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Chemoenzymatic Epoxidation of Terpenes by Lyophilized Mycelium of Psychrophilic *Cladosporium cladosporioides* 01

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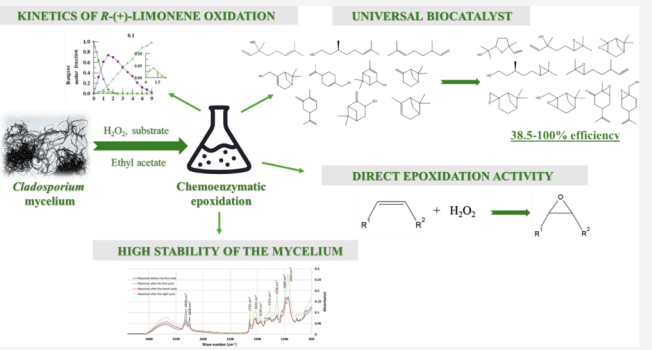
Cite This: *Ind. Eng. Chem. Res.* 2024, 63, 17790−17802

ABSTRACT: Terpene epoxides constitute a group of compounds of particular importance due to their biological activity or use in the production of polymers. It is important to develop a universal, inexpensive, and sustainable method to obtain these compounds from readily available terpenes. Chemoenzymatic epoxidation by the freeze-dried mycelium of *Cladosporium cladosporioides*01 is an alternative to processes based on the well-known and expensive lipase B from*Candida antarctica*. In the present work, we studied the kinetics of limonene epoxidation by the fungal biocatalyst and found the highest epoxidation activity in the "green" solvent ethyl acetate with a 4-fold excess of H_2O_2 relative to the substrate. Epoxidation followed a two-way sequential mechanism in which the major initial product limonene 1,2-epoxide and the minor intermediate product limonene 8,9-epoxide were further converted to limonene diepoxide. Substrate specificity studies revealed that a wide number of linear and cyclic monoterpenes, e.g., linalool, citronellene, citronellal, α-pinene, β-pinene, myrtenol, and perillyl alcohol, were efficiently converted to their respective epoxides by this biocatalyst. The biocatalyst may show activity in the direct oxidation of a double bond without the use of a peracid. The freeze-dried mycelium exhibited higher biocatalytic activity after defatting and high stability in ethyl acetate and in the presence of an oxidant. No decrease in its activity was observed after eight biocatalytic cycles. Therefore, the mycelium can be successfully used for the sustainable large-scale production of terpene epoxides.



INTRODUCTION

Terpenes are a readily available group of compounds with many applications in both industry and everyday life. Large quantities of hydrocarbon monoterpenes are obtained from waste biomass from the forestry (a source of α- and β-pinene) and from agricultural (a source of limonene) industries.[1] Turpentine (containing 70.4% α-pinene and 20.5% β-pinene[2]) is the most common byproduct of kraft paper pulping, making it the most important renewable monoterpene resource. Its annual output is estimated to be 230,000 metric tonnes of crude sulfate turpentine (CST). Additionally, approximately 100,000 tonnes of gum turpentine is produced annually from the distillation of resin from forest trees.[1,3] In turn, the main source of limonene is a pomace from citrus juice production. The annual production of limonene is approximately 70,000 tons per year.[4] These readily available and inexpensive terpenes are important resources with a variety of uses in pharmacy, perfumes, and flavorings.[5] Bearing in mind the availability, terpenes could be regarded as good substrates for the preparation of value-added chemicals, of which epoxides are particularly important.[6]

Epoxides are used to produce cyclic carbonates or monomers through ring-opening (ROP)[7] and ring-opening copolymerization (ROCOP) reactions, leading to polyethers, polyesters and polycarbonates.[8] Copolymerization of 1,2-limonene epoxide with carbon dioxide produces poly(limonene carbonate). This biodegradable polymer has remarkable hardness, transparency, and heat resistance.[9] In turn, the isomerization of α-pinene epoxide can be used to produce (+)-campholenic aldehyde, a crucial component in the fragrance industry.[10,11] Moreover, terpene epoxides can be used in the pharmaceutical industry as active ingredients in drugs due to their proven anticancer activity against human osteosarcoma cells (MG-63) ((−)-isopulgeol epoxide)[12] or anticonvulsant activity (epoxy-carvone).[13] Therefore, it is important to develop a universal and efficient method for the epoxidation of various terpene compounds.

Among the known epoxidation methods, chemoenzymatic epoxidation using lipases is of particular interest due to the

Received: June 14, 2024
Revised: September 20, 2024
Accepted: October 8, 2024
Published: October 11, 2024

https://doi.org/10.1021/acs.iecr.4c02228
Ind. Eng. Chem. Res. 2024, 63, 17790−17802

environmentally friendly conditions used for this transformation, such as moderate temperature, atmospheric pressure and a "green" solvent like ethyl acetate. In this process, lipases utilize their perhydrolase capacity to catalyze the conversion of acids into peracids, which then chemically oxidize the double bond in the substrate.[14−17] Direct epoxidation of the double bond in an α,β-unsaturated aldehyde by a single-point mutant of lipase B from *Candida antarctica* (CalB) in the presence of H_2O_2 in acetonitrile or phosphate buffer is also known.[18] In addition to CalB, other commercial biocatalysts have been used in chemoenzymatic epoxidation of citronellol and β-caryophyllene, such as lipase from*Burkholderia cepacia*,*Rhizopus oryzae*,*Mucor miehei*,*Candida rugosa*, *Pseudomonas fluorescens*,*Aspergillus niger*and*Mucor javanicus*.[19,20] The activity in the epoxidation reaction of the above lipases is relatively low, and in practice, the only biocatalyst used in this reaction is CalB in free or immobilized form (Novozyme-435). This lipase has a high catalytic activity although its major drawbacks are the high cost of enzyme preparation, the need for immobilization for reusability, sensitivity to H_2O_2, and relatively low operational stability.[21,22] For these reasons, a search for new biocatalysts for the chemoenzymatic epoxidation of alkenes needs to be undertaken.

Previous studies have shown that freeze-dried*Cladosporium cladosporioides*01 mycelium is an efficient biocatalyst for the chemoenzymatic epoxidation of limonene to 1,2-limonene oxide and limonene diepoxide. We have optimized the process and showed that with this biocatalyst the epoxidation is cost-effective due to its low price.[16] As a follow-up of the study, it was decided to determine the kinetics of the chemoenzymatic epoxidation of limonene and the influence of both the amount of H_2O_2 and the type of solvent on the catalytic activity of the mycelium. This study also aimed to determine the operational stability of the fungal biocatalyst and its ability to mediate the epoxidation of different terpene substrates. Attempts were made to reduce the *E*-factor by reusing the solvent and mycelium.

EXPERIMENTAL SECTION

Chemicals. (*R*)-(+)-limonene (98%), linalool (97%), (*S*)-(−)-β-citronellol (≥98%), (+)-β-citronellene (≥98.5%), myrcene (≥90%), (+)-α-pinene (98%), (−)-β-pinene (99%), (+)-limonene oxide (97%), (1*R*)-(−)-myrtenol (95%), (+)-carvone (≥96%), (*S*)-(−)-perillaldehyde (≥92%), (*S*)-(−)-citronellal (96%), (−)-*trans*-pinocarveol (96%), (+)-*trans*-p-menth-2-ene (≥98.5%), (+)-p-menth-1-ene (≥97%), (*S*)-(−)-perillyl alcohol (96%), (*S*)-*cis*-verbenol (96%), (1*S*)-(−)-verbenone (94%), (−)-dihydrocarveol (≥95%), (*D*)-glucose (99.5%), $CaCO_3$ (≥99%), 14% boron trifluoride-methanol solution (BF_3 · MeOH) and yeast extract were obtained from Sigma-Aldrich, USA. Ethyl acetate, 1,4-dioxane, 1-propanol, 2-propanol, acetone, acetonitrile, benzene, chloroform, dichloromethane, dimethylacetamide (DMAC), dimethylformamide (DMF), ethanol, hexane, isooctane, methanol, *tert*-butyl methyl ether (MTBE), *N*-methyl-2-pyrrolidone (NMP), toluene, acetic acid, Tween 80, $MgSO_4$-$7H_2O$, $(NH_4)_2SO_4$ and K_2HPO_4 (purity >99%) were purchased from POCH, Poland. 30% H_2O_2 was obtained from CHEMPUR, Poland.

