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ScienceDirect
Journal of Molecular Catalysis B: Enzymatic 51 (2008) 24–35
JOURNAL OF MOLECULAR CATALYSIS B: ENZYMATIC
www.elsevier.com/locate/molcatb

Enhanced catalytic performance and stability of chloroperoxidase from *Caldariomyces fumago* in surfactant free ternary water–organic solvent systems

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Received 7 May 2007; received in revised form 17 September 2007; accepted 11 October 2007
Available online 23 October 2007

Abstract

Surfactant free ternary systems formulated with organic solvents such as α-pinene or *n*-hexane, short chain alcohols and water, were used as new reaction media to maximize the catalytic performance of chloroperoxidase from *Caldariomyces fumago* (CPO) towards the oxidation of hydrophobic compounds including olefins and terpenols. The influence of the composition and the hydration state of the system, the nature of the oxidizing agent as well as substrate availability on the catalytic behavior and stability of the enzyme was investigated, using the chlorination of monochlorodimedone (MCD) and the epoxidation of styrene as model reactions. Based on residual chlorination activity measurements, which were in accordance with differential scanning calorimetry (DSC) findings, it was concluded that in low water systems formulated with α-pinene CPO stability is significantly increased compared to other reaction media (up to 65% residual activity after 24 h of incubation at 30 °C). Electron paramagnetic resonance (EPR) spectroscopy indicated that in low water content systems the ferric environment of the active site of CPO does not undergo severe modifications. Total turnover numbers (TTN) obtained for the oxidation of various olefins and terpenols in α-pinene-based systems, were up to 25,000 which is 2–170 times higher than previously reported values. The results indicate that such ternary systems can be efficiently employed as reaction media leading to highly enhanced stability and increase CPO potential as practical catalyst for oxidation of large amounts of hydrophobic compounds in a single-step biocatalytic process.

Keywords: Chloroperoxidase; Ternary systems; Surfactant free microemulsions; Stability; Oxidation

1. Introduction

Chloroperoxidase from *Caldariomyces fumago* (CPO; EC 1.11.1.10) is recognized as one of the most promising for synthetic applications heme peroxidases with respect to its broad substrate specificity and catalytic features, including reactions such as classical peroxidation, oxidative halogenation, epoxidation, enantioselective sulfoxidation, hydroxylation of aromatic compounds, as well as oxidation of dienes and alcohols [1–11]. The transformation of a broad spectrum of substrates by CPO leads to valuable compounds for the synthesis of biologically active molecules [12–14].

The versatile catalytic activities of CPO have attracted much interest in understanding the structural properties of the enzyme [15,16]. A key characteristic of CPO is the presence of a heme group at the active site [17], enabling the enzyme to catalyze various biotransformations using a cytochrome P450 monooxygenase-like mechanism in oxygen-transfer reactions [6,8,18]. The main difference between CPO and P450 monooxygenases is that the later use molecular oxygen as substrate and cofactor NAD(P)H that must be regenerated during the biotransformation process, thus limiting their industrial applications [6,8,19]. The heme center of CPO has been extensively studied by EPR spectroscopy techniques [20,21].

Despite the remarkable synthetic potentialities of CPO, its application in various biotransformation processes of industrial interest has been hindered by the frequent formation of side products, the low aqueous solubility of organic substrates, as

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doi:10.1016/j.molcatb.2007.10.006

well as by its limited stability due to inactivation by hydrogen peroxide [8,22–24] or other factors [25,26]. Moreover, many chloroperoxidase biotransformations involve both hydrophobic substrates and hydrophilic oxidizing agent (such as H_2O_2) that are insoluble in the same reaction system.

The solubility of substrates was improved by performing the CPO-catalyzed biotransformations in aqueous co-solvent mixtures [2,14,27–29] as well as in various macroheterogeneous biphasic systems, such as liquid–liquid systems composed of a water immiscible organic solvent and water [30–32]. In addition, nearly anhydrous systems where the enzyme is usually suspended as a powder or added after immobilization onto a suitable carrier in various low water media, have been used [27,33–38], but the catalytic efficiency and selectivity remained low.

