChCl/sorbitol (1:1 M ratio) as a solvent and was similar to that (95.4%) after 12 h obtained under the optimized reaction conditions (Fig. 2d). This result suggests that the production of ESO could be scaled up for potential industrial applications. For

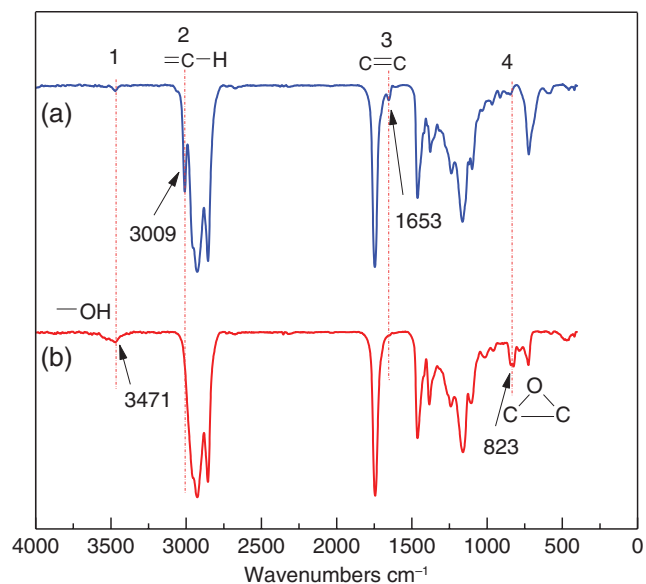


**Fig. 3** Time course of an enzymatic epoxidation reaction of soybean oil under optimized reaction conditions. *General conditions:* soybean oil, 1000 g; ChCl/sorbitol (1:1 M ratio), 1400 g; molar ratio of octanoic acid/H<sub>2</sub>O<sub>2</sub>/C=C-bonds, 0.3:1.5:1; H<sub>2</sub>O<sub>2</sub> concentration, 2.1 mol L<sup>-1</sup>; and enzyme loading 15 U g<sup>-1</sup> of soybean oil. The reaction mixture was stirred at 500 rpm (in a 5000 mL beaker) at 50°C

comparison, ESO was produced under the same reaction conditions in the absence of ChCl/sorbitol (1:1 M ratio), and the conversion was below 10%. The conversion was remarkably improved using ChCl/sorbitol (1:1 M ratio) instead of a buffer as a reaction medium, which may be due to the stabilization effect and higher soybean oil-NADES interfacial area. Our previous study showed that enzymes exhibited significantly higher activity at the oil-deep eutectic solvent interface compared to the oil–water interface and that the interfacial surface area was directly related to the overall rate of the reaction (Lan et al., 2017). Therefore, ChCl/sorbitol (1:1 M ratio) has the potential to be used a reaction medium in the AOL-catalyzed synthesis of ESO due to its high efficiency and environmental friendliness.

The product (ESO) and the starting material were analyzed by FT-IR to confirm the formation of the desired epoxide product. As shown in Fig. 4, the absorption peak at 3471 cm<sup>-1</sup> is assigned to the –OH stretching vibration of free fatty acids present in the substrate (soybean oil). The intensity of this peak was somewhat enhanced in the final

**Fig. 2** Effects of the reaction temperature, molar ratio of octanoic acid/H<sub>2</sub>O<sub>2</sub>/C=C-bonds, and enzyme loading on the epoxidation of soybean oil. (a) Effect of the reaction temperature on the epoxidation of soybean oil. *Reaction conditions:* soybean oil, 3 g; ChCl/sorbitol (1:1 M ratio), 4.2 g; molar ratio of octanoic acid/H<sub>2</sub>O<sub>2</sub>/C=C-bonds, 0.3:1:1; and enzyme loading 15 U g<sup>-1</sup> of soybean oil. (b) Effect of the molar ratio of H<sub>2</sub>O<sub>2</sub>/C=C-bonds on the epoxidation of soybean oil. *Reaction conditions:* soybean oil, 3 g; ChCl/sorbitol (1:1 M ratio), 4.2 g; the molar ratio of octanoic acid/C=C-bonds, 0.3:1; enzyme loading 15 U g<sup>-1</sup> of soybean oil, 50°C. (c) Effect of the molar ratio of octanoic acid/C=C-bonds on the epoxidation of soybean oil. *Reaction conditions:* soybean oil, 3 g; ChCl/sorbitol (1:1 M ratio), 4.2 g; molar ratio of H<sub>2</sub>O<sub>2</sub>/C=C-bonds, 1.5:1; enzyme loading 15 U g<sup>-1</sup> of soybean oil, 50°C. (d) Effect of enzyme loading on the epoxidation of soybean oil. *Reaction conditions:* soybean oil, 3 g; ChCl/sorbitol (1:1 M ratio), 4.2 g; molar ratio of octanoic acid/H<sub>2</sub>O<sub>2</sub>/C=C-bonds, 0.3:1.5:1, 50°C. (e) Effect of ChCl/sorbitol (1:1 M ratio) content on the epoxidation of soybean oil. *Reaction conditions:* soybean oil, 3 g; molar ratio of octanoic acid/H<sub>2</sub>O<sub>2</sub>/C=C-bonds, 0.3:1.5:1 (the weight of hydrogen peroxide solution [hydrophilic phase] was approximately 1.8 g); and enzyme loading 15 U g<sup>-1</sup> of soybean oil, 50°C. Full epoxidation of all C=C-double bonds corresponds to an *OO*<sub>the</sub> value of 7.34%