Biocatalyst Preparation. To obtain the biocatalyst for the chemoenzymatic epoxidation of (*R*)-(+)-limonene, the psychrophilic fungus *C. cladosporioides* 01 was cultured in 300 mL Erlenmeyer flasks containing 100 mL of liquid medium (olive oil 10 g/L, Tween 80 7 g/L, glucose 2.5 g/L, yeast extract 1 g/L, $(NH_4)_2SO_4$ 5 g/L, K_2HPO_4 1 g/L, $MgSO_4$-$7H_2O$ 0.2 g/L, and $CaCO_3$ 5 g/L, pH 6) for 4 days at 20 °C on a rotary shaker (150 rpm).[23] After completion of the culture, the mycelium was separated from the postculture liquid by vacuum filtration and washed with 150 mL of distilled water. The rinsed mycelium was frozen overnight at −20 °C and lyophilized for 48 h. Freeze-dried mycelium was milled and used for epoxidation.

Defatting the Mycelium. The obtained crude mycelium (14.7632 g) was placed in an Erlenmeyer flask (500 mL) and ethyl acetate (200 mL) was added. The obtained suspension was stirred at room temperature using magnetic stirrer for 16 h. Then, the mycelium was separated from the organic phase by a filtration and placed again in an Erlenmeyer flask (500 mL). Hexane (200 mL) was added to the flask and the obtained suspension was stirred at room temperature using magnetic stirrer for another 16 h. The mycelium was separated from the organic phase by filtration and left to dry in an open air for overnight.

General Chemoenzymatic Epoxidation Procedure. Chemoenzymatic epoxidation of terpenes was carried out in sealed 25 mL Erlenmeyer flasks. The substrates (58 μL; 71 mM) were dissolved in ethyl acetate (5 mL) containing lyophilized mycelium (287 mg), 30% H_2O_2 (164 μL; 319 mM) and acetic acid (88 μL). The reaction conditions were optimized in a previous work.[16] The reaction was performed for 3 h at 55 °C.

Kinetics Study. Reactions were carried out for 9 h for the systems with different quantitative ratios (*R*) of (*R*)-(+)-limonene to biocatalyst, as described in Table S1.

The values of the other reactants, ethyl acetate (5 mL), acetic acid (85 μL), and H_2O_2 (385 μL), remained constant. The limonene epoxidation course was tested after 0.5, 1, 1.5, 2, 2.5, 3, 3.5, 4, 5, 6, 7, 8, and 9 h of reaction.

Basic statistical parameters were determined for kinetic models. The symbols COD, corr, and MSC represent the coefficient of determination, correlation coefficient, and model selection criterion, respectively, of which the coefficient of determination is a measure of the total variance consistent with the model. The correlation coefficient provides information on how changes in one variable are related to changes in the other, and a standardized quantity called the model selection criterion relates the coefficient of determination to the number of parameters (coefficients were calculated using the PSI-Plot v. 10.5 & ProStat v. 6.5 program).

These coefficients are defined by the equations:

$$COD = \frac{\sum_{i=1}^{n} w_i^2 (y_i^{obs} - \bar{y}^{obs})^2 - \sum_{i=1}^{n} w_i^2 (y_i^{obs} - \bar{y}^{cal})^2}{\sum_{i=1}^{n} w_i^2 (y_i^{obs} - \bar{y}^{obs})^2} \quad (1)$$

$$corr = \frac{\sum_{i=1}^{n} w_i^2 (y_i^{obs} - \bar{y}^{obs})^2 (y_i^{cal} - \bar{y}^{cal})}{\left[\sum_{i=1}^{n} w_i^2 (y_i^{obs} - \bar{y}^{obs})^2 - \sum_{i=1}^{n} w_i^2 (y_i^{obs} - \bar{y}^{cal})^2\right]^{0.5}} \quad (2)$$

https://doi.org/10.1021/acs.iecr.4c02228
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$$MSC = \ln \frac{\sum_{i=1}^{n} w_i^2 (y_i^{obs} - \bar{y}^{obs})^2}{\sum_{i=1}^{n} w_i^2 (y_i^{obs} - y^{cal})^2} - \frac{2p}{n} \quad (3)$$

where w is the statistical weight, n is the number of experimental points, p is the number of parameters to be fitted, and the indices *obs*, $\overline{obs}$, *cal* and $\overline{cal}$ refer to the experimental magnitude, mean experimental values, calculated values, and mean calculated values, respectively.

The Effect of the Amount of Oxidant. Limonene epoxidation was carried out for 48 h in five systems containing different quantitative molar ratios (4:1, 2:1, 1:1, 1:2, 1:4) of substrate to H_2O_2 under constant conditions (temperature, 55 °C; ethyl acetate (5 mL), acetic acid (88 μL), limonene (58 μL), and biocatalyst (287 mg). Limonene epoxidation performance was tested after 0.5, 1, 1.5, 2, 2.5, 3, 4, 6, and 8 h.

Solvent Effect. Mycelium (287 mg) was weighed into glass flasks, and 5 mL of solvent was added. The following organic solvents were used: ethyl acetate, benzene, chloroform, *n*-hexane, 1-propanol, dichloromethane, MTBE, 2-propanol, 1,4-dioxane, methanol, acetone, toluene, DMF, NMP, DMAC, and acetonitrile. Then, 30% H_2O_2 (164 μL, 319 mM), acetic acid (88 μL, 309 mM), and limonene (58 μL, 71 mM) were added to each flask. The flasks were sealed with silicone stoppers and incubated at 55 °C for 3 h.

Epoxidation of Other Terpene Substrates. Mycelium (287 mg) was weighed into 25 mL glass flasks. Then, ethyl acetate (5 mL), H_2O_2 (164 μL), acetic acid (88 μL), and tested terpene (71 mM) were added. Linalool, (*S*)-(−)-*β*-citronellol, (+)-*β*-citronellene, myrcene, (+)-*α*-pinene, (−)-*β*-pinene, (1*R*)-(−)-myrtenol, (+)-carvone, (*S*)-(−)-perillaldehyde, (*S*)-(−)-citronellal, (−)-*trans*-pinocarveol, (+)-*trans*-*p*-menth-2-ene, (+)-*p*-menth-1-ene, (*S*)-(−)-perillyl alcohol, (*S*)-*cis*-verbenol, (1*S*)-(−)-verbenone, and (−)-dihydrocarveol were used for epoxidation.

Recycling of Fungal Biocatalyst. Mycelium (287 mg) was weighed into 25 mL glass flasks. Then ethyl acetate (5 mL), H_2O_2 (164 μL), acetic acid (88 μL), and limonene (58 μL) were added. The flasks were sealed with silicone stoppers and incubated for 3 h at 55 °C. After the reaction was ceased, 25 μL of the reaction mixture was added to 225 μL ethyl acetate and analyzed by GC-MS. The remaining contents of the flasks were transferred to thimbles and centrifuged (10 min/10,000 rpm). After centrifugation, the supernatant was decanted, and the mycelium was rinsed twice with 10 mL of ethyl acetate. The supernatant was then centrifuged again (10 min/10,000 rpm) and discarded. The mycelium was suspended in 5 mL of ethyl acetate and transferred to reaction flasks. A fresh batch of reagents was then added, and the flasks were sealed and incubated again for 3 h at 55 °C. This procedure was repeated eight times. After each cycle, qualitative changes in the mycelia were examined using ATR-IR.

Efficiency of Limonene Epoxidation without the Addition of Acetic Acid. The reaction was carried out under standard conditions in ethyl acetate and toluene in the following systems: with and without acetic acid and with nondefatted and defatted biocatalysts. After 3 h, the samples were analyzed using GC−MS.