Alternatively, microheterogeneous reaction systems, such as water-in-oil (w/o) microemulsions formulated with various surfactant molecules, have been used as media for the CPO-catalyzed transformation of hydrophobic substrates [39,40]. However, larger scale application of these enzyme-containing w/o microemulsions may be considerably hindered by the necessity for separating the surfactant from the enzyme and the reaction products. These difficulties may be avoided by using ternary systems consisting of a hydrocarbon, a short chain alcohol and water. These so-called surfactant free microemulsion-like systems tend to form stable and optically transparent dispersions of aqueous microdroplets or aggregates in the hydrocarbon solvent, that provide a microheterogeneous medium, into which both hydrophobic and hydrophilic substrates may be enzymatically transformed [41–44]. The phase behavior and the physical properties of such systems have been investigated by conductivity measurements or using spectroscopic and ultracentrifugation techniques [44,45].

In the present contribution, we report the use of surfactant free ternary systems as new reaction media for CPO-mediated oxidations involving both hydrophobic and hydrophilic substrates. The reaction media used included ternary mixtures of *n*-hexane, short chain alcohols and water, as well as a novel reaction system, where *n*-hexane was replaced by α-pinene, a natural compound that is the main component of several essential oils [46]. α-Pinene-containing systems were used in the present study so as to explore the performance of CPO in an essential-oil simulated environment. This approach could enrich CPO applications spectrum with the transformation of biologically active compounds, such as terpenols found in essential oils, in a natural-like environment, and overcome drawbacks usually associated with the application of conventional chemical methods or organic solvents [14].

The effect of various reaction parameters such as composition, and water content of the systems, nature of the oxidizing agent as well as substrate availability on enzyme activity and stability was investigated. Optimum bioconversion conditions were applied to improve total turnover numbers (TTN, defined as the amount of product formed over the catalytic lifetime of the enzyme), in CPO-containing ternary systems with respect to the oxyfunctionalization of linear and cyclic olefins as well as the oxidation of terpenols to the corresponding aldehydes.

2. Experimental

2.1. Materials

Chloroperoxidase (EC 1.11.1.10) from *C. fumago* and monochlorodimedone (1,1-dimethyl-4-chloro-3,5-cyclohexanedione, MCD) were purchased from Sigma Chemicals (USA). CPO activity was determined at 25 °C using the method described by Morris and Hager [47]. One unit of CPO catalyzed the conversion of 1.0 μmole of MCD to dichlorodimedone (DCD) per minute at the above experimental conditions. The enzyme preparation had an activity of approximately 21.500 U ml^{-1}. The molarity of CPO solutions was determined spectrophotometrically using a molar absorption coefficient (ε_{400}) of 91.2 mM^{-1} cm^{-1} [27,48].

t-Butyl hydroperoxide (*t*-BHP) either as an aqueous solution (70%, v/v) or as a solution in *n*-decane were from Aldrich (USA). Hydrogen peroxide (30%, w/w, aqueous solution), styrene, α-pinene, olefins, terpenols and the corresponding aldehydes as well as all other chemicals were of the highest purity commercially available.

2.2. Ternary phase diagrams

The ternary phase diagrams were constructed on the basis of isotropic properties of various compositions of the organic solvent (either *n*-hexane or α-pinene), alcohol (2-propanol or *t*-butanol) and water at 25 °C [44]. Solutions of organic solvent and alcohol were prepared, and then titrated with 100 mM citrate–phosphate buffer (pH 2.75) or 50 mM citrate–phosphate buffer (pH 5.0). Visual observations were made after each addition of water until phase separation, which determined the phase limiting line. The compositions of the ternary systems used for CPO-catalyzed reactions were chosen to correspond to the monophasic area (area B) of the phase diagram that is presented in Fig. 1.

2.3. Chlorination activity in ternary systems

The chlorinating activity of CPO was determined at 25 °C by monitoring the decrease in absorbance at 278 nm due to the conversion of MCD (ε_{278} = 12,200) to DCD [27,48]. After mixing the appropriate volumes of organic solvent and alcohol, the aqueous phase (100 mM citrate–phosphate buffer, pH 2.75), containing the enzyme (2–10 U), MCD (0.1 mM) and KCl (6.67 mM) was added. The final water content was adjusted by adding a suitable amount of the above-mentioned buffer. In all cases, the final volume of the reaction system was 1 ml. The mixture was shaken using a vortex until transparency achieved. The reaction was initiated with the addition of *t*-BHP (38.5 mM). Experiments were carried out in triplicate.

