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

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Industrial strategy to efficiently produce epoxidized oils: natural deep eutectic solvent-based enzymatic epoxidation

Running Title: Production of epoxidized oils

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Abstract: Whether enzymes show good H_2O_2-resistance is a key bottleneck for the epoxidized process of oil by enzymatic process. In this study, the stability of three lipases against H_2O_2 was evaluated in different types of natural deep eutectic solvents (NADES). The lipases are from *Aspergillus oryzae* (AOL), *Aspergillus fumigatus* lipase B (AflB) and marine *Janibacter* (MAJ1), respectively. The poor robustness of lipases against H_2O_2 was strengthened significantly under the NADES. Specifically, AOL retained 84.7% of its initial activity in the presence of choline chloride/sorbitol and 3 mol/L H_2O_2 after 24 h incubation. The epoxidation process was further optimized by AOL lipase in ChCl/sorbitol as follows: molar ratio of octanoic acid/H_2O_2/C=C-bonds = 0.3:1.5:1, enzyme loading 15 U/g substrate, ChCl/sorbitol content 70.0% of the weight of hydrophilic phase, reaction temperature of 50 °C. Under the optimized conditions, up to 96.8% conversion was achieved. Moreover, the lipase immobilized in NADES retained approximately 66% of its initial activity after being used for seven batch cycles. Overall, NADES-based enzymatic epoxidation is a promising strategy for the synthesis of epoxidized oils.

Keywords: Epoxidation; Lipase; Natural deep eutectic solvent; Soybean oil; Enzyme catalysis

1. Introduction

Epoxidized oils have gained widespread attentions as bio-based and toxicologically less-questionable substitutes for phthalates which are potentially toxic to human health and the environment (Liu et al., 2016), their production has been reported by many researchers. Chemical methods have more disadvantages, such as environmental burdens (e.g., salt wastes originating from the neutralization of the catalysts or the formation of undesirable side-products), acute operational risks and corrosive deterioration of reaction vessels. In recent years, a biocatalytic alternative to the aforementioned chemical synthesis that uses lipases has attracted considerable interest from academic researchers (Sun et al., 2011; Liu et al., 2016; Chen et al., 2017). However, to the best of our knowledge, this reaction has not yet been adapted on an industrial scale.

In the epoxidized process of oils, H_2O_2 is not only a substrate but also shows a strong detrimental effect on the chemical structure of enzymes because of its ability to oxidize amino acids (e.g., Arg, Lys, Met, His). To solve the problem, strategies, such as enzyme immobilization and protein engineering, have been used to improve the H_2O_2-tolerance of enzymes (Ogola et al., 2010). Alternatively, reaction media also play a very important role in efficient epoxidation by a biocatalyst. Aqueous reaction media are not good for per-hydrolysis reactions. Here, the desired perhydrolysis activity of lipases competes with their natural hydrolytic activity. Hence, not only are the ester functionalities of the oils cleaved (leading to more complex, low-quality products), but *in situ* concentration of peracids is also reduced, resulting in lower epoxidation rates. Some organic solvents (e.g., toluene, benzene and chloromethane) have proven to be excellent media in epoxidation, but their potential toxicity 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 toxicity issues, occasional poor biodegradability and manufacturing costs (due to lengthy preparation) (Zhang et al., 2012). To overcome the

above-mentioned disadvantages, a new generation of solvents, the so-called deep eutectic solvents, have achieved growing interest in past years (Abbott et al., 2003). Deep eutectic solvents generally refer to a eutectic mixture of two or more, preferably cheap and safe components. Compared to ionic liquids, deep eutectic solvents have the advantage of easier preparation (also translating to lower production costs) and, provided they are synthesized from natural components, toxicological innocuousness and biodegradability. Since then, deep eutectic solvents have been variously applied in catalysis, as extraction solvents, in material chemistry and in organic synthesis (Zhang et al., 2012). In 2011, Choi and co-workers 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-based enzymatic epoxidation.

Therefore, in our ongoing efforts to establish NADES-based enzymatic epoxidations, we report the application of this concept to evaluate lipases against H_2O_2 in the different kinds of NADES, and the potential for the preparation of epoxidized soybean oil (ESO).

2. Materials and Methods

2.1 Materials

The recombinant *Pichia pastoris* X-33, containing pGAPZαA-AOL, AflB and the MAJ1 expression strain, was stored at −80 °C in the laboratory. Soybean oil with an iodine value of 125 g I_2/100 g (acid value = 0.17 mg KOH/g) 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 analytical grade.