Study of the Peroxygenase Activity of the Fungal Biocatalyst. The reaction was carried out with and without a nondefatted or defatted biocatalyst under standard conditions without the addition of acetic acid. For the study of peroxygenase activity, acetic acid was not used in the reaction, and instead of ethyl acetate, 5 mL of phosphate buffer (10 mM, pH 7) containing 4%, 30% or 60% acetone was used as a solubilizer.[24] After 3 h, the samples were extracted with 5 mL of hexane. After phase separation, hexane was collected and the samples were analyzed by GC−MS.

Attenuated Total Reflection Infrared Spectroscopy. After each biocatalytic cycle, the dry mycelium was analyzed by attenuated total reflection infrared spectroscopy (ATR-IR) to examine the qualitative differences between the IR spectra of the biocatalysts. The spectra were recorded using a TENSOR 27 Bruker spectrometer equipped with a diamond onto which the tested mycelium was applied. The spectra were recorded in the wavenumber range of 4000−600 cm^{-1} with 60 scans per spectrum at a resolution of 1 cm^{-1}.

Analysis of Fatty Acid Composition in Mycelium. Ethyl acetate and hexane fractions obtained in mycelium defatting step were combined and evaporated under reduced pressure using rotary evaporator affording an oily residue (1.6172 g). The analysis of the fatty acid composition in the mycelium was carried out using the methylation process. Derivatization was performed by taking 5 mg of the tested sample, which was introduced into 1 mL of a $BF_3 \cdot MeOH$ solution. Then the vessel with the sample and reagent was placed in the thermostat at 50 °C for 30 min. After the derivatization process was completed, the vessel was cooled and 1 mL of distilled water and 5 mL of isooctane were added. The mixture was thoroughly mixed and then left to separate the phases. The upper layer was then taken and subjected to chromatographic analysis.

Qualitative and quantitative analyzes of methyl fatty acid derivatives were conducted using a gas chromatograph hyphenated with a triple quadruple tandem mass spectrometer detector (GCMS-TQ8040; Shimadzu, Kyoto, Japan). GC−MS conditions were as follows: capillary column: Zebron ZB5-MSi (30 m x 0.25 mm i.d., 0.25 μm film thickness; Phenomenex, Torrance, CA, USA); carrier gas: helium (grade 5.0); flow rate: 1.5 mL/min; high-pressure injection mode: 250.0 kPa for 1.5 min; injector temperature: 325 C; injection volume: 1 μL; temperature program: initial temperature 60 °C held for 3 min, ramp at a rate of 12 °C/min to the temperature 300 °C and held for 15 min; tandem mass spectrometer operated with normalized electron energy of 70 eV; ion source temperature: 185 °C.

For qualitative and quantitative purposes the full scan mode with range 40−550 m/z was used. The content of fatty acids in the tested sample was determined using the area normalization method.

Analysis of Biocatalysis Efficiency. After the specified epoxidation time, 100 μL aliquots were taken from the reaction mixture, dried over anhydrous sodium sulfate, and diluted 10-fold with ethyl acetate. The samples were subjected to GC-FID and GC-MS analyses. GC-FID and GC-MS analyses of the limonene oxidation products were performed according to the method described earlier.[23] The chemoenzymatic epoxidation efficiency was expressed as the molar conversion (%) of terpene to the corresponding epoxides.

RESULTS

Kinetics Study. Two reaction variants were considered.

The first (a) and second (b) variants are for parallel consecutive reactions and they are the only ones viable (Figure 1, Figure 2). This can be seen in Figure 3 for substrate/biocatalyst ratios R = 0.1 and 0.2, where the maxima in the

https://doi.org/10.1021/acs.iecr.4c02228
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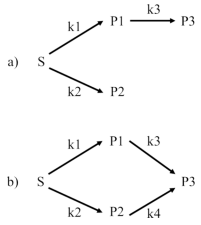

Figure 1. Possible transformations of limonene in chemoenzymatic epoxidation catalyzed by the mycelium of *C. cladosporioides*. S – substrate: limonene, P1–limonene 1,2-epoxide, P2–limonene 8,9-epoxide, P3–limonene diepoxide, k1, k2, k3, k4 - rate constants.

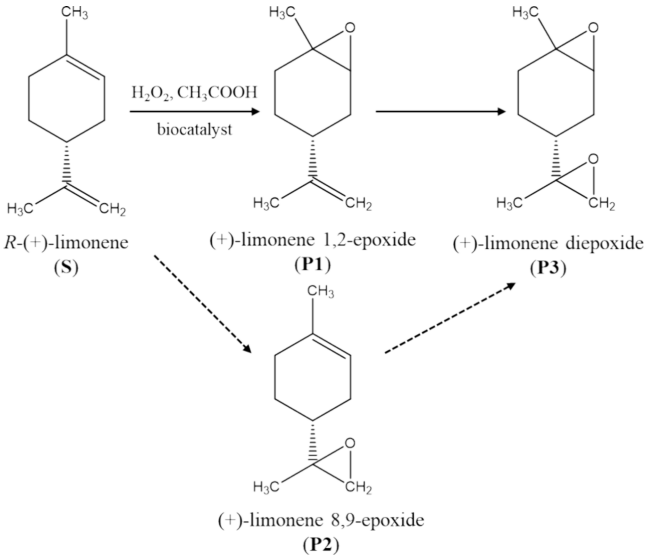

Figure 2. Reactants participating in the limonene transformation.

'run' of P2 and P1 concentration values over time are clearly visible. Thus, P2 may be transformed into P3. In contrast, the 'depletion' of substrate S and product P2 does not inhibit the increase in concentration of P3, so transformation of P1 into P3 must occur; this is confirmed by the decrease in concentration of P1 seen in Figure 3 after reaching a maximum at 1.5–2.5 h.

Due to the "depletion" of the substrate S, only two products P1 and P3 remain in the system and in relation to this system, the transformation of P1 into P3, the well-known first-order reaction model can therefore be applied according to the following:

$$\ln[P1] = -k3t + P1_0 \tag{4}$$

where $P1_0$ is the concentration of P1 at the beginning of the reaction, t is time. This is a rectilinear relation, and it is easy to see that it is also fulfilled for the substrate S, that is, for the relation ln[S] vs t. (Figure 3). The substrate transforms simultaneously into products P1 and P2, so one can only think that this transformation is a first-order reaction; however, it is impossible to separate the kinetics of the formation of P2 from P1. Let us now analyze the reaction kinetics in detail according to model (a) for higher values of the substrate/catalyst ratio R. Substrate S transforms into products P1 and P2 in two parallel reactions, and then product P1 transforms into P3. If we assume that, as in the case with R = 0.1 and 0.2, these are first-order reactions, then the course of their rate changes can be characterized by the following equations:

$$\frac{dS}{dt} = -k1[S] - k2[S] \tag{5}$$

$$\frac{dP1}{dt} = k1[S] - k3[P1] \tag{6}$$

$$\frac{dP2}{dt} = k2[S] \tag{7}$$

This is the case for the combination of parallel and consecutive reactions.[25] For parallel reactions, the change in substrate concentration S is given by the following equation:

$$[S] = [S_0]e^{-(k1+k2)t} \tag{8}$$

where $[S_0]$ is the substrate concentration S at the beginning of the reaction. All reactant concentrations are expressed in molar fractions, which, as is known, are equivalent to the ratio of the molar concentration of a given reactant to the sum of the molar concentrations of all reactants considered. At the start of the reaction, the only reactant was substrate S; hence, $[S_0] = 1$.

