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RESEARCH ARTICLE

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Study on green extraction of limonene from orange peel and cascade catalysis to produce carvol and carvone in deep eutectic solvents

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Abstract

Carvol and carvone are oxidation products from the natural product limonene. They are important raw materials for the flavours and fragrances industry and also act as pharmaceutical active ingredients. Orange waste peels possibly represent an attractive source for limonene, but studies on valorizing orange peel wastes are rare. In this study, we report a new enzymatic cascade system for the in-situ conversion of limonene from orange peel into valued-added carvol and carvone. The use of deep eutectic solvents (DES) allows for efficient in-situ extraction of limonene from waste orange peels. We propose a dual function use of DES as solvent for the extraction and the biocatalytic oxidation of limonene as well as cosubstrate to promote the oxidation reaction. Using ChCl-Pro-H_2O DES for the extraction of limonene from waste orange peels, approximately 17 milligrams of limonene per gram of orange peel was achieved at 40°C for 24 h. Then, with ChCl-Pro-H_2O DES as the extractant and reaction medium, a cascade reaction system of choline oxidase (ChOx) and unspecific peroxygenase (UPO) was established to catalyse the conversion of limonene into carvol and carvone. The concentration of the final products was up to about 1.6 mmol L^{-1}. This study showed a biocatalytic transformation pathway and provides technical support for the high-value utilization of waste in orange peel.

KEYWORDS
Carvol, carvone, Cascade reaction, deep eutectic solvents, Orange peel waste

1 | INTRODUCTION

Orange peel waste (OPW) is a common agricultural waste product.[1] The European Union alone produces several million tonnes of orange peel waste every year.[2] At present, more than 70% of the OPW is directly discarded, and only a very small fraction is used for products such as traditional Chinese medicine tangerine peel.[3,4] OPW, however, is rich in limonene (about 2%–5% of the dry weight) and thereby represents an interesting renewable source for limonene.[5,6] Common techniques for the extraction of limonene from orange peel comprise steam distillation, physical pressing, solvent extraction, supercritical fluid CO_2 extraction and microwave-assisted extraction.[7] Aiming at environmentally more acceptable extraction methods for limonene from OPW, a range of solvents have been investigated,[8] amongst them so-called Deep Eutectic Solvents (DESs).[9–11,12] DESs are promising alternatives to conventional organic solvents considering their toxicological properties, their biodegradability and, most importantly, their tuneable extracting properties.[13] Recently, Sun et al. have demonstrated the feasibility of using DES to develop an easy-to-operate one-pot method for simultaneous extraction and

conversion of polydatin to resveratrol from *P. cuspidatum* powder.[14] This method has a higher extraction rate than traditional organic solvents.

While limonene itself is relevant as flavour and fragrance ingredient, its oxidation products such as carvol and carvone have a wide range of applications,[15] such as in flavours and fragrances,[16] as antimicrobial agents[17] or as active pharmaceutical ingredients.[18]

Therefore, it is rather astonishing that relatively few reports deal with the valorization of OPW-derived limonene. The Mihovilovic group proposed a mixed culture approach comprising *Pseudomonas putida* and *Escherichia coli* to convert limonene into carvone followed by reduction of the conjugated C=C-double bond and Baeyer-Villiger oxidation.[5] Recently, we have reported a dual use of choline-based DES serving as extractant for limonene and as sacrificial cosubstrates for the in-situ generation of H_2O_2 to promote lipase-mediated, chemoenzymatic Prilezhaev reactions.[10] The latter reaction suffers from the actual oxidation step being non-enzymatic (i.e. lacking enzyme-induced selectivity). More elegantly, selective H_2O_2-driven oxidation reactions can be performed by peroxygenases.[10,19,20]

Deep Eutectic Solvents (DES) are increasingly receiving interest as alternative reaction media especially for biocatalytic reactions. Often, a beneficial effect of DES on the reaction or on the biocatalyst have been reported.[21] Therefore, DESs have a significant potential for the production of high-value compounds.[22]

We, therefore, set out to evaluate if DESs can also serve a dual function for the valorization of OPW as limonene extractant and as smart solvent for the selective oxyfunctionalization of limonene. As biocatalysts for the biochemical transformation of limonene to carvol and carvone we used the recombinant, evolved unspecific peroxygenase from *Agrocybe aegerita* (*Aae*UPO)[23–26] catalysing the H_2O_2-driven oxyfunctionalization of limonene. As *Aae*UPO, just like any heme-dependent enzyme, suffers from poor stability against H_2O_2, we aimed at in-situ generation of H_2O_2. For this we envisioned the choline oxidase from *Arthrobacter nicotianae* (*An*ChOx).[27,28] *An*ChOx catalyses the oxidation of choline to betaine while reducing O_2 to H_2O_2 (Scheme 1).