2.2 Production of AOL lipase, MAJ1 lipase and AflB lipase

The expression strain was used to produce AOL lipase, MAJ1 lipase and AflB lipase. The fermentation inoculums was 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), 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, and then the recombinant lipase in the supernatant was concentrated and buffer-exchanged to buffer A (20 mmol/L sodium phosphate, pH 8.0) through a 10 kDa molecular mass membrane (Viva ow 200, Sartorius, Germany). The lipases were purified in a Q Sepharose Fast Flow column. Finally, the purified lipase was freeze-dried in a freeze dryer (Christ ALPHA 1-2 LD plus, Osterode, Germany) for subsequent reactions.

2.3 Preparation of NADESs

Choline chloride was mixed with sorbitol, xylitol or glycerol in a molar ratio of 1:1. These mixtures were heated to 80 °C while continuously stirred until colorless, homogeneous liquids were obtained.

2.4 Assay on perhydrolysis activity of lipase

The perohydrolysis activity of lipase was tested using the monochlordimedone (MCD) assay (Bernhardt et al., 2005). Here, the formation of peracids is detected indirectly through the formation of hypobromite (via oxidation of bromide by the peracid), which itself reacts with MCD. The latter is quantified via its characteristic absorption at 290 nm. The test was performed at 40 °C with pentanoic acid as the substrate (in 0.1 mol/L pentanoic acid buffer at pH 6.0 containing 90 mmol/L NaBr and 180 μmol/L MCD) in the presence of 100 mmol/L H_2O_2. The activity was determined spectrophotometrically and expressed in specific activity

units, where 1 U represents the amount of enzyme required to produce 1 μmol of MCD per minute under the reaction conditions.

2.5 Enzymatic epoxidation of soybean oil

All reactions were performed in a 25 mL conical flask submerged in a thermostatic water bath for temperature control. The reaction was conducted in two liquid phases comprising 3 g of soybean oil (as hydrophobic phase) and 4.2 g NADES. The reaction mixture was supplemented with hydrogen peroxide and carboxylic acid, and then the reaction was initiated by addition of the enzyme. The reaction mixture was stirred (500 rpm) for 24 h. Stirring was stopped after the end of reaction, after which the oil sample and the NADES were allowed to stand to enable stratification; and the upper oil sample was removed for subsequent detection.

2.6 Evaluation of enzymatic epoxidation of soybean oil

Iodine values and oxirane values were determined following previously described titration methods (Monono et al., 2015). The experimentally determined oxirane oxygen content (OO_{exp}) was calculated using the following equation:

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

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_{the}) is defined as the maximum oxirane content in 100 g soybean oil (Zhang et al., 2017):

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

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

In this study, the relative conversion to oxirane was calculated as follows:

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

2.7 FT-IR analysis of the final ESO product

One milligram of the purified sample with 100 mg KBr were initially mixed, ground, and mortar. 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 cm^{-1} during 128 scans, with the resolution at 2 cm^{-1}. The absorption peaks were identified from the spectra (Gogoi et al., 2017).

2.8 Statistical analysis

All experiments were performed in triplicate. The results are presented as the means ± standard deviations. The differences among mean values were evaluated in SPSS 19.0 through significant difference tests and variance analysis.

3. Results and Discussion

3.1 Influence of different NADES on lipase stability against H_2O_2

NADES were employed as reaction media and H_2O_2 was employed as an oxygen supplier in the epoxidation reaction. However, H_2O_2 is a known inactivator of enzymes. Therefore, the stabilities of AOL lipase, AflB lipase and MAJ1 lipase against H_2O_2 were investigated, and results are shown in Table 1. Interestingly, the high resistance of the three lipases against H_2O_2 were observed in the presence of three types of NADES (ChCl/sorbitol, ChCl/xylitol and ChCl/glycerol). For example, 3.0 mol/L H_2O_2 almost completely inactivated the lipases in the buffer, whereas under the same conditions, albeit in the presence of ChCl/xylitol, AOL retained 65.6% of its initial activity, AflB retained 33.8% and MAJ1 retained 33.1%. Particularly in ChCl/sorbitol media, AOL almost completely retained its initial activity after incubation in the presence of 0.5 mol/L H_2O_2 for 24 h, and retained 84.7% of its initial activity after incubation in the presence of 3.0 mol/L H_2O_2 for 24 h. The reason for this enormous stabilization is not yet fully understood. Previously, H-bond donating ionic liquids and polyols including sorbitol were demonstrated to stabilize enzyme structures (Kotlewska et al., 2011; Diego et al., 2004). Our previous study reported that an H-bond

donating deep eutectic solvents could stabilize the enzyme structure (Zhou et al., 2016). Similarly, the H-bond networks in polyol-based NADES used in this study possibly alleviate the attack of H_2O_2 on the catalytic center of the enzyme and played a role in the stability of the enzyme. Therefore, the use of a certain kind of NADES as reaction system may be a promising future approach for biocatalysis due to the stabilization effect. The effect of ChCl/sorbitol as a reaction medium on AOL-catalyzed lipase was investigated in subsequent experiments.