Equation 6 ultimately takes the form:

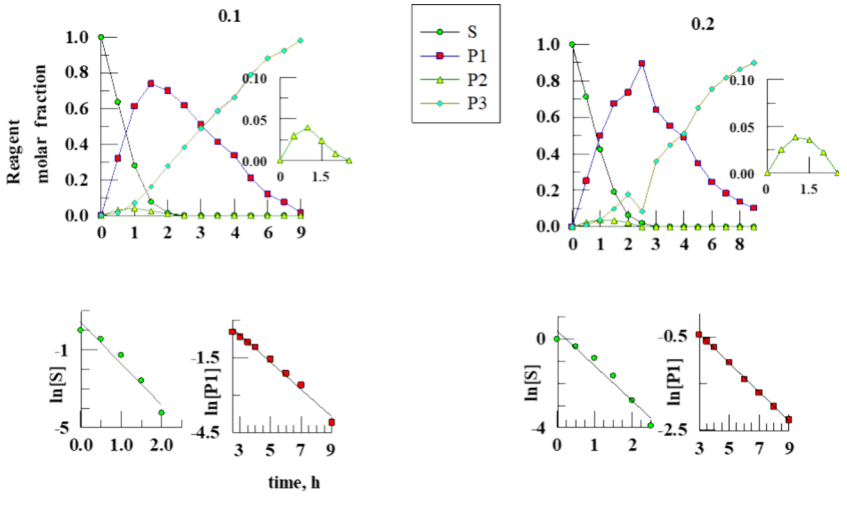

Figure 3. Change in the concentrations of S, P1, P2, P3 during the oxidation of limonene (R = 0.1, 0.2).

https://doi.org/10.1021/acs.iecr.4c02228
Ind. Eng. Chem. Res. 2024, 63, 17790–17802

Table 1. Values of the Reaction Rate Constants for the Oxidation of Limonene to 1,2-Epoxide, 8,9-Epoxide and Diepoxide along with Standard Deviations

	rate constants, [1/h]					statistical parameters of the model
R	model	k1	k2	k3	k4	
0.1	(a)			0.54 ± 0.026		COD = 0.986 corr = 0.993 MSC = 3.79
0.2	(a)			0.31 ± 0.004		COD = 0.999 corr = 0.999 MSC = 2.38
0.5	(a)	0.53 ± 0.015	0.006 ± 0.005	0.093 ± 0.003		COD = 0.986 corr = 0.996 MSC = 6.49
0.5	(b)	0.515 ± 0.016	0.032 ± 0.014	0.081 ± 0.007	0.466 ± 0.307	COD = 0.987 corr = 0.994 MSC = 4.20
0.75	(a)	0.307 ± 0.004	0.015 ± 0.002	0.039 ± 0.001		COD = 0.997 corr = 0.998 MSC = 5.71
0.75	(b)	0.290 ± 0.003	0.038 ± 0.003	0.020 ± 0.002	0.329 ± 0.003	COD = 0.998 corr = 0.999 MSC = 6.312
1	(a)	0.190 ± 0.003	0.014 ± 0.002	0.028 ± 0.003		COD = 0.994 corr = 0.997 MSC-4.981
1.5	(a)	0.16 ± 0.001	0.011 ± 0.001	0.024 ± 0.001		COD = 0.998 corr = 0.999 MSC = 6.92
1.5	(b)	0.151 ± 0.001	0.020 ± 0.001	0.009 ± 0.002	0.204 ± 0.025	COD = 0.999 corr = 1 MSC = 7.628
2	(a)	0.104 ± 0.001	0.009 ± 0.001	0.019 ± 0.002		COD = 0.999 corr = 1 MSC = 7.065

$$\frac{d[P1]}{dt} + k3[P1] = k1e^{-(k1+k2)t} \tag{9}$$

Multiplication of both sides of this equation by the integral factor e^{k3t} gives

$$\frac{e^{k3t}d[P1]}{dt} + k3[P1]e^{k3t} = k1e^{(k3-k1-k2)t} \tag{10}$$

The left-hand side of the equation is a derivative of the product and therefore

$$\frac{d([P1]e^{k3t})}{dt} = k1e^{(k3-k1-k2)t} \tag{11}$$

Integration of both sides of this equation according to

$$\int d([P1]e^{k3t}) = \int k1e^{(k3-k1-k2)t}dt \tag{12}$$

gives:

$$[P1]e^{k3t} = \frac{k1}{k3-k1-k2}e^{(k3-k1-k2)t} + c1 \tag{13}$$

where the integration constant $c1$ for the condition $[P1] = 0$ and $t = 0$ is defined by the equation:

$$c1 = \frac{-k1}{k3-k1-k2} \tag{14}$$

The concentration [P1] is ultimately governed by the equation:

$$[P1] = \frac{k1}{k3-k1-k2}e^{-(k1+k2)t} + c1e^{-k3t} \tag{15}$$

The concentration of P2 after considering that $[S_0] = 1$ is given by the equation:

$$[P2] = \frac{k2}{(k1+k2)}[1 - e^{-(k1+k2)t}] \tag{16}$$

which is consistent with the parallel reaction model (a).

For model (b) reactions by analogy with eq 15, the concentration [P2] and constant c2 are described by

$$[P2] = \frac{k2}{k4-k1-k2}e^{-(k1+k2)t} + c2e^{-k4t} \tag{17}$$

$$c2 = \frac{-k2}{k4-k1-k2} \tag{18}$$

The concentration [P3] for reaction runs (a) and (b) can be calculated from the relationship:

$$[P3] = 1 - [P2] - [P1] - [S] \tag{19}$$

Based on eqs 8, 15, 16, 17, and 19, the rate constants for the different reaction steps were calculated (Table 1) using the PSI PLOT calculation program. The changes in reactant

https://doi.org/10.1021/acs.iecr.4c02228
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concentration during the limonene oxidation reaction are shown in Figure 4 and Figure 5.

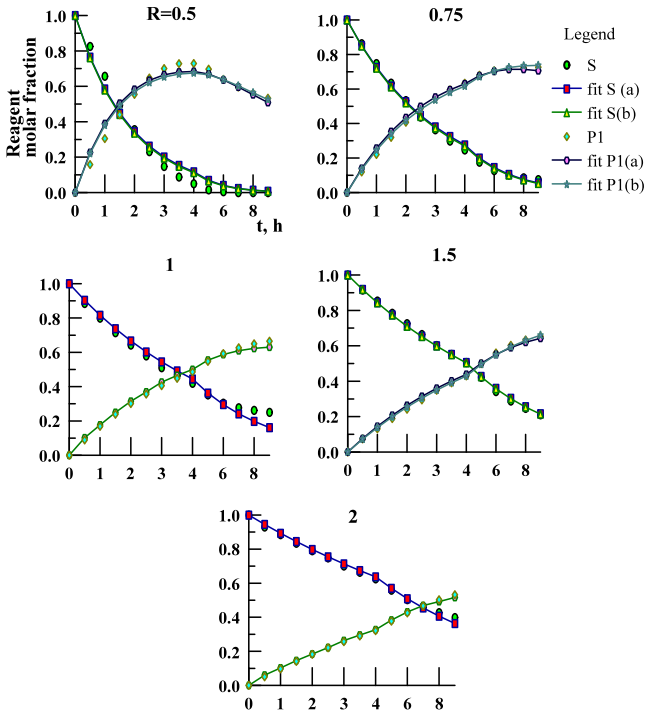

Figure 4. Change in concentrations of S and P1 during the oxidation of limonene.

For most of the tested systems, the COD determination coefficient reached a value above 0.99, which indicates a good fit of models (a) and (b) to the experimental data (Table 1).

The Effect of the Amount of Oxidant. At a 4:1 ratio of substrate to oxidant, the amount of limonene 1,2-epoxide (P1) remained constant for 8 h. Under these conditions, limonene was oxidized for less than 5% (Figure 6). At 2:1 ratio, the amount of substrate in the analyzed samples decreased to 85% after 8 h. P1 was formed with 9−12% conversion and its concentration did not increase with time. No diepoxide (P3) was formed although limonene 8,9-epoxide (P2) began to appear. At 1:1 ratio, the amount of limonene decreased more significantly and continued for 4 h yielding 26% of P1 and 4% of P2. At 1:2 ratio, there was a steady decrease in the concentration of limonene up to 4 h in favor of P1 whose concentration reached almost 63%. During this time frame, about 8% of P2 and 4% of P3 were formed successively. At 1:4 limonene:oxidant ratio, the substrate was completely converted within 3h. During the first hour of reaction there was a strong increase in the concentration of P1 (up to 50%), while a minor amount of P2 was also formed. The highest concentration of P1 was observed after 2 h after which a significant increase in the concentration of P3 occurred owing to the epoxidation of P1 and P2.