2.4. Epoxidation activity in ternary systems

Determination of epoxidation activity was based on the CPO-catalyzed oxidation of styrene into styrene oxide at 25 °C. The appropriate amounts of solvent and alcohol, were mixed with

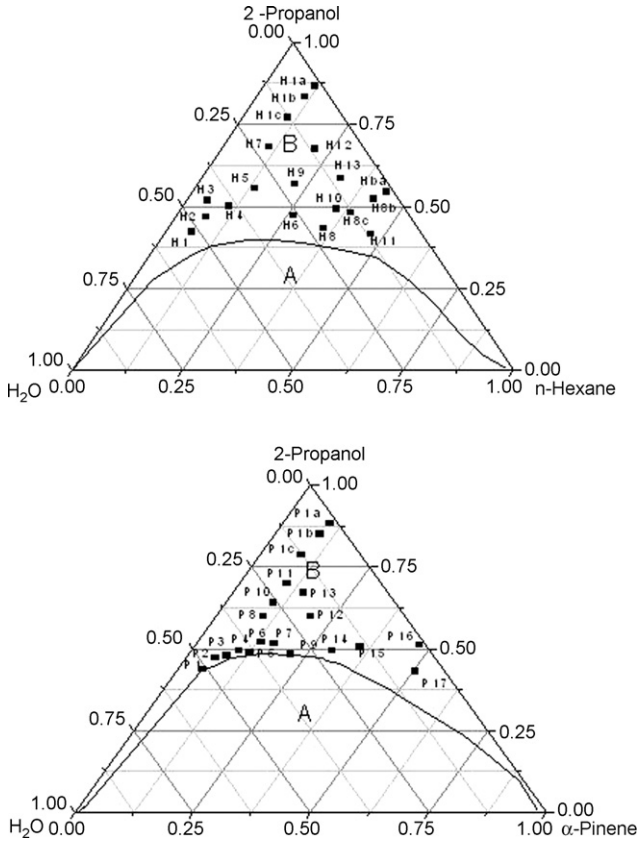


Fig. 1. Triangle phase diagrams of (a) *n*-hexane/2-propanol/water and (b) α-pinene/2-propanol/water given in molar fractions. Symbols (■) within region B correspond to monophasic ternary systems examined.

50 mM citrate–phosphate buffer (pH 5.0) containing 20 U of CPO resulting in a total volume of 1 ml. The final concentration of styrene was 200 mM. The enzymatic reaction was initiated by adding the oxidative agent *t*-BHP (200 mM). Samples were withdrawn at different time intervals and analyzed by gas chromatography (GC) as described. The GC chromatogram showed separated peaks for *R*- and *S*-styrene epoxide. Experiments were carried out in triplicate. Controls were performed for each reaction set in the absence of CPO or *t*-BHP.

2.5. CPO-catalyzed oxidation reactions

The ability of CPO to catalyze halide independent oxidations of hydrophobic substrates in ternary systems was examined using olefins such as 1-methyl-cyclohexene and *cis*-2-heptene, as well as terpenols (nerol, geraniol and perillyl alcohol). In a typical reaction, the substrate (50–200 mM) was added via a syringe into a septum-covered vial containing the appropriate amounts of organic solvent, alcohol and 50 mM citrate–phosphate buffer, pH 5.0 containing 140 U ml^{-1} of CPO. The reaction was initiated with the addition of *t*-BHP (concentration equal to that of substrate) and the reaction mixture was incubated at 25 °C. Samples were withdrawn periodically, extracted with dichloromethane and analyzed by GC or HPLC. Controls were performed in the absence of CPO or *t*-BHP.

Reaction products were isolated by column chromatography and preparative TLC in a manner similar to this described by Skouridou et al. [49], in 20–30% yield. Highly purified oxidized products were also obtained by semi-preparative HPLC. A Discovery C_{18} column (particle size 5 μm, length 250 mm, diameter 10 mm) was used. The elution was performed at 27 °C with acetonitrile at a flow rate of 4 ml min^{-1}. Products were detected at 220 or 240 nm. Each product-containing organic solvent aliquot was dried and analyzed by GC–MS. Identification of products was based on commercially available standards. The GC–MS apparatus was equipped with a split/splitless injector and a VF-5 ms column (Varian) was used for separation (30 m × 0.25 mm i.d., 0.25 μm film thickness). The temperature program for the GC-CP3900 interfaced with a Saturn 2000 MS. The initial oven temperature was 50 °C. After 4 min the temperature of the column increased at 230 °C (15 °C min^{-1} rate) and finally at 285 °C (10 °C min^{-1} rate). The total run time was 31.5 min. The control of the GC–MS system and the evaluation of the chromatograms received were carried out by means of MSWS Chromatography Software (Varian).