As such, we envisioned multiple synergistic functions for the DES in the overall process: as substrate (choline chloride), extraction of starting material (limonene), and reaction medium.

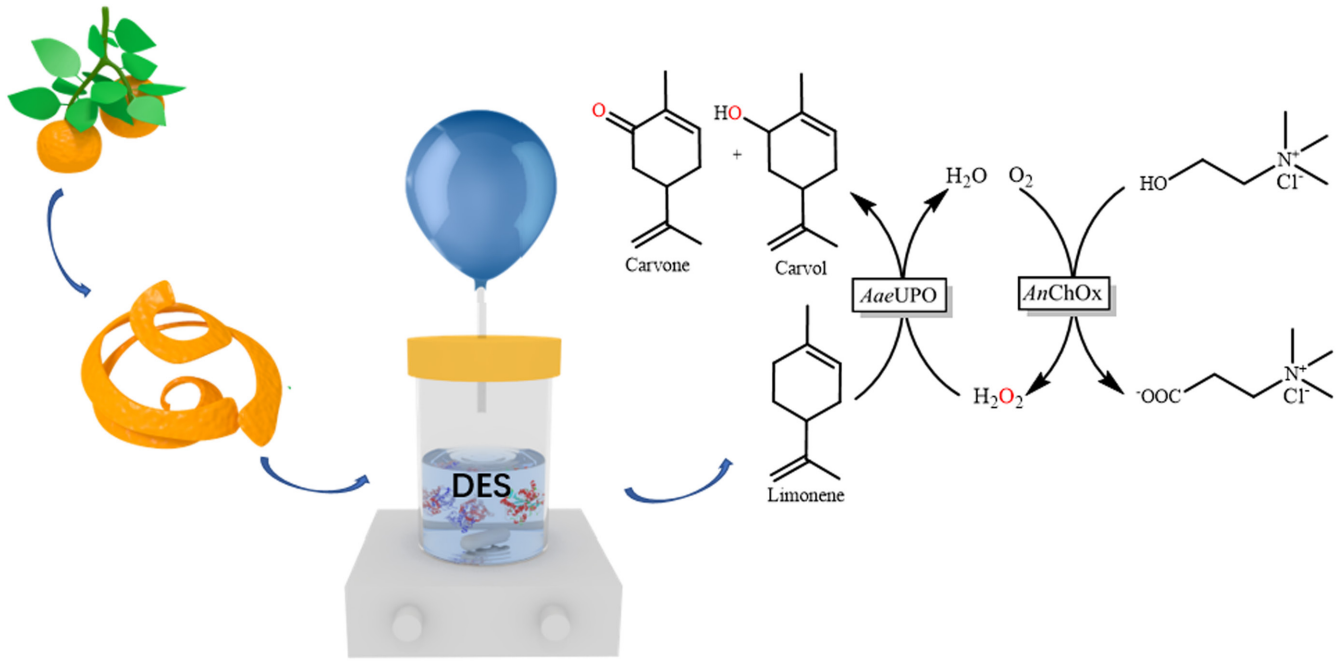
2 | MATERIALS AND METHODS

2.1 | Chemical reagents and materials

All chemicals were purchased from Sigma-Aldrich, TCI, Acros or Aladdin with the highest purity available and used without further treatment. All reagents were analytical grade and used as received. Fresh orange was purchased from a whole-sale fruit market in Guangzhou (Guangdong, China). We pulverized the orange peel with a crusher, then the orange peel samples were stored at 4°C temperature.

2.2 | Experimental setup and operating conditions

For analysis of the biocatalytic reactions an Agilent 7890B gas chromatography system (Agilent Technologies, Palo Alto, CA, USA) was used. Column used: CP-Sil 88 gas chromatography column (Agilent Technologies, Palo Alto, CA, USA: 60 m × 0.25 mm, 0.20 μm). Authentic standards of limonene, carvol and carvone were used for



SCHEME 1 Envisioned valorization of OPW-derived limonene to carvol and carvone. Deep Eutectic Solvents (DESs) serve as extractant of limonene from OPW, as solvent and as sacrificial electron donor for the biocatalytic transformation to carvol and carvone

qualitative chromatographic peak time, and standard solutions of different concentrations were prepared with the above standards and a standard curve was obtained through gas phase detection for quantitative calculation.