Solvent Effect. The effect of solvents was studied using a substrate:H_2O_2 ratio of 1:4. The results showed that ethyl acetate is by far the most efficient solvent (Figure 7). In agreement with the results depicted in Figure 6E, limonene is converted almost completely to epoxide P1 and diepoxide P3. After 3 h of reaction, 26% of the diepoxide is formed. A dramatic difference is observed in comparison with other solvents where much lower product yields than with ethyl acetate were obtained. Apart from ethyl acetate, benzene and toluene appeared as alternative solvents for the epoxidation reaction. In benzene, about 27% of 1,2-epoxide, 3% of 8,9-epoxide, and a trace amount of diepoxide were formed. In toluene, on the other hand, 20% of 1,2-epoxide, 2.5% of 8,9-

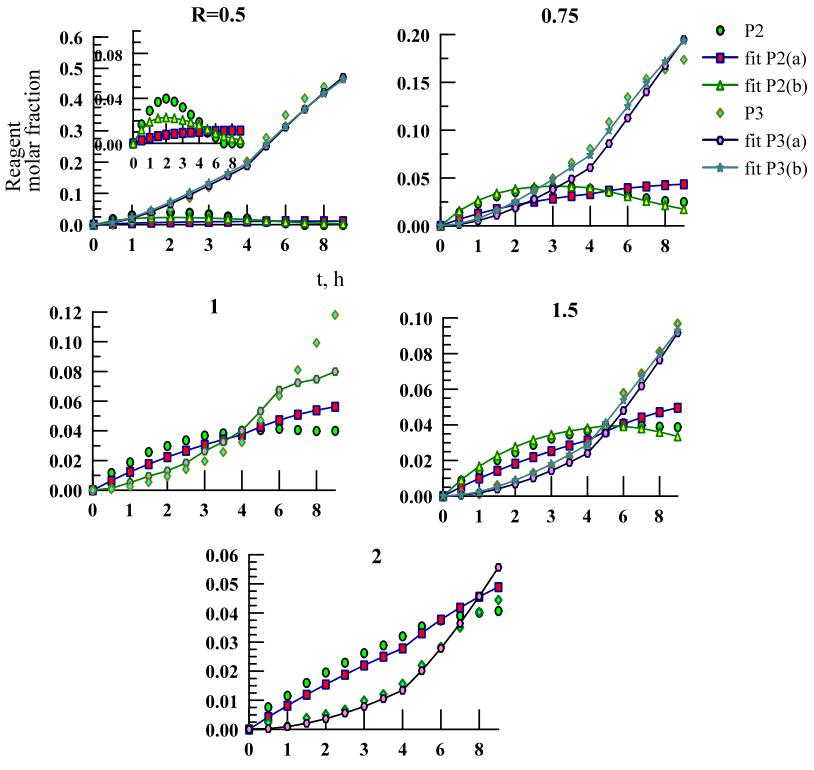

Figure 5. Change in concentrations of P2 and P3 during the oxidation of limonene.

https://doi.org/10.1021/acs.iecr.4c02228
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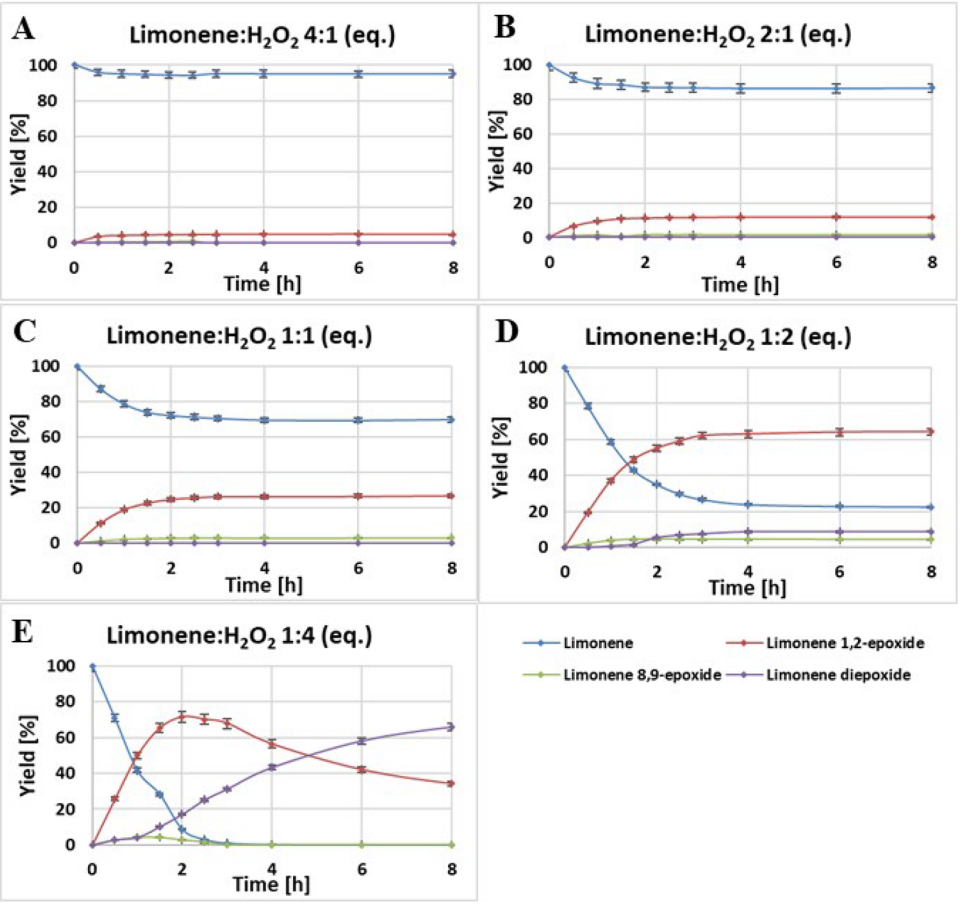

Figure 6. Effect of the molar ratio of substrate to H_2O_2 (A, 4:1, B, 2:1, C, 1:1, D, 1:2, E, 1:4) on the course of the chemoenzymatic epoxidation of limonene.

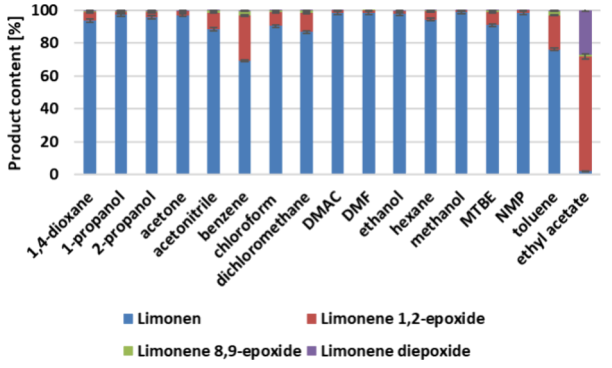

Figure 7. Effect of solvent type on the efficiency of the chemoenzymatic epoxidation of limonene.

epoxide, and a small amount of diepoxide (0.5%) were formed. In 1,4-dioxane, acetonitrile, chloroform, dichloromethane, hexane or MTBE, the reaction proceeds with low yield (4–11%). In the more polar solvents tested, i.e. 1-propanol, 2-propanol, acetone, DMAC, DMF, ethanol, methanol, and NMP, the reaction was very slow with trace amounts of products.

Recycling of the Biocatalyst. In the first biocatalytic cycle, 99.3% of limonene was oxidized to 1,2-epoxide (64%) and diepoxide (35.3%). In agreement with the above experiments, no 8,9-epoxide formation was observed (cf. Figure 6E). After the first incubation for 3h at 55 °C, the biocatalyst was already activated which resulted in an acceleration of the reaction in the second incubation affording quantitative conversion of limonene to 1,2-epoxide (57%) and diepoxide (43%). The increased biocatalytic activity of the *C. cladosporioides* 01 mycelium was maintained until the eighth incubation. In the last cycle studied, the oxidation of limonene was 100% and yielded 1,2-epoxide (59%) and diepoxide (41%). The total relative activity of mycelium after each cycle is presented in Figure 8.