2.6. Analytical methods

Gas chromatography (GC) analysis was performed by a Shimatzu GC 17A device, which was equipped with a β-DEX™ 120, fused silica capillary column (30 m × 0.25 mm × 0.25 μm film thickness, Supelco) and a FID detector which was kept at 300 °C. Helium was the carrier gas used at a flow rate of 1.2 ml min^{-1}. Monitoring of styrene oxidation progress was performed using a temperature program where the initial temperature of the oven (100 °C) was kept constant for 30 s and then it was increased at a steady rate (15 °C min^{-1}) to a final temperature of 180 °C. Slightly modified conditions were applied for GC analysis concerning oxidation of olefins; the initial temperature of the oven was 80 °C, and it was kept constant for 1 min, after which it was increased at a steady rate (15 °C min^{-1}) to a final temperature of 180 °C. The reaction products were quantified on the basis of their response in relation to an internal standard (i.e. *n*-undecane).

HPLC analysis of terpenols and their aldehydes was performed by using a μBondapack C_{18} column, particle size 10 μm, length 300 mm, diameter 3.9 mm and a diode array UV detector. Linear gradient from 50 to 100% acetonitrile in water (containing 0.1% acetic acid) was employed for 22 min. The elution was performed at 27 °C with a flow rate of 1 ml min^{-1}. Calibration curves were constructed with different concentrations of terpenols and the corresponding aldehydes. Products were detected at 220 or 240 nm.

2.7. Enzyme stability

The effect of the water content on enzyme stability was investigated through comparison of systems where the aqueous phase ranged between 0.4 and 18.9% (v/v), and a constant alcohol/organic solvent ratio was maintained. After preparing the organic solvent and 2-propanol mixtures, the appropriate amount of 100 mM citrate–phosphate buffer, pH 2.75 containing 2–10 U

of CPO was added. Ternary mixtures were then incubated for different time intervals (0–72 h) at various temperatures ranging from 25 to 50 °C. The residual chlorination activity of CPO was measured after increasing the water content of the mixture with the addition of a solution of MCD (0.1 mM) and KCl (6.67 mM) prepared with the same buffer and *t*-BHP (38.5 mM), resulting in 1 ml final volume. Experiments were carried out in triplicate. The inactivation rate constants (k_d) of enzymes were calculated through first order inactivation kinetics [50].

In order to investigate the effect of substrate on CPO stability, 1 ml ternary mixtures containing the enzyme (20 U) and styrene (200 mM final concentration) were prepared. Following incubation of the mixtures at 25–50 °C the residual enzyme activity was measured. The reaction was initiated with the addition of *t*-BHP (200 mM). Samples were withdrawn periodically, and the amount of styrene and products was determined by GC as described.

2.8. Determination of kinetic parameters

Kinetic studies for enzyme performance in ternary systems were based on the CPO-catalyzed oxidation of styrene into styrene oxide at 25 °C. The reaction volume (1 ml) consisted of the appropriate amounts of solvent, 2-propanol, citrate–phosphate buffer (50 mM, pH 5.0) containing 20 U of CPO and styrene. The concentration of substrate varied over the 0–1000 mM range. Reactions were initiated by adding the oxidative agent (*t*-BHP) at 200 mM final concentration. Samples were withdrawn at different time intervals and analyzed by GC. Experiments were carried out in triplicate. Determination of initial reaction rates was based on the rate of styrene epoxide formation. The equation for initial reaction velocity was used for parametric identification of apparent maximum velocity (V_{max}^{app}) and apparent Michaelis constant (K_m^{app}) using the Enzfitter software (Biosoft Corp.). The k_{cat}^{app} values were calculated assuming 100% active enzyme.

2.9. Differential scanning calorimetry

A differential scanning calorimetry DSC 131 (Setaram) device was used to measure the denaturation temperature (T_d) of CPO-entrapped in ternary systems formulated with *n*-hexane or α-pinene, 2-propanol and water (100 mM citrate–phosphate buffer, pH 2.75). Aliquots (100 μl) of CPO solutions were transferred to DCS aluminum cells. The cells were pressure sealed and weighted before heating. All experiments were carried out in triplicate at 0.1 mg ml^{-1} protein concentration. The same media in the absence of protein were used as reference cells. Samples were kept at 35 °C for 15 min and then heated in air up to 100 °C, at a rate of 0.3 °C min^{-1}.