Gas detection method: sample volume: 1 μL; the injector temperature, 250°C; splitl mode(30:1); detector temperature 280°C; GC oven temperature for working solutions and samples were as follows: initial 50 to 200°C at a ramp rate of 10°C/min, hold for 3 min, from 200 to 230°C at a ramp rate of 5°C/min, then hold for 2 min.

2.3 | Analysis of limonene and its oxyfunctionalization products

Limonene and its oxyfunctionalization products were analysed by gas chromatography. The results are shown in Figure 1. The standard of limonene and its oxyfunctionalization products can be separated by gas chromatography. The peak time of each substance was: limonene 8.95 min, carvone 16.7 min, carvol 16.6 min and 16.8 min. The conversion ratios in this study were calculated using the ratio of the limonene concentration in the DES-extracted orange peel weight to produce carvol and carvone in the cascade reaction. The specific formula is as follows:

$$\text{Conversion}\,(\%) = \frac{c(\text{carvol}) + c(\text{carvone})}{c(\text{limonene})} \times 100$$

2.4 | Experimental procedures

2.4.1 | Preparation of DES

ChCl-based deep eutectic solvent can be easily prepared by thermally treating the admixed precursors. The ChCl-based DES used in this study (Table 1) were synthesized as described previously,[29,30] choline chloride was mixed with others at an appropriate molar ratio at 80°C under rigorous agitation for 2 h continuous stirring until colourless, homogeneous liquids were obtained.

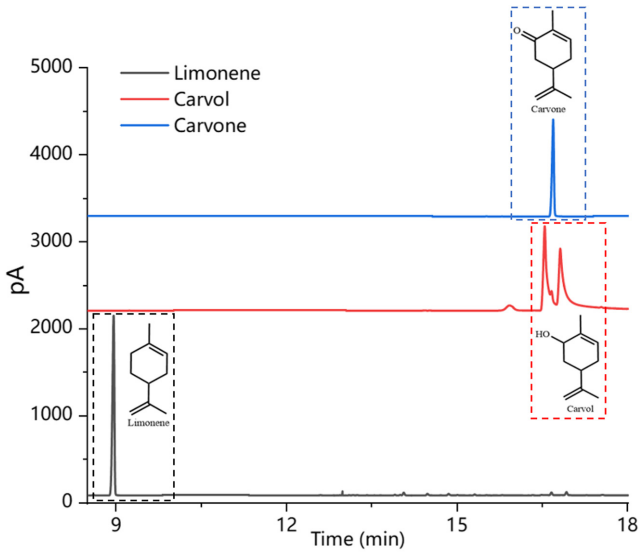


FIGURE 1 GC chromatogram of limonene standards and its products

2.4.2 | Preparation of *An*ChOx and *Aae*UPO

Preparation of AnChOx

The plasmid of ChOx from *Arthrobacter nicotianae* was kindly provided by Dr. Doris Ribitsch.

Cultivation protocol. Proteins of *An*ChOx was expressed in *E.coli* BL21 (DE3) gold cells. Precultures in 25 mL LB-medium (10 g/L tryptone, 5 g/L yeast extract, 10 g/L NaCl, 50 mg/L kanamycin) were incubated overnight (12 h, 37°C, 200 rpm) and used to inoculate the main cultures. The main cultures (500 mL LB-medium) were mixed with the inoculum until an optical density of 0.01 was reached. Main cultures were cultivated until an OD_{600} of 0.8 (4 h, 37°C, 200 rpm) was be observed. Protein overexpression was induced by supplementing isopropyl β-D-1-thiogalactopyranoside (IPTG; 0.05 mmol L^{-1} final concentration). Upon induction, the cultivation temperature was reduced to 20°C. Cells were harvested after 5 h by centrifugation (4000 rpm, 20 min, 4°C). The obtained cell pellets were suspended in 20 mmol L^{-1} sodium phosphate buffer, 500 mmol L^{-1} NaCl, pH 7.5 and disrupted by sonication on ice. Soluble proteins were separated from cell fragments and insoluble proteins by centrifugation (10 000 rpm, 40 min, 4°C). The supernatant was filtered through a 0.45 μm cellulose-acetate filter and subsequently used for further purification.