After the first reaction cycle, a decrease in the intensity of the band with wavenumber 2920–2850 was visible in the infrared absorption spectrum. In the spectrum of the

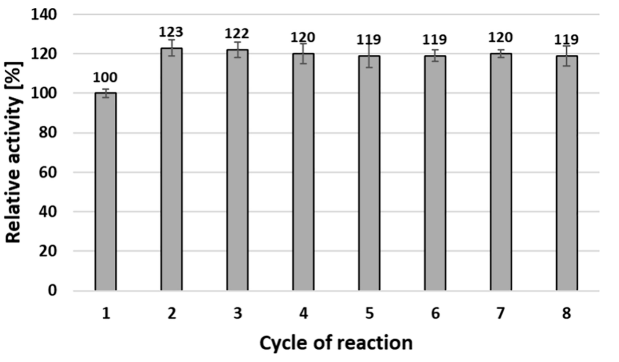

Figure 8. Reusability of*C. cladosporioides* 01 in a chemoenzymatic reaction cycle for the epoxidation of limonene

https://doi.org/10.1021/acs.iecr.4c02228
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biocatalyst after the eighth cycle, a clear increase in the intensity of the bands in the range from 1735 to 600 cm^{-1} was observed (Figure S1).

Analysis of Fatty Acid Composition of the Mycelium. During cultivation, the mycelium accumulated fatty acids and defatting with ethyl acetate and hexane allowed their content to be estimated at 11% (m/m) level. Among the fatty acids, 10-octadecenoic acid was the most abundant (74.37%) (Table 2). The mycelium also contained palmitic acid (11.78%), 9,12-octadecadienoic acid (6.05%) and stearic acid (6%). Behenic acid (1.03%), 18-methylnonadecanoic acid (0.51%) and 11-hexadecanoic acid (0.27%) were also present in small amounts.

Table 2. Analysis of the Composition of Fatty Acids Extracted from *C. cladosporioides* Mycelium

no.	retention time [min]	name of compounds*	relative conc. [%]
1	25.487	11-hexadecenoic acid, methyl ester C16:1	0.27
2	25.759	methyl palmitate C16:0	11.78
3	28.181	9,12-octadecadienoic acid, methyl ester C18:2	6.05
4	28.310	10-octadecenoic acid, methyl ester C18:1	74.37
5	28.572	methyl stearate C18:0	6.00
6	30.874	methyl 18-methylnonadecanoate C21:0	0.51
7	31.164	behenic acid, methyl ester C22:0	1.02

*Data from MS NIST base.

Epoxidation Efficiency without the Addition of Acetic Acid. In ethyl acetate, neither negative nor positive effects of acetic acid addition were observed. In the case of epoxidation using nondefatted mycelium, approximately 22% of the substrate remained after 3 h (Table S2). Defatting of the mycelium resulted in more efficient oxidation of limonene (96%). In the case of epoxidation in toluene, a slightly positive effect of acetic acid on the reaction was observed. With the addition of acetic acid, both with the nondefatted and defatted biocatalyst, the conversion of limonene into its epoxide was ca. 7%, whereas in the absence of acetic acid, the nondefatted biocatalyst performed less efficiently (conversion ca. 3%, which is equivalent to the autoxidation of limonene without a catalyst) than the defatted biocatalyst (conversion ca. 6%). None of the reaction systems were stereospecific as the *cis/trans* isomers of limonene 1,2-epoxide were formed in a 53:47 ratio.

Peroxygenase Activity of Fungal Biocatalyst. Under conditions promoting peroxygenase activity (water conditions with acetone as a solubilizer, without the addition of acids),[24] conversion of limonene to limonene oxide was 22% using nondefatted mycelium in a pH 7 phosphate buffer with 30% acetone. For defatted mycelium the conversion of limonene was remarkably lower under these conditions (10.6%). Epoxidation in buffers containing 4% and 60% acetone resulted in 3.6%−7.8% conversion (Tab. S3). In this case also none of the reaction systems were stereospecific.

Epoxidation of Other Terpene Substrates. We found that chemoenzymatic epoxidation using freeze-dried mycelium of *C. cladosporioides* 01 is an efficient method for various terpene compounds. The oxidation efficiency depended on the structure of the substrate molecule and, by applying a substrate to oxidant ratio of 1:4, ranged from 100% for *trans*-p-menth-1-ene, 92.8% for linalool, 90.1% for citronellol, 89.4% for myrcene, 78.3% for perillyl alcohol, 78% for α-pinene, 50.2% for citronellal, and 38.5% for carvone. The freeze-dried mycelium was not active with the bicyclic ketone verbenone. Detailed efficiencies of chemoenzymatic epoxidation of terpene compounds and main products are presented in Table 3. All chromatograms and mass spectra of oxidized products are included in Figures S2−S48.

Table 3. Oxidation Efficiency of Terpenes in Chemoenzymatic Epoxidation Catalyzed by Freeze-Dried Mycelium of *C. cladosporioides* 01

substrate	product yield (%)
linalool	linalool 3,6-oxide −21.1%
	6,7-epoxylinalool −71.7%
citronellol	citronellol epoxide −90.1%
citronellene	citronellene monoepoxide −89.3%
myrcene	myrcene monoepoxide −64.6%
	myrcene diepoxide −24.8%
α-pinene	α-pinene oxide −78%
β-pinene	β-pinene oxide −45.5%
myrtenol	myrtenol epoxide −81.5%
carvone	carvone epoxide −38.5%
citronellal	citronellal epoxide −50.2%
perillyl alcohol	perillyl alcohol monoepoxide −46.4%
	perillyl alcohol diepoxide −31.9%
werbenol	werbenol epoxide −77.8%
werbenone	0%
dihydrocarveol	dihydrocarveol epoxide −66.1%
pinocarveol	pinocarveol epoxide −39.7%
perillylaldehyde	perillyl aldehyde epoxide −53.2%
(+)-*trans*-p-menth-2-ene	2,3-epoxy-p-menthane −63.7%
(+)-*p*-menth-1-ene	1,2-epoxy menthane −100%

Epoxidation conditions: ethyl acetate, 5 mL; 30% H_2O_2, 164 μL; acetic acid, 88 μL; substrate, 71 mM; reaction time 3 h at 55 °C.

***E*-Factor Analysis.** Repeated use (8 cycles) of the biocatalyst (284 mg) as described in detail above for the conversion of limonene, resulted in the formation of product (limonene epoxides) (427.68 mg) with traces of unreacted substrate (0.161 mg). To achieve this level of conversion, 2047.2 mg of 30% H_2O_2 and 8000 mg of ethyl acetate were required which allowed us to estimate the *E*-factor as high as 24.1.

DISCUSSION

Terpene epoxides are valuable nature-derived renewable monomers for the synthesis of biodegradable polymers with thermal resistance, hardness and transparency.[26] Several types of enzymes, including cytochrome P450 monooxygenases, flavoprotein monooxygenases, heme peroxygenases and chloroperoxidases, are known to catalyze epoxidation reactions. However, these enzymes have limited practical applications due to the need for costly cofactors, their sensitivity toward high concentrations of H_2O_2, and/or the inability to sustain catalytic activity in organic solvents. The use of catalysts, such as lipases, is one method of addressing these issues.[27] Among the available catalysts for the chemoenzymatic epoxidation of limonene, the freeze-dried mycelium of *C. cladosporioides* 01 is particularly active in ethyl acetate.[16]

In the first stage of this study, the kinetics of the chemoenzymatic epoxidation of limonene by freeze-dried

https://doi.org/10.1021/acs.iecr.4c02228
Ind. Eng. Chem. Res. 2024, 63, 17790−17802

mycelia of *C. cladosporioides* 01 were determined for two possible reaction models, (a) and (b) (Figures 1 and 2). It can be seen from Figure 4 that the fit of models (a) and (b) to the data describing the changes in [S] and [P1] is almost identical, while Figure 5 shows that for values R = 0.5, 0.75, and 1.5, model (b) better describes the change in P2 product concentrations. For values R = 1 and R = 2, the change in P2 and P3 concentrations could only be described based on model (a), as model (b) obtained negative and therefore physically meaningless k4 values.