2.10. EPR spectroscopy

X-band EPR spectra were recorded with an ER-200D Bruker spectrometer equipped with an Oxford ESR-9 cryostat, an Anritsu microwave frequency counter and a Bruker NMR Gaussmeter. Theoretical simulations were done with software provided by Prof. M.P. Hendrich, Dept. of Chemistry, Carnegie Mellon University, Pittsburgh, PA, USA.

2.11. Conductivity measurements

The conductivity measurements in ternary systems were carried out with a Metrohm 644 conductometer at 25 °C as described elsewhere [44]. The cell constant, *c*, was equal to 0.1 cm^{-1}.

3. Results and discussion

3.1. Phase diagrams of surfactant free ternary systems

Two types of surfactant free ternary systems were investigated as media for CPO-catalyzed halide-dependent and halide-independent reactions. The first type included ternary mixtures of *n*-hexane, alcohol (2-propanol or *t*-butanol) and water whereas in the second type *n*-hexane was replaced by α-pinene. The phase diagrams of the ternary systems composed of organic solvent, 2-propanol and water (100 mM citrate–phosphate buffer, pH 2.75) at 25 °C are depicted in Fig. 1a and b, respectively. Similar phase diagrams were determined for analogous ternary systems, where the aqueous phase consisted of 50 mM citrate–phosphate buffer pH 5.0 (data not shown). Region A of phase diagrams corresponds to ternary mixtures which tend to separate rapidly into two phases upon standing, while compositions that constitute the remainder of the diagram (region B), are homogeneous, optically transparent and show no phase separation. As it can be seen from Fig. 1, the phase diagrams which were obtained for the two types of ternary systems examined exhibited the same pattern. However, the replacement of *n*-hexane with α-pinene reduced the monophasic area of the phase diagram. Based on previous works, region B compositions correspond to systems consisted of aqueous microdroplets dispersed in continuous organic solvent rich phase, or systems where no microstructures could be detected [44,45,51]. Conductivity measurements in α-pinene-rich systems of the monophasic area showed a non-linear increase in electrical conductivity with increasing water content up to 3% by volume (data not shown). This observation is consistent with the existence of a percolation phenomenon that is attributed to microdispersions of water in an organic-continuous medium [52].

Various ternary systems that correspond to the monophasic region B (Fig. 1), were chosen as media in order to investigate the catalytic performance of CPO. The composition (% volume) of such systems as well as the initial reaction rates observed during CPO-mediated halogenation and peroxidation reactions are presented in Tables 1 and 2.

3.2. Halogenation activity of CPO—effect of oxidizing agent

As it can be seen in Tables 1 and 2, the enzyme kept its chlorination activity in ternary systems, while the highest activity was observed in high water content systems. However, in low water

Table 1
Initial reaction rates for the chlorination of MCD and epoxidation of styrene catalyzed by CPO in *n*-hexane-based ternary systems

System	*n*-Hexane	2-Propanol (% volume)	Aqueous buffer	Initial reaction rate (μM min^{-1})	
				Chlorination	Epoxidation
H1	15.8	65.3	18.9	320 ± 26	129 ± 10
H2	17.2	67.2	15.6	410 ± 21	139 ± 6
H3	11.7	73.7	14.6	377 ± 27	104 ± 6
H4	23.4	64.6	12.0	333 ± 25	92 ± 11
H5	27.1	64.4	8.5	349 ± 26	76 ± 9
H6	46.0	47.7	6.3	442 ± 28	56 ± 5
H7	19.9	74.6	5.5	330 ± 26	40 ± 6
H8	37.8	57.2	5.0	369 ± 27	28 ± 2
H9	55.8	39.6	4.6	195 ± 16	37 ± 3
H10	53.4	43.4	3.2	111 ± 17	25 ± 2
H11	34.1	63.4	2.5	n.d	35 ± 2
H12	64.5	33.3	2.2	n.d	33 ± 2
H13	47.3	50.7	2.0	n.d	28 ± 2

n.d: No reaction could be detected. Reaction conditions for epoxidation: CPO 20 U; *t*-BHP 200 mM; styrene 200 mM. Reaction conditions for chlorination: CPO 20 U; *t*-BHP 38.5 mM; MCD 0.1 mM; KCl 6.64 mM.