Protein purification. *An*ChOx was purified using an GE Chromatography system (Biorad). At first, the crude enzyme was injected into a His Prep™ FF16/10 column balanced by washing buffer A (20 mmol L^{-1} sodium phosphate buffer, 500 mmol L^{-1} NaCl, pH 7.5) at a flow rate of 5 mL min^{-1}. After equilibrated by washing buffer A, the binding protein was eluted by elution buffer B (20 mmol L^{-1} sodium phosphate buffer, 500 mmol L^{-1} NaCl, 500 mmol L^{-1} imidazole, pH 7.5) at a flow rate of 5 mL min^{-1}. After elution, the target protein was desalting by column HiPrep™

TABLE 1 Composition of DES[29,30]

Name	Combination	molar ratio
ChCl-Pro-H_2O	choline chloride/propanediol/H_2O	1:1:1
ChCl-Sor	choline chloride/sorbitol	1:1
ChCl-Gly	choline chloride/glycerol	1:2
ChCl-EG	choline chloride/ethylene glycol	1:2
ChCl-Urea	choline chloride/urea	1:2
ChCl-Urea-Gly	choline chloride/urea/glycerol	1:1:1
ChCl-Xyl-H_2O	choline chloride/xylitol/H_2O	5:2:5
ChCl-Glu-H_2O	choline chloride/glucose/H_2O	1:1:3

26/10 with desalting buffer (20 mmol L^{-1} sodium phosphate buffer, pH 7.5) at a flow rate of 5 mL min^{-1}. The purified protein was stored at 4°C.

Preparation of AaeUPO

*Aae*UPO used stemmed from a previous pilot-scale fermentation of the enzyme.[26]

2.4.3 | Limonene extraction

Extracts of orange peels were produced using ChCl-Pro-H_2O DES. Initially the peels were chopped finely with the help of a mixer and dissolved into the DES. The content was stirred and heated in a closed vessel for the effective extraction of limonene into the DES. Orange peel (1 g) in a glass vial filled with ChCl-Pro-H_2O DES system (4 mL) for extraction. The solution was filtered and then extracted by n-hexane, to be analysed via GC (Figure 2).

2.4.4 | Study on the factors affecting the extraction of limonene from orange peel by DES

The effects of water content of DES, extraction temperature, the ratio of orange peel weight to DES volume and extraction time on the extraction effect were investigated. According to the standard system described in 2.4.3, the variable conditions of moisture content in DES system are 0, 10, 25, 50, 75 and 100%. The extraction temperature variables were 30, 40 and 50°C. The ratio of orange peel weight to DES volume was selected as 0.5:4, 1.0:4, 1.5:4 and 2.0:4. The extraction time variables were 2, 6, 12, 24, 36 and 48 h.

2.4.5 | Enzyme cascade reaction

In this study, an experimental device for in-situ extraction and enzymatic transformation of limonene in orange peel to prepare oxyfunctionalization products was designed (Figure 3). As shown in the