It can be seen that the reaction rates k1 and k4 for the oxidation of limonene decrease as the value of the substrate/catalyst ratio R increases. Moreover, for R = 0.5, 0.75, and 1.5, the k4 value is greater than k3, so the process of converting P2 to P3 occurs faster than the conversion of P1 to P3. Thus, the epoxidation of the double bond in the isopropenyl moiety of limonene is much more difficult than the epoxidation of the endocyclic double bond. For the same reason, the values of k1 and k4 were many times higher than those of k2 and k3.

It is worth mentioning here that, based on theoretical calculations, Jiang et al. (2010)[28] and Wang et al. (2023)[29] came to the conclusion about the preferred attachment of O_3 to the endocyclic double bond of limonene. Although this is a different system than the one presented in our work, the endocyclic double bond in limonene is undoubtedly more reactive toward oxygen from peracetic acid than the double bond in the isopropenyl group.

From the reaction models (a) and (b) and the experimental data, it can be concluded that both oxidation processes proceed simultaneously, except that the contribution of process (b) to the overall course of the oxidation reaction decreases as the value of the *R*-substrate/catalyst ratio increases. Thus, with a small amount of catalyst, the transformation of P2 into P3 is minimal, i.e. the reaction is limited only to the transition of S into P2 and, as a result, no clear maximum is observed in the course of P2 concentration changes for R = 1, 1.5, 2. It may also be due to H_2O_2 depletion and insufficient quantity in the reaction systems with high substrate concentrations. Overall, it can be concluded that under all experimental conditions tested, P2 never reaches molar fractions higher than 10%, which is consistent with the reaction rate constants collected in Table 1.

Studies performed with R = 0.2 showed that significant excess of the oxidant H_2O_2 is required to efficiently oxidize the limonene molecule. A 2-fold excess of peroxide relative to the substrate did not result in the complete oxidation of limonene after 4 h. In contrast, a 4-fold excess of the oxidant resulted in the complete oxidation of limonene after 3 h but further oxidation from 1,2-epoxide to diepoxide occurred at a lower rate which is consistent with the lower reaction rate constant k3 compared to k1. This might also to an certain extent be related to an insufficient amount of H_2O_2 due to its low stability at high reaction temperatures and its possible partial decomposition by other enzymes from the mycelium. Significant excess of H_2O_2 relative to the substrate for an effective chemoenzymatic reaction has also been described before. Zanette et al. (2014) used a 71-fold excess of H_2O_2 relative to oleic acid in chemoenzymatic epoxidation using CalB immobilized in microemulsion-based organogels.[30] In contrast, complete epoxidation of β-caryophyllene by CalB was possible with a more than 6-fold excess of H_2O_2.[19]

The reaction microenvironment, especially the type of organic solvent, is crucial for the catalytic activity of enzymes. *C. cladosporioides* 01 mycelia biocatalyst showed the highest epoxidation activity in ethyl acetate and was much less active in benzene and toluene. This demonstrates the narrow range of organic solvents that can be used on an industrial scale in processes using mycelia. Da Silva et al. (2013) carried out comprehensive studies on the effect of organic solvents on the activity of CalB in the chemoenzymatic epoxidation of β-caryophyllene.[19] Among 16 organic solvents (*n*-hexane, cyclohexane, toluene, chloroform, *t*-butanol, MTBE, dichloromethane, ethyl ether, ethyl acetate, THF, acetone, ethanol, acetonitrile, methanol, DMF, and DMSO), CalB showed low or no activity only in methanol, DMF, and DMSO. Furthermore, the solvent used clearly influenced the selectivity of the epoxidation toward mono- and diepoxide of β-caryophyllene, for example in toluene and ethyl acetate, oxidation of the substrate to diepoxide was achieved after only 8 h of reaction. The use of hexane, THF, and ethanol resulted in complete oxidation to monoepoxide after 8 h and no further oxidation to the diepoxide after 24 h. This indicates that it is necessary to determine the optimal solvent for a new biocatalyst to be used in epoxidation. The most commonly used solvent in chemoenzymatic epoxidation is toluene.[22,31,32] However, the high catalytic activity of freeze-dried *C. cladosporioides* 01 mycelium in ethyl acetate is advantageous because it has low toxicity, is environmentally friendly and can be described as a "green solvent",[33] unlike toluene.[34]

Freeze-dried mycelia biocatalyst from *C. cladosporioides* 01 is characterized by high stability in ethyl acetate at high temperature (55 °C) in the presence of H_2O_2 and acetic acid. This was evidenced by the sustained catalytic activity in eight consecutive biocatalytic cycles and slight changes in the mycelium ATR-IR spectrum. Compared to literature data, the mycelium of *C. cladosporioides* 01 is characterized by one of the highest stabilities in chemoenzymatic epoxidation reactions and can be successfully used to design a process in flow (even on the industrial scale) as supported by a preliminary cost analysis.[16] The Novozyme-435 biocatalyst (immobilized CalB) retained 90% of its initial chemoenzymatic activity for oleic acid epoxidation after seven biocatalytic cycles at room temperature.[35] The same Novozyme-435 biocatalyst was characterized by equally high stability in epoxidation using UHP (urea-H_2O_2) as an oxidant. After seven biocatalytic cycles, 90% of its initial activity was achieved. Interestingly, the use of H_2O_2 resulted in complete inhibition of the enzyme activity after the second biocatalytic cycle.[36] Bhalerao et al. (2018) reported that Novozyme-435 used in the ultrasound-assisted chemoenzymatic epoxidation of soybean oil retained only 35% of its initial activity after seven biocatalytic cycles.[22] Novozyme-435 used in α-pinene epoxidation in a two-phase reaction system retained 51% of its initial activity after seven biocatalytic cycles.[37] In contrast, the stability of Novozyme-435 in α-pinene epoxidation in a single-phase system is 57% after six biocatalytic cycles.[38] Nonimmobilized CalB retained only 30% of its initial activity after seven biocatalytic cycles in the chemoenzymatic epoxidation of oleic acid.[30]

Filamentous fungi have the ability to accumulate fatty acids during cultivation.[39,40] The mycelium of *C. cladosporioides* 01 accumulates 11% (m/m) fatty acids relative to its dry weight during cultivation. It appeared that removal of fatty acids from the mycelium was responsible for the increased chemoenzymatic epoxidation of limonene after the first biocatalytic cycle. This is supported by a decrease in the intensity of the 2920 cm^{-1} band in the ATR-IR spectrum after the first biocatalytic cycle, which is responsible for the stretching

https://doi.org/10.1021/acs.iecr.4c02228
Ind. Eng. Chem. Res. 2024, 63, 17790−17802

vibrations of the OH groups in acids (the band intensity changes in the range of 1735 cm^{-1} and 3100−3600 cm^{-1} are caused by the accumulation of acetic acid in subsequent biocatalytic cycles[41]). This was also confirmed by the higher reaction efficiency with mycelium after degreasing in hexane and ethyl acetate. This is due to the lack of competing reactions, i.e. transesterification of acids and epoxidation of unsaturated fatty acids catalyzed by the same enzyme. The presence of fatty acids in fungal biocatalysts has both advantages and disadvantages. It allows chemoenzymatic epoxidation to take place in toluene or benzene without the need to add an acid to the system but, on the other hand, it also produces a number of other byproducts that make the purification of the desired product difficult. The most rational approach seems to be the chemoenzymatic epoxidation of the fatty acids present in the mycelium in ethyl acetate to epoxy fatty acid esters and then using of mycelium for epoxidation of the desired compound in the second cycle. Epoxy fatty acid esters can be used as poly(lactic acid) plasticizers.[42]