3.2 Enzymatic epoxidation of soybean oil by AOL lipase

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

3.2.1 Effect of temperature

The effect of 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 varied from 30 to 50 °C, and the maximum conversion (83.5%) was obtained at 50 °C. However, there was a slight decrease in the conversion when the reaction temperature was changed from 50 to 60 °C. With a further increase from 60 to 70 °C the overall conversion decreased rapidly, which may be attributed to a decreasing intrinsic stability of AOL. Therefore, the reaction temperature of 50 °C was selected in subsequent experiments. Apparently, this temperature provided the highest conversions of the reactions (for both the perhydrolysis and epoxidation reactions).

3.2.2 Effect of H_2O_2/C=C-bonds molar ratio

Figure 2b shows the effect of hydrogen peroxide addition on the relative conversion of the enzymatic epoxidation reaction. The conversion increased when the H_2O_2/C=C-bonds molar ratio varied from 0.5:1 to 1.5:1. The maximum conversion (94.1%) was observed at a H_2O_2/C=C-bonds molar ratio of 1.5:1. In addition, the reaction rate of the AOL-catalyzed perohydrolysis also depended on the concentration of the H_2O_2 substrate. Above a molar ratio of H_2O_2/C=C-bonds of 1.5:1, the initial reaction rate and the final conversion rapidly decreased rapidly, which was probably attributed to the inactivation of AOL by excess H_2O_2 and/or peracids. Therefore, the H_2O_2 concentration was fixed to molar ratio of H_2O_2/C=C-bonds of 1.5:1 for further experiments.

In a previous study, the relative conversion to oxirane from alkene was 77.0%. The reaction was catalyzed by immobilized lipase Novozyme 435 and the molar ratio was used 1.5:1 of H_2O_2/C=C-bonds, using ionic liquid [Bmim]PF_6 as a reaction medium (Sun et al., 2014). However, we used the same molar ratio concentration for this study, the conversion was very high (94.1%). A lower conversion was obtained in the previous study, which may because the activity of the enzyme was decreased by high concentration of hydrogen peroxide in ionic liquid. Alternatively, in the NAEDS system, enzymes showed excellent activity as well as stability under high concentration of hydrogen peroxide (Table 1). Therefore, this characteristic behaviour of NADES system may make it an excellent choice for enzymatically producing epoxidized oils with high yield.

3.2.3 Effect of 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 Figure 2c. The conversion increased from 58.8% to 94.9% when the octanoic acid/C=C-bonds molar ratio varied 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 by 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 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, the over-loaded octanoic acid will lead to time- 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.

3.2.4 Effect of enzyme loading

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

3.2.5 Effect of ChCl/sorbitol content

Finally, the effect of ChCl/sorbitol content was investigated as shown in Figure 2e. This reaction system contained 3.0 g soybean, and the molar ratio of octanoic acid/H_2O_2/C=C-bonds = 0.3:1.5:1, which mean that the weight of hydrogen peroxide solution (hydrophilic phase) was approximately 1.8 g. 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 reduced from 95.1% to 56.2%. This may be possibly because of the H-bond networks in NADES were damaged by excessive water content (Hammond et al., 2017) (Figure S1 shows the effect of the content of water), thereby resulting in a lower conversion. The conversion was similar or lower when the content of ChCl/sorbitol was increased to 7.2 g (80.0 wt. %) and 16.2 g (88.9 wt. %). Therefore, the ChCl/sorbitol content was fixed to 70.0 wt.% (in hydrophilic phase) for further experiments.

3.3 Upscaling of epoxidation reaction

The scale-up of epoxidation reaction was performed under the optimized reaction conditions. As shown in Figure 3, the conversion was 96.8% after 12 h of scale-up reaction using ChCl/sorbitol as solvent and was similar to that (95.4%) after 12 h obtained under the optimized reaction conditions (Figure 2d). The result suggests that the production of ESO could be scaled up for potential industrial applications. For comparison, ESO was produced under the same reaction conditions in the absence of ChCl/sorbitol, and the conversion was below 10%. The conversion was remarkably improved using ChCl/sorbitol instead of a buffer as a reaction media, which may be due to the stabilizing effect and a higher interfacial area. Our previous study showed that interface-activated enzymes exhibit significantly higher activity in a deep eutectic solvents system than in a buffer and that the interfacial surface area was directly related to the overall rate of the reaction (Lan et al., 2017). Therefore, using ChCl/sorbitol as reaction media has potential 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 Figure 4, the absorption peak at 3471 cm^{-1} is assigned to the -OH stretching vibration of free fatty acids present in the raw soybean oil. The intensity of this peak was somewhat enhanced in the final 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 cm^{-1} and the C=C bond stretching at 1653 cm^{-1}) were showed in the spectrum of unsaturated starting material (soybean oil), but they were disappeared completely in the final product. The characteristic epoxy group absorption at 823 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}H NMR (Figure S2).