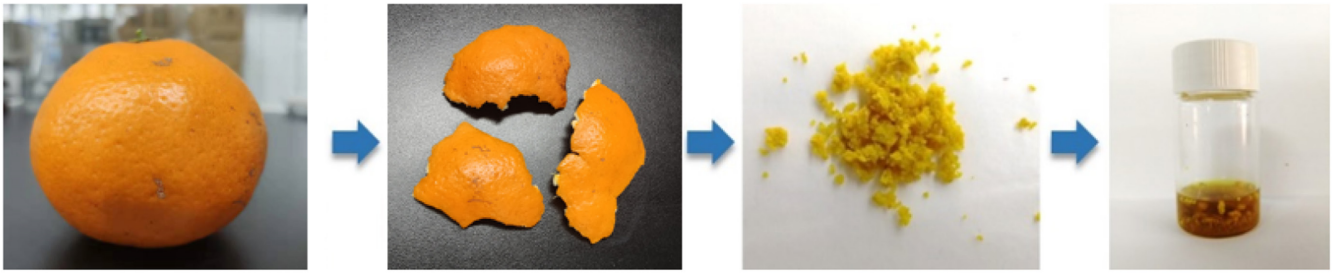


FIGURE 2 Pretreatment and extraction of orange peel

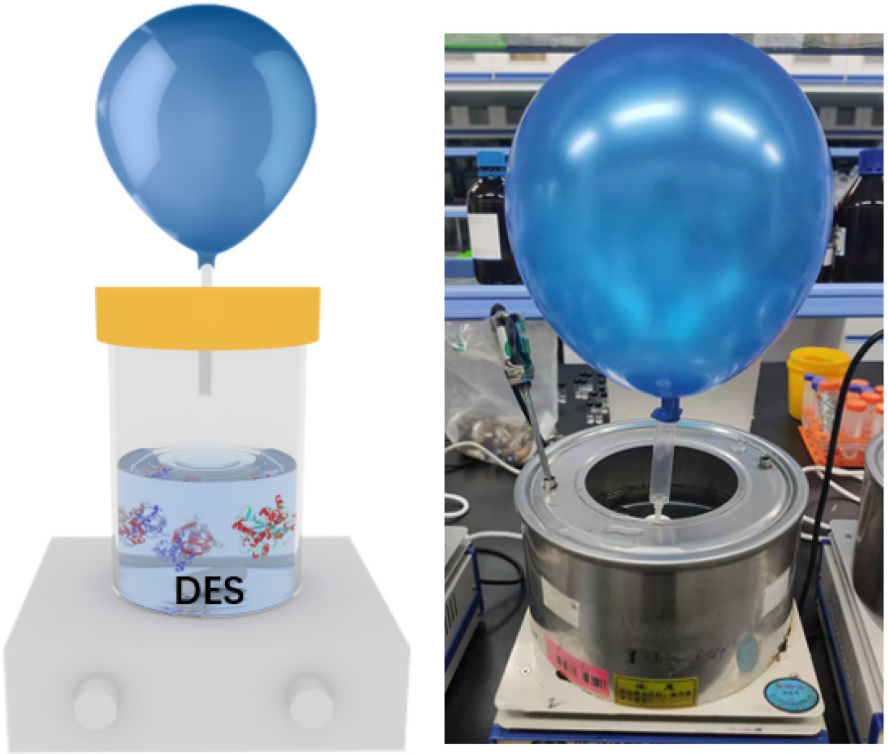


FIGURE 3 Image of homemade one-pot biocatalytic cascade reaction setup used in the experiments

figure, the reaction device was composed of a balloon providing O_2, a reaction glass bottle with spacer at the top, and a heat collecting constant temperature heating magnetic stirrer. All reactions were carried out in a 20 mL conical flask submerged in a thermostatic oil bath for temperature control. One-pot biocatalytic cascade reaction using and *An*ChOx and *Aae*UPO were performed at 40°C in 2 mL of DES and 2 mL sodium phosphate buffer (NaPi, pH 8, 50 mmol L^{-1}). Unless mentioned otherwise, 1 g orange peel were then added to the suspension. *An*ChOx and *Aae*UPO were then added to the suspension. At intervals, aliquots were withdrawn, extracted with n-hexane and analysed by gas chromatography.

3 | RESULTS AND DISCUSSION

3.1 | Selection of the DES

In a previous study we studied the extraction of limonene from OPW using 8 different DES.[10] Here, we evaluated these DES as possible reaction media for the envisioned bienzymatic transformation of limonene into carvol or carvone. As shown in Figure 4, ChCl-Pro-H_2O and ChCl-Sor excelled in enabling the selective oxyfunctionalization of limonene into the products of interest and a total of 1.5 and 1.6 mmol L^{-1} of limonene oxyfunctionalization