To explain the influence of fatty acids on the efficiency of limonene epoxidation, a reaction was carried out with defatted and nondefatted mycelia, with or without the addition of acid, in ethyl acetate or toluene. The greatest effect of fatty acids was observed in ethyl acetate because the mycelium had highest catalytic activity in this solvent. The use of fatty acid contaminated mycelium resulted in a lower efficiency of limonene epoxidation by 18%. On the other hand, the absence of acetic acid did not lead to a reduction in the reaction rate which proves the hydrolysis of ethyl acetate to ethanol and acetic acid, which is then converted to peracid, used for chemical oxidation of limonene. In turn, in toluene, there was no difference in the efficiency of limonene epoxidation between degreased and nondegreased mycelia, provided that acetic acid was added. This demonstrates that fungal biocatalysts have a greater tendency to oxidize short-chain acids than long-chain acids during perhydrolysis. Epoxidation run with defatted mycelia in toluene with no added acetic acid resulted in slightly lower activity compared to the acid-containing system. The occurrence of the chemoepoxidation reaction without an intermediate factor may indicate that the biocatalyst can oxidize the double bond directly with H_2O_2. Hydrolytic enzymes may be responsible for this activity. The involvement of unspecific peroxygenase (UPO) is less obvious due to their sensitivity to high concentrations of H_2O_2.[43] Svedendahl et al. (2008) reported the direct epoxidation of but-2-enal with H_2O_2 catalyzed by a single-point mutant of CalB. The yield of the reaction, carried out in acetonitrile for 17.5 days, was 44%.[18] The mycelium of*C. cladosporioides* 01 showed a direct epoxidation efficiency of limonene in toluene at a level of 6.5% after only 3 h.

To determine the involvement of peroxygenase activity, the reaction was performed in phosphate buffer with the addition of acetone as a solubilizing agent. The optimal concentration of the solubilizing agent was 30%. The presence of fatty acids in the mycelium accelerated the chemoenzymatic reaction by 11% compared to the reaction with defatted mycelium. This may indicate the perhydrolytic transformation of fatty acids into peracids which then chemically oxidize the limonene molecule. Importantly, it was about twice as fast in a buffer containing 30% acetone as in toluene alone. This reaction is not stereospecific and gives two stereoisomers of limonene 1,2-epoxide (*cis* and *trans*). No other products were observed. Biondi et al. (2021) used oat seed peroxygenase for the stereospecific epoxidation of limonene in a phosphate buffer with *tert*-butyl hydroperoxide. After 2 h, the conversion of the substrate was 99% affording both (*R*)-(+)-limonene *trans*-1,2-epoxide (79%) and (+)-*trans*-carveol (15%).[44] The use of unspecific UPO from*Agrocybe aegerita* in the transformation of α-pinene resulted in a range of products—verbenol (44%), verbenone (9%), α-pinene oxide (1%), myrtenal (3%), γ-terpinene (1%) and p-cymene (1%).[45] The biotransformation of β-pinene by whole cells of *Chrysosporium pannorum* A-1, in turn, resulted in the production of *trans*-pinocarveol and remarkable amounts of other unidentified products.[46] Therefore, the best solution for exclusive formation of epoxides is to use a lipolytic catalyst.

Chemoenzymatic epoxidation using freeze-dried mycelium of*C. cladosporioides* 01 is a universal method for the oxidation of terpene compounds. The process of chemoenzymatic epoxidation of terpenes using mycelium as a biocatalyst is characterized by the same or even higher efficiency than systems using the well-known and commonly used CalB. After 8 h of reaction in ethyl acetate using CalB, the efficiency of citronellol epoxidation was 23%,[20] while using *C. cladosporioides* 01 mycelium it was 90% after 3 h. In turn, α-pinene oxidation in the presence of CalB resulted in an efficiency of 95% after 5.5 h of reaction,[36] whereas the mycelium oxidizes α-pinene with 78% efficiency within 3 h. To overcome the problem of inactivation of the expensive lipase enzyme in large-scale enzymatic epoxidation (due to the use of H_2O_2 and peracids at high concentrations), glutaraldehyde-cross-linked *Rhizopus oryzae* whole cells were used in chemoenzymatic epoxidation of α-pinene. After 6 h of reaction in ethyl acetate with stirring (180 rpm) at 30 °C, 41.2% conversion of the substrate to α-pinene oxide was achieved.[47] This is one of the few reports on the use of a biocatalyst in the form of whole mycelium cells for the chemoenzymatic epoxidation of terpenes. Unfortunately, the authors did not investigate the stability of the biocatalyst in subsequent biocatalytic cycles.

Compared to other chemoenzymatic epoxidation processes, the use of*C. cladosporioides* 01 mycelium has a low environmental impact. Reusing the mycelium eight times and recovering ethyl acetate resulted in an *E*-factor of 24.1. Single use of the mycelium gave an *E*-factor = 48 when the solvent was reused.[16] In chemoenzymatic epoxidation processes presented by other authors, the *E*-factor ranged from 32.8 to 349.6.[32,48−50] It should also be noted that these processes were carried out in toluene which has a negative impact on the environment. It is also worth paying attention to the production of the biocatalyst itself and its impact on the environment, and not only to the impact of the chemoenzymatic catalysis process. The *E*-factor in the case of obtaining a pure enzyme preparation is 38,8 $g_{waste} \cdot g_{enzyme}^{-1}$.[51] In the case of obtaining the biocatalyst in the form of lyophilized mycelium of *C. cladosporioides* 01, the *E*-factor is only 46.3 $g_{waste} \cdot g_{biocatalyst}^{-1}$ (per one liter of medium: biocatalyst, 22 g, waste, 1017.5 g (water, 985.8 g, Tween 80, 7 g, yeast extract, 1 g, glucose, 2.5 g, $(NH_4)_2SO_4$, 5 g, K_2HPO_4, 1 g, $MgSO_4-H_2O$, 0.2 g, olive oil, 10 g, $CaCO_3$, 5 g)). This proves that the negative impact on the environment is low both during the preparation of the biocatalyst and during prolonged (multiple-use) catalysis.

CONCLUSIONS

This study has provided insight into both theoretical and practical aspects of using a new lipolytic biocatalyst for terpene

https://doi.org/10.1021/acs.iecr.4c02228
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oxidation. Freeze-dried mycelium of*C. cladosporioides* 01 emerged as an efficient, inexpensive and universal biocatalyst for the chemoenzymatic epoxidation of terpenes. The mycelium showed the highest catalytic activity in ethyl acetate with a 4-fold excess of oxidant and was stable during epoxidation without loss of activity for up to eight biocatalytic cycles. The high stability of the mycelium during the process greatly enhances its potential for use on an industrial scale. Activation of the mycelium after the first biocatalytic cycle is caused by washing out the fatty acids and eliminating competitive reactions such as epoxidation of unsaturated fatty acids and their esterification. More insight into the molecular details of the chemoenzymatic oxidation mechanism will require the purification and characterization of the enzyme(s) from*C. cladosporioides* 01 involved in the efficient (perhydrolytic) epoxidation of terpenes.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acs.iecr.4c02228.

Quantitative ratios of limonene to biocatalyst (Table S1); comparison of the chemoenzymatic efficiency of limonene epoxidation depending on the type of mycelium used (defatted and nonfatted), the addition of acetic acid, and the type of solvent (ethyl acetate and toluene) (Table S2); comparison of limonene epoxidation efficiency depending on the type of mycelium used (defatted and nonfatted) and phosphate buffer with 4, 30, and 60% acetone content (Table S3); comparison of ATR-IR spectra of mycelium before the first catalytic cycle, after the first cycle, after the fourth cycle, and after the eight cycle (Figure S1); mass spectra of terpene oxidation products and ATR-IR spectra of the biocatalyst after biocatalytic cycles are included (PDF)

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Funding

No additional funding from outside the university was involved in the work.

Notes

The authors declare no competing financial interest.

■ ACKNOWLEDGMENTS

The authors would like to thank Maria Curie-Skłodowska University in Lublin, Poland, for providing institutional funds to support this work.

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