3.4 Reusability of catalyst

The recycling of catalysts was extremely critical when considering the economically and environmentally friendly factors. In this study, the oil phase (upper layer) was separated from the lower layer (including NADES, enzyme and water) by centrifugation after epoxidation reaction. So the purification of ESO was relatively simple compared with organic solvent systems, eliminating some steps such as organic extraction and vacuum distillation, thus reducing energy consumption and avoiding the potential threats of organic solvents to human health and the environment. The water present in the lower layer was removed under vacuum at 50 °C and −90 kPa (vacuum pressure) and the remaining part (including NADES and enzyme, we called "Whole") was recycled and reused. Figure 5 shows the results for the reusability of "Whole" for ESO production. The conversion after the first reaction was 96.8%. When used in the 2nd 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. Although immobilization of the enzyme (e.g., with resin and nanoparticles) was used to achieve catalyst recovery (Cui et al., 2016), economic factors must be considered. Therefore, we propose the concept of "stabilization of the enzyme with NADES" to achieve enzyme recycling and reuse.

4. Conclusion

In summary, this study offers an industrial strategy to efficiently produce of epoxidized oils by focusing on the application of a non-conventional reaction medium, NADES. The tolerance of lipase against H_2O_2 was reinforced through the combination with NADES. Under the optimized conditions, up to 96.8% conversion was achieved. Finally, NADES-based enzymatic epoxidation serves as a promising protocol for facilitating product separations and the recycling of catalysts.

Conflict of Interest

There are no conflicts to declare.

References

Abbott, A. P., Capper, G., Davies, D. L., Rasheed, R. K., & Tambyrajah, V. (2003). Novel solvent properties of choline chloride/urea mixtures. *Chemical Communications*, **1:**70-71.

Bernhardt, P., Hult, K., & Kazlauskas, R. J. (2005). Molecular basis of perhydrolase activity in serine hydrolases. *Angewandte Chemie-International Edition***, 44:**2742-2746.

Cui, J. D., Cui, L. L., Jia, S. R., Su, Z. G., & Zhang, S. P. (2016). Hybrid cross-linked lipase aggregates with magnetic nanoparticles: A robust and recyclable biocatalysis for the epoxidation of oleic acid. *Journal of Agricultural and Food Chemistry*, **64:**7179-7187.

Choi, Y. H., van Spronsen, J., Dai, Y. T., Verberne, M., Hollmann, F., Arends, I. W. C. E., Witkamp, G. J., & Verpoorte, R. (2011). Are natural deep eutectic solvents the missing link in understanding cellular metabolism and physiology? *Plant Physiology*, **156:**1701-1705.

Chen, J. N., Zhou, J. F., Liu, W., Bi, Y. L., & Peng, D. (2017). Enzymatic epoxidation of soybean oil in the presence of perbutyric acid. *Chemical Papers*, **71:**2139-2144.

Dai, Y. T., van Sprinsen, J., Witkamp, G. J., Verpoorte, R., & Choi, Y. H. (2013). Natural deep eutectic solvents as new potential media for green technology. *Analytica Chimica Acta*, **766:**61-68.

Diego, T. D., Lozano, P., Gmouh, S., Vaultier, M., & Iborra, J. L. (2004). Fluorescence and CD spectroscopic analysis of the α-chymotrypsin stabilization by the ionic liquid, 1-ethyl-3-methylimidazolium bis[(trifluoromethyl)sulfonyl]amide. *Biotechnology Bioengineering*, **88:**916-924.

Gogoi, P., Boruah, M., Sharma, S., & Dolui, S. K. (2015). Blends of epoxidized alkyd resins based on jatropha oil and the epoxidized oil cured with aqueous citric acid solution: A green technology approach. *ACS Sustainable Chemistry & Engineering*, **3:**261-268.

Hammond, O. S., Bowron, D. T., & Edler, K. J. (2017). The effect of water upon deep eutectic solvent nanostructure: An unusual transition from ionic mixture to aqueous solution. *Angewandte Chemie-International Edition*, **56:**9782-9785.

Kotlewska, A. J., van Rantwijk, F., Sheldon, R. A., & Arends, I. W. C. E. (2011). Epoxidation and Baeyer–Villiger oxidation using hydrogen peroxide and a lipase dissolved in ionic liquids. *Green Chemistry*, **13:**2154-2160.