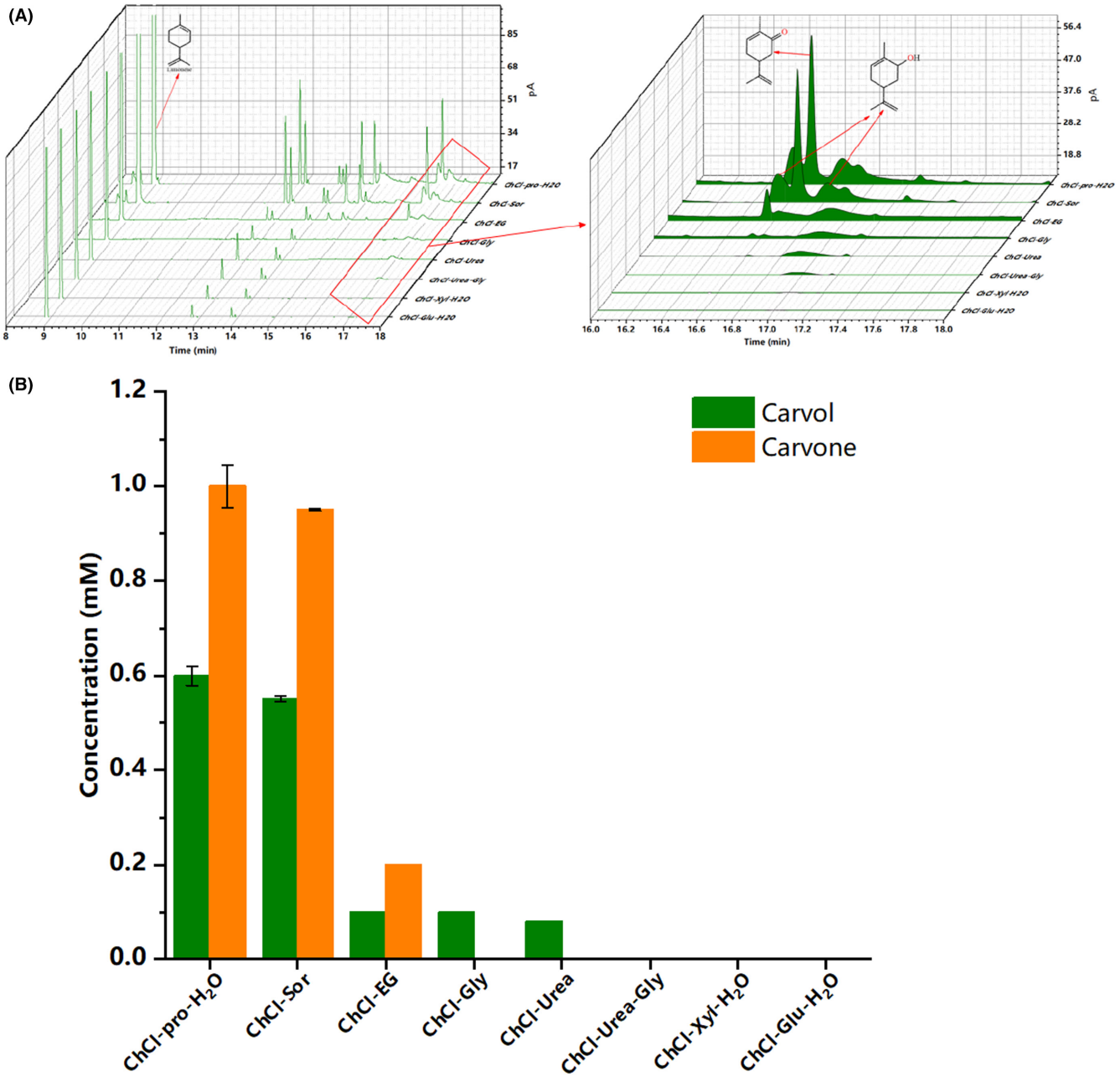


FIGURE 4 Effects of different components of DES on the cascade catalytic conversion of limonene to carvol and carvone ((A) gas chromatogram; (B) product quantitative analysis). Reaction conditions: orange peel (1 g) in a glass vial filled with DES system (4 mL) for reaction, *An*ChOx (5 μmol L^{-1}), *Aae*UPO (1 μmol L^{-1}), sodium phosphate buffer (NaPi, pH 8, 50 mmol L^{-1}), 40°C, 24 h, 500 rpm

products (conversion was about 3%) were observed in ChCl-Sor or ChCl-Pro-H_2O, respectively. It should be mentioned that the selectivity of the peroxygenase-catalysed oxyfunctionalization reaction was not exclusive (Figure 4) as also some epoxidation products and the corresponding epoxide hydrolysis products were observed. We are confident that more selective peroxygenase variants will enable more selective transformations.[24]

3.2 | Factors influencing the limonene extraction efficiency of DES from OPW

We decided to continue our investigation focusing on ChCl-Pro-H_2O as multi-purpose solvent. First, we investigated the influence of several process conditions on the efficiency of limonene extraction from OPW (Figure 5). The highest limonene extraction was observed using a water content of 50% (v/v) (Figure 5A). Especially at lower water contents the extraction efficiency was lower, which we attribute to the increasing viscosity of the extraction mixture and the resulting diffusion limitation.[31,32]

Another key factor that affects the extraction efficiency is temperature. It can be seen from Figure 5B that the extraction efficiency increases with increasing temperature. One plausible explanation for this observation may be that the viscosity of the extraction mixture decreases at increasing temperatures thereby accelerating diffusion. It should, however, also be kept in mind that too elevated temperatures may also negatively influence the stability of the biocatalysts. Furthermore, the solubility of O_2 (as a cosubstrates for the enzymatic conversion) also decreases with increasing temperature.