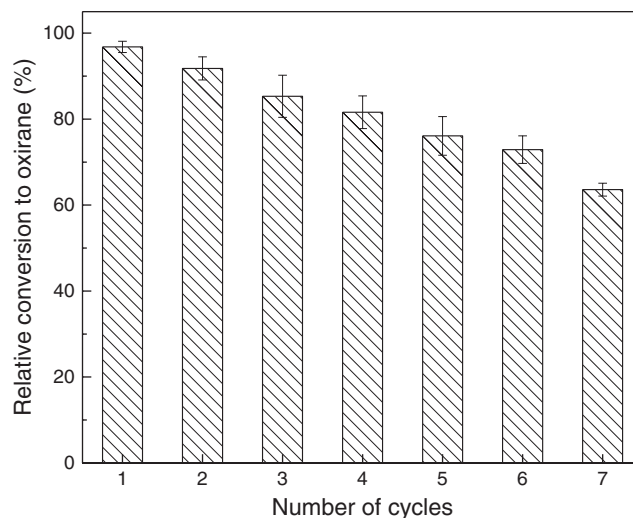


**Fig. 4** FT-IR spectra of soybean oil (a) before and (b) after reaction for 12 h

product, which may be due to a minor contribution of residual octanoic acid. More importantly, two characteristic absorption peaks ( $=C-H$  stretching vibration at  $3009\text{ cm}^{-1}$  and the  $C=C$  bond stretching at  $1653\text{ cm}^{-1}$ ) appeared in the spectrum of unsaturated starting material (soybean oil), but they completely disappeared in the final product. The characteristic epoxy group absorption at  $823\text{ cm}^{-1}$  was only found in the product, indicating that the successful conversion of soybean oil to ESO was achieved. The successful conversion of soybean oil to ESO was also confirmed by  $^1\text{H}$  NMR (Fig. S2).

### Reusability of AOL

The recycling of catalysts is extremely critical when considering the economical and environmental performance. In this study, the oil phase (upper layer) was separated from the lower layer (including NADES, enzyme, and water) by centrifugation after the epoxidation reaction. Thereby, the purification of ESO was relatively simple compared to organic solvent systems, eliminating some steps such as organic solvent extraction and vacuum distillation. Consequently, the use of NADES instead of organic solvent systems can avoid potential threats of organic solvents to human health and the environment. The water (about 30.0 wt.% of the lower layer) present in the lower layer was completely removed under vacuum at  $50^\circ\text{C}$  for 20 min and the residue (including NADES and enzyme) was recycled and reused. Fig. 5 shows the results for the reusability of the catalysts and NADES for ESO production. The conversion after the first reaction was 96.8%. When used in the



**Fig. 5** The reusability of catalysts and NADES during epoxidation. The water present in the lower layer was completely removed under vacuum at  $50^\circ\text{C}$  for 20 min and the residue (including NADES and enzyme) was recycled and reused. *Reaction conditions:* soybean oil, 3 g; the residue (including NADES and enzyme), 4.2 g; the molar ratio of octanoic acid/ $\text{H}_2\text{O}_2$ / $C=C$ -bonds, 0.3:1.5:1; and  $\text{H}_2\text{O}_2$  concentration,  $2.1\text{ mol L}^{-1}$ . The reaction mixture (500 rpm, in a 25 mL conical flask) for 12 h at  $50^\circ\text{C}$

second run, the conversion was 90.3% and AOL retained 93.2% of its initial activity. After seven cycles, the conversion decreased to 63.6%, which was about 65.7% of its initial activity. Possibly, sorbitol esters may be produced as a by-product during the reaction and accumulate with increasing the number of reuses. Whether the amount of sorbitol esters produced has an effect on the reusability of the lipase will be explored in a future study. Although the immobilization of the enzyme (e.g., with resin and nanoparticles) was used to achieve catalyst recovery (Cui et al., 2016), the high cost of the immobilization matrix must be considered. Therefore, we propose the concept of “stabilization of the enzyme with NADES” to achieve enzyme recycling and reuse.

### Conclusions

This study offers a feasible strategy to efficiently produce epoxidized oils by applying a nonconventional reaction medium, NADES. The tolerance of the lipase against  $\text{H}_2\text{O}_2$  was reinforced through the stabilization effect of NADES. Up to 96.8% conversion was achieved under the optimized conditions. Finally, NADES-based enzymatic epoxidation serves as a promising protocol for facilitating product separations and the recycling of catalysts.

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**Conflict of Interest** The authors declare that they have no conflict of interest.

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