Lan, D. M., Wang, X. P., Zhou, P. F., Hollmann, F., & Wang, Y. H. (2017). Deep eutectic solvents as performance additives in biphasic reactions. *RSC Advances*, **7:**40367-40370.

Liu, W., Chen, J. N., Liu, R. L., & Bi, Y. L. (2016). Revisiting the enzymatic epoxidation of vegetable oils by perfatty acid: perbutyric acid effect on the oil with low acid value. *Journal of the American Oil Chemists' Society*, **93:**1479-1486.

Monono, E. M., Haagenson, D. M., & Wiesenborn, D. P. (2015). Characterizing the epoxidation process conditions of canola oil for reactor scale-up. *Industrial Crops and Products*, **67:**364-372.

Ogola, H. J. O., Hashimoto, N., Miyabe, S., Ashida, H., Ishikawa, T., Shibata, H., & Sawa, Y. (2010) Enhancement of hydrogen peroxide stability of a novel *Anabaena* sp. DyP-type peroxidase by site-directed mutagenesis of methionine residues. *Applied Microbiology and Biotechnology*, **87:**1727-1736.

Sun, S. D., Ke, X. Q., Cui, L. L., Yang, G. L., Bi, Y. L., Song, F. F., & Xu, X. D. (2011). Enzymatic epoxidation of *Sapindus mukorossi* seed oil by perstearic acid optimized using response surface methodology. *Industrial Crops and Products*, **33:**676-682.

Sun, S. D., Li, P., Bi, Y. L., & Xiao, F. A. (2014). Enzymatic epoxidation of soybean oil using lonic liquid as reaction media. *Journal of Oleo Science*, **63:**383-390.

Zhang, Q. H., Vigier, K. D., Royer, S., & Jérôme, F. (2012). Deep eutectic solvents: syntheses, properties and applications. *Chemical Society Reviews*, **41:**7108-7146.

Zhou, P. F., Wang, X. P., Zeng, C. X., Wang, W. F., Yang, B., Hollmann, F., & Wang, Y. H. (2016). Deep eutectic solvents enable more robust chemoenzymatic epoxidation reactions. *ChemCatChem*, **9:**934-936.

Zhang, X., Wan, X. H., Cao, H., Dewil, R., Deng, L., Wang, F., Tan, T. W., & Nie, K. L. (2017). Chemo-enzymatic epoxidation of *Sapindus mukurossi* fatty acids catalyzed with *Candida* sp. 99-125 lipase in a solvent-free system. *Industrial Crops and Products*, **98:**10-18.

Table 1. Residual perhydrolysis activity of enzyme after incubation with H_2O_2 in different media [a].

Enzyme	Media (molar ratio)	Residual enzyme activity after 24 h (%)		
		0.5 mol/L H_2O_2	1 mol/L H_2O_2	3 mol/L H_2O_2
AOL	Buffer (pH=6, 20 mM phosphate)	53.2±3.13	33.2±1.82	5.1±1.1
	ChCl/sorbitol (1:1)	97.3±2.67	90.3±2.13	84.7±1.52
	ChCl/glycerol (1:1)	86.6±1.60	76.4±1.22	56.2±2.03
	ChCl/xylitol (1:1)	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)	85.3±2.67	79.3±3.22	63.3±1.93
	ChCl/glycerol (1:1)	61.6±1.76	46.6±1.94	31.6±0.74
	ChCl/xylitol (1:1)	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)	81.3±4.68	70.5±2.52	52.3±2.38
	ChCl/glycerol (1:1)	69.6±3.83	45.2±2.16	35.6±1.73
	ChCl/xylitol (1:1)	63.2±3.06	43.5±3.11	33.1±2.69

[a] General conditions: One milliliter of NADES was supplemented with 75 μL, 150 μL or 450 μL 30% H_2O_2 (for 0.5 mol/L, 1 mol/L and 3 mol/L final, respectively) and appropriate amount of buffer (pH=6, 20 mmol/L phosphate) to a total volume of 1.5 mL, the enzyme loading of 15 U/mL and the mixture was incubated at room temperature for 24 h.

Figure legends

Figure 1. The substrate specificity of AOL to carboxylic acid.