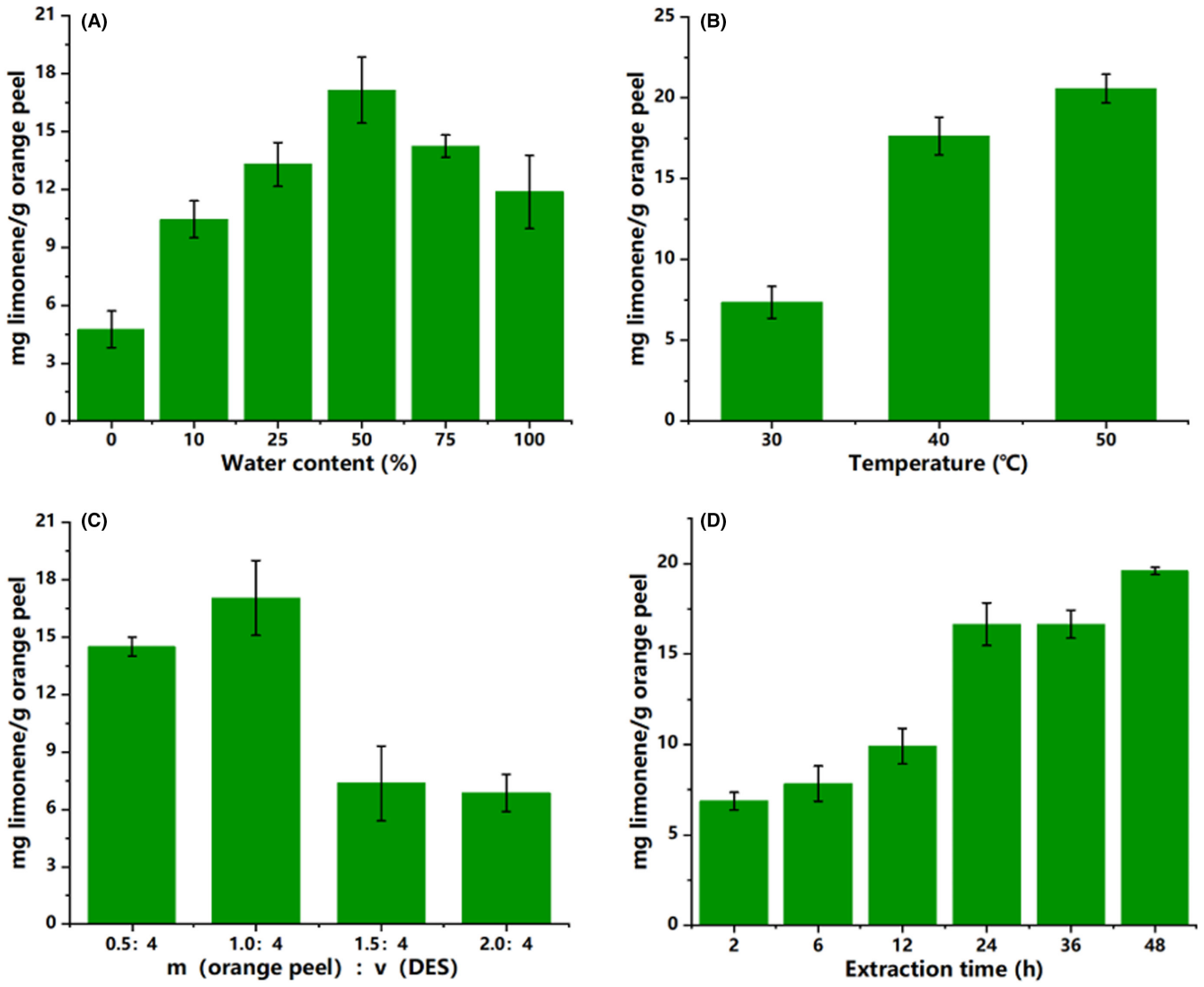


FIGURE 5 Effects of key factors on extraction of limonene from orange peels by ChCl-Pro-H_2O DES ((A) water content; (B) temperature; (C) ratio of orange peel weight to DES volume; (D) extraction time). Reaction conditions: according to the standard system described in 2.4.3, the variable conditions of water content in DES system are 0, 10, 25, 50, 75 and 100%; the extraction temperature variables were 30, 40 and 50°C; the ratio of orange peel weight to DES volume was selected as 0.5:4, 1.0:4, 1.5:4 and 2.0:4; the extraction time variables were 2, 6, 12, 24, 36 and 48 h, 500 rpm

Varying the ratio of OPW to DES, we found that a ratio of 25 (w/v) of OPW to DES gave the highest limonene extraction efficiency. It can be seen from Figure 5C that when using 4 mL DES extraction system, the optimal orange peel weight was 1 g. When the amount of orange peel is greater than 1 g, the extraction effect is significantly reduced. A possible reason is that when the amount of orange peel is too high, the volume of DES is too small, and the extraction system becomes very viscous, a large amount of orange peel adheres to it. On the extraction flask, the extraction and stirring are uneven or even difficult to stir at the same time, resulting in poor extraction effect.