Figure 2. Effects of the reaction temperature, molar ratio of octanoic acid/H_2O_2/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: molar ratio of octanoic acid/H_2O_2/C=C-bonds = 0.3:1:1, enzyme loading of 15 U/g substrate. (b) Effect of molar ratio of H_2O_2/C=C-bonds on the epoxidation of soybean oil. Reaction conditions: molar ratio of octanoic acid/C=C-bonds = 0.3:1, enzyme loading of 15 U/g substrate, at 50 °C. (c) Effect of molar ratio of octanoic acid/C=C-bonds on the epoxidation of soybean oil. Reaction conditions: molar ratio of H_2O_2/C=C-bonds = 1.5:1, enzyme loading of 15 U/g substrate, at 50 °C. (d) Effect of enzyme loading on the epoxidation of soybean oil. Reaction conditions: molar ratio of octanoic acid/H_2O_2/C=C-bonds = 0.3:1.5:1, at 50 °C. (e) Effect of ChCl/sorbitol content on the epoxidation of soybean oil. Reaction conditions: molar ratio of octanoic acid/H_2O_2/C=C-bonds = 0.3:1.5:1, enzyme loading of 15 U/g substrate, at 50 °C. Full expoxidation of all C=C-double bonds corresponds to an OO_{the} value of 7.34%.

Figure 3. Time course of an enzymatic epoxidation reaction of soybean oil under optimized reaction conditions. General conditions: The reaction was conducted in two liquid phases comprising 1000 g of soybean oil (as hydrophobic phase) and 1400 g ChCl/sorbitol, molar ratio of octanoic acid/H_2O_2/C=C-bonds = 0.3:1.5:1, enzyme loading of 15 U/g substrate. The reaction mixture was stirred at 500 rpm at 50 °C.

Figure 4. FT-IR spectra of soybean oil (A) and the oil after reaction 12 h (B).

Figure 5. Reusability of the "Whole" during epoxidation. Reaction conditions: molar ratio of octanoic acid/H_2O_2/C=C-bonds = 0.3:1.5:1, 3 g soybean oil, 4.2 g "Whole", reaction temperature of 50 °C, reaction for 12 h.

Graphical abstract

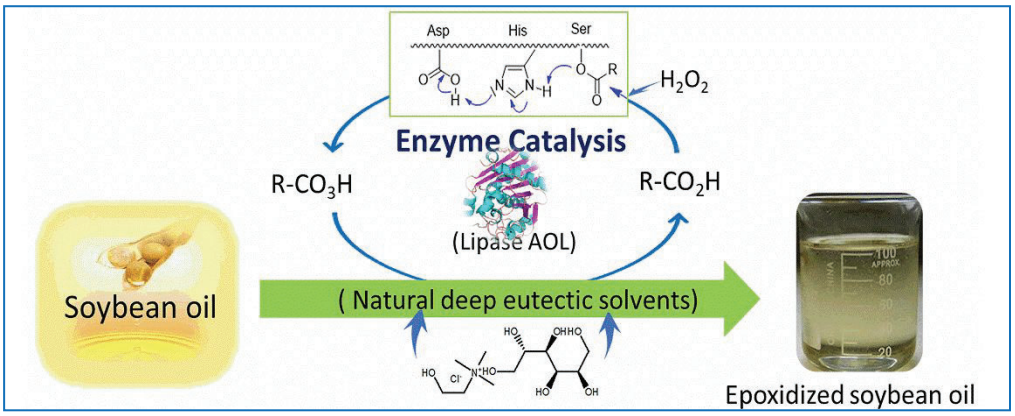


Figure 1.

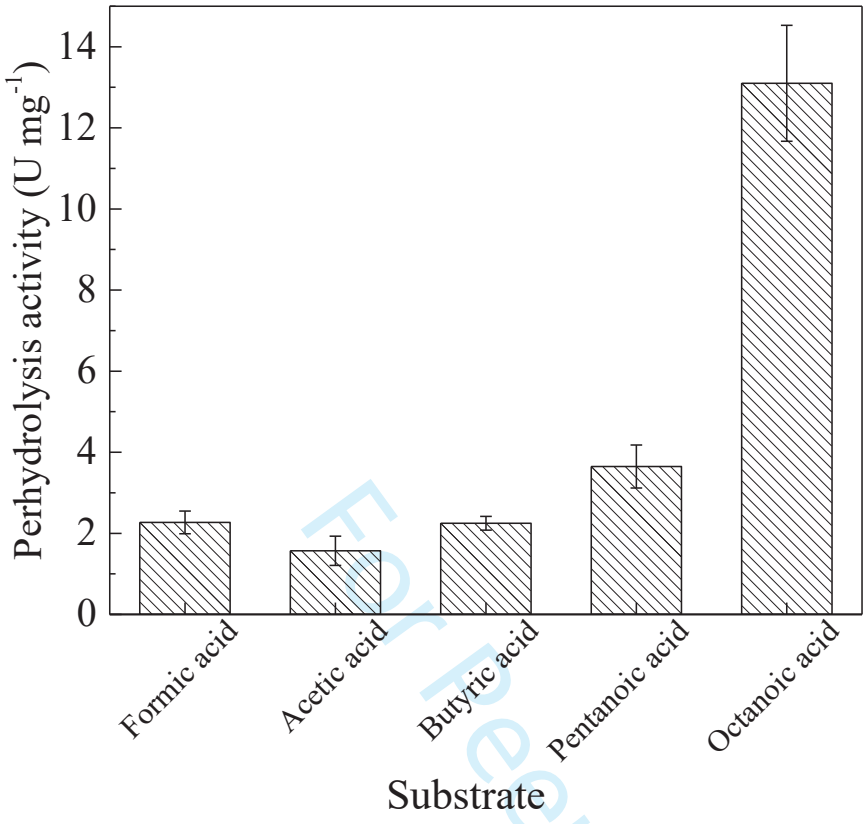
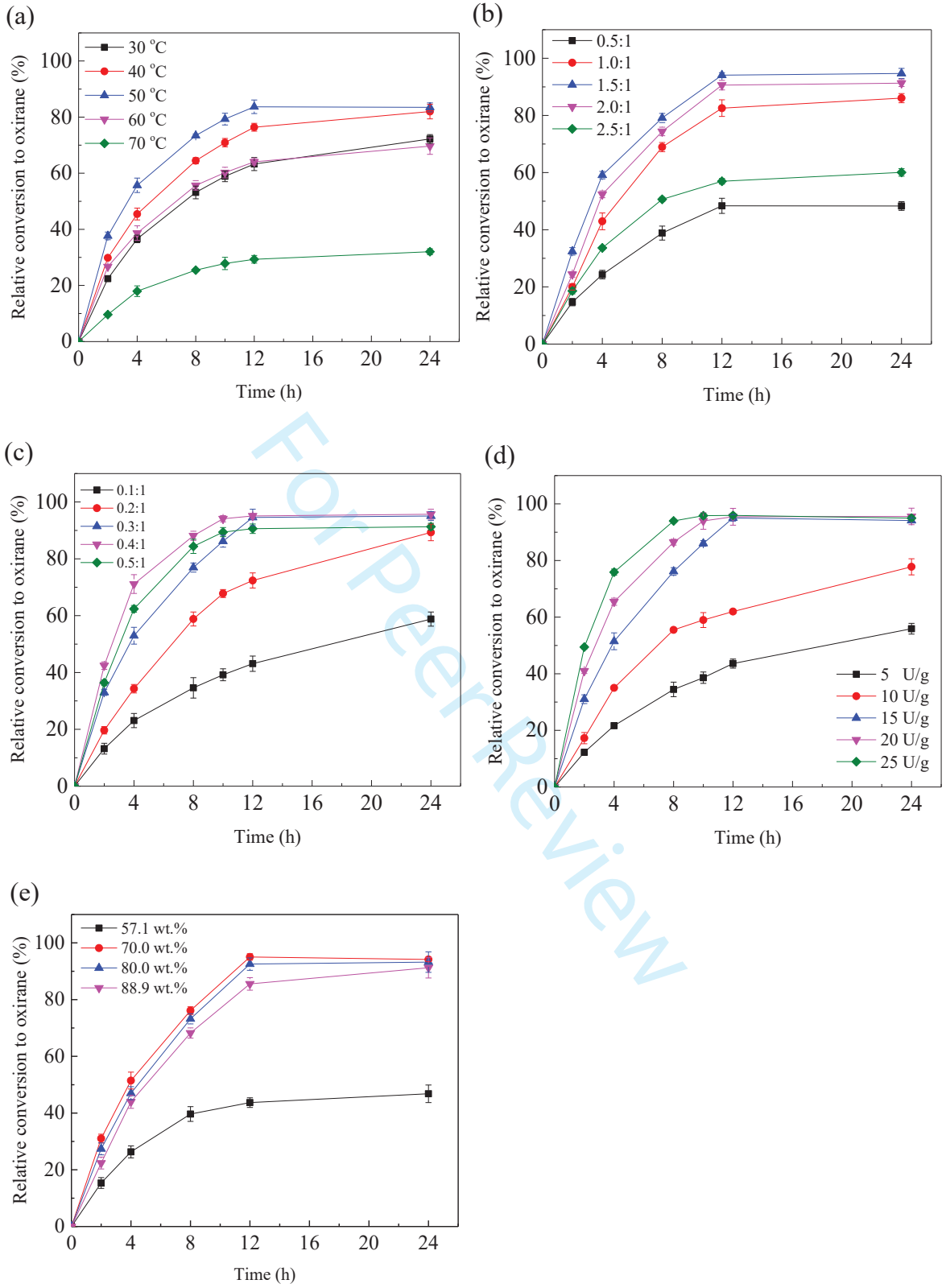


Figure 2.