Finally, the extraction time is also an important factor affecting the extraction efficiency. We confirmed that the extraction kinetics played a decisive role (Figure 5D). By examining the extraction time of the ChCl-Pro-H_2O DES system it can be seen that as the extraction time increases, the amount of extraction increases. However, when the extraction time is longer than 24 h, the extraction volume increases slowly, indicating that the extraction process is close to equilibrium. At this time, the extraction of limonene in orange peel was almost complete.

3.3 | The cascade of *An*ChOx and *Aae*UPO catalyses the conversion of limonene to carvol and carvone

To provide a proof-of-concept for the valorization of OPW-derived limonene (Scheme 1) we combined the ChCl-Pro-H_2O limonene extraction from orange peels with the bienzymatic oxyfunctionalization cascade (Figure 6). Pleasantly, 1.6 mmol L^{-1} of limonene oxidation products (0.6 mmol L^{-1} carvol and 1 mmol L^{-1} carvone) was obtained after 24 h (Figure 6). The product concentration increased steadily for 24 h after which no further product accumulation was observable; the final limonene conversion was approximately 3%. This rather poor conversion may be assigned to a poor stability of one of the biocatalysts under the reaction conditions chosen for this experiment. Particularly, the ratio of *An*ChOx to *Aae*UPO will be investigated further in future studies. Possibly, *An*ChOx produced more H_2O_2 than *Aae*UPO could convert. The resulting increasing levels of H_2O_2 may have irreversibly inactivated *Aae*UPO thereby resulting in relatively poor robustness of the overall process. We are convinced that balancing the *An*ChOx-catalysed H_2O_2 generation with the *Aae*UPO-catalysed H_2O_2-consumption will result in more robust processes and will enable full conversion of the OPW-derived limonene.

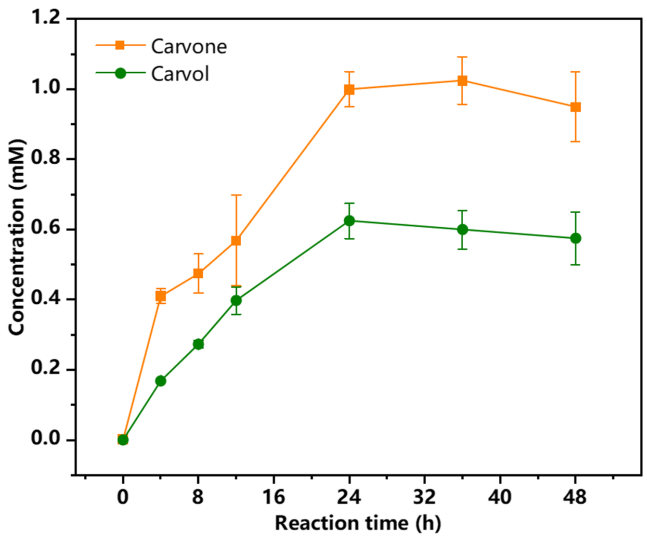


FIGURE 6 Time course of cascade catalytic conversion of limonene into carvol and carvone (Reaction conditions: orange peel (1 g) in a glass vial filled with ChCl-Pro-H_2O DES system (4 mL) for reaction, *An*ChOx (5 μmol L^{-1}), *Aae*UPO (1 μmol L^{-1}), sodium phosphate buffer (NaPi, pH 8.0, 50 mmol L^{-1}), 40°C, 500 rpm)

4 | CONCLUSION

In this study, we evaluated the influence of DES content, extraction temperature and time on the extraction effect. On this basis, we further studied the use of choline oxidase and unspecific peroxygenase cascades to catalyse the conversion of limonene into carvone and carvone. In general, DES, as an in-situ extraction solvent and enzyme-catalysed reaction medium, has shown satisfactory results in both aspects.

Overall, we have demonstrated a new enzymatic cascade approach to oxidize the natural limonene readily to carvol and carvone. The use of DES allows for efficient in-situ extraction of limonene from waste orange peel. Next to the roles of DES as cosubstrate for H_2O_2 generation and reaction reservoir, this study showed a biocatalytic transformation pathway for the high-value utilization of waste in orange peel. Participation of the reaction solvent as a cosubstrate has been proven a robust approach in biocatalytic reactions.[33,34]

Admittedly, there are still many questions worthy of our in-depth study, such as the key rate-limiting steps of enzymatic cascade catalysis and the stability of enzymes during the reaction. These aspects are currently being explored in our laboratory.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

DATA AVAILABILITY STATEMENT

All data are available from the corresponding author upon reasonable request.

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