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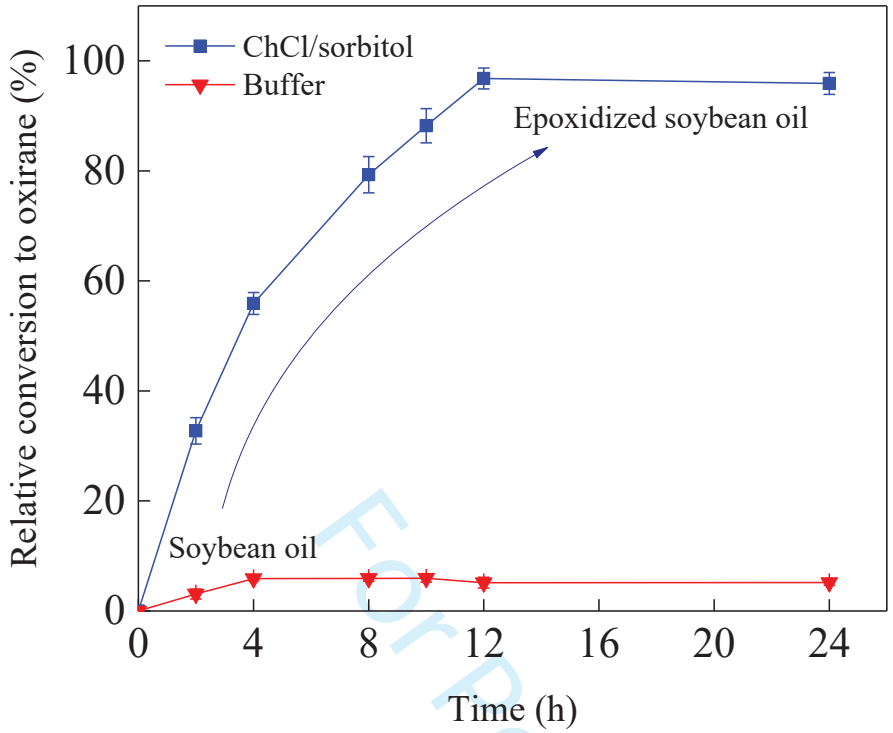

Figure 4.

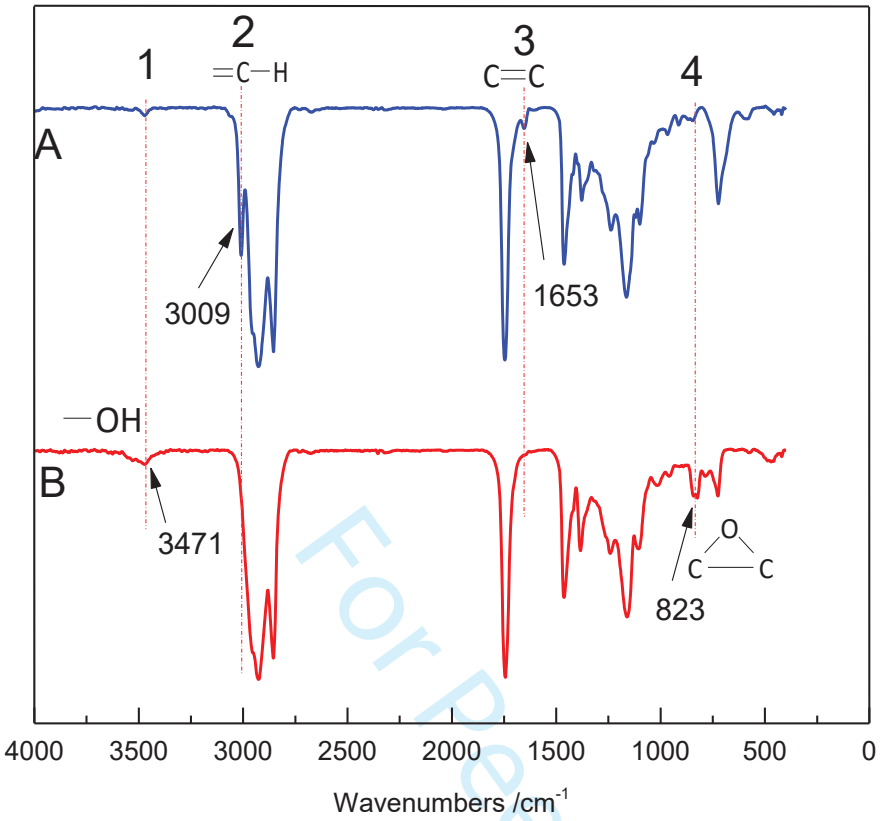

Figure 5.