Table 2
Initial reaction rates for the chlorination of MCD and epoxidation of styrene catalyzed by CPO in α-pinene-based ternary systems

System	α-Pinene	2-Propanol (% volume)	Aqueous buffer	Initial reaction rate (μM min^{-1})	
				Chlorination	Epoxidation
P1	16.0	65.1	18.9	280 ± 22	138 ± 13
P2	17.6	66.9	15.5	254 ± 21	140 ± 14
P3	22.9	63.4	13.7	265 ± 24	105 ± 10
P4	26.0	62.0	12.0	255 ± 19	89 ± 7
P5	31.3	58.0	10.7	201 ± 18	87 ± 6
P6	31.8	59.0	9.2	169 ± 14	73 ± 3
P7	36.5	55.3	8.2	75 ± 9	79 ± 9
P8	23.6	68.4	8.0	61 ± 8	63 ± 2
P9	44.9	48.1	7.0	23 ± 3	81 ± 5
P10	22.8	70.4	6.8	25 ± 3	47 ± 2
P11	21.7	73.3	5.0	32 ± 4	43 ± 3
P12	39.1	56.5	4.4	n.d	52 ± 2
P13	30.4	65.5	4.1	n.d	49 ± 2
P14	53.2	42.8	4.0	n.d	45 ± 5
P15	58.4	39.0	2.6	n.d	42 ± 3
P16	64.6	33.4	2.0	n.d	43 ± 3
P17	71.2	27.7	1.1	n.d	41 ± 2

n.d: No reaction could be detected. Reaction conditions as described in Table 1.

content systems the spectroscopic determination of chlorination activity was not possible since these systems became turbid at experimental conditions applied.

Interestingly, when α-pinene-based ternary systems were used as reaction media, in addition to MCD chlorination products, some oxygenated derivatives of α-pinene such as α-pinene oxide, verbenol and verbenone were also detected and identified by HPLC and GC–MS analysis. As it has recently been reported, α-pinene could be accommodated by the catalytic center of CPO, and served as a substrate in chlorination reactions [14]. Moreover, no oxidation of 2-propanol was detected in all reaction systems used.

The effect of the concentration of two oxidizing agents such as H_2O_2 or *t*-BHP, which are commonly used with CPO, on its chlorination activity, was investigated in various ternary systems. As it can be seen in Fig. 2, at low overall concentration of the oxidative agent, the reaction rate was significantly higher

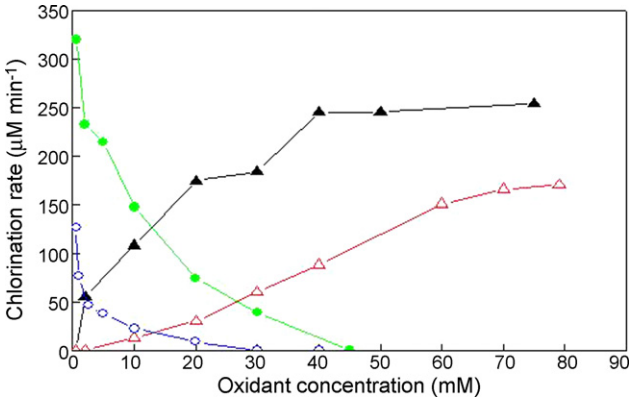


Fig. 2. Effect of concentration of *t*-BHP (triangles) and H_2O_2 (circles) on initial rate of MCD chlorination catalyzed by CPO in *n*-hexane-based (H1, closed symbols) and α-pinene-based (P1, open symbols) ternary systems. Reaction conditions: CPO 20 U; MCD 0.1 mM; KCl 6.64 mM.

with H_2O_2 than with *t*-BHP. However, an increase in H_2O_2 concentration resulted in sharp decline of the reaction rate, while at 30 mM H_2O_2 concentration the catalytic activity of CPO was almost completely inhibited in both reaction systems used. The strong inhibitory effect of H_2O_2 was associated with heme degradation and/or protein denaturation [24], and was also observed in different reaction media [23,32,38]. On the other hand, an increase in *t*-BHP concentration (added either as an aqueous solution or as a solution in *n*-decane), did not inhibit the catalytic action of CPO even when the oxidizing agent was present at 80 mM concentration (Fig. 2).

The CPO catalytic behavior observed in chlorination experiments, could be attributed to the different partition and therefore the availability of oxidizing agents (H_2O_2 and *t*-BHP) in CPO microenvironment. It is expected that the hydrophilic substrates (MCD, Cl^-) as well as the water-soluble H_2O_2 will be fully encapsulated in the aqueous microphase of the ternary system, located in close vicinity with enzyme molecules. Thus, the decreased catalytic activity of CPO observed at higher overall concentrations of H_2O_2 (Fig. 2), could be attributed to the increased concentration of the oxidant in the enzyme microenvironment, which has been shown to accelerate enzyme inactivation [23,38]. On the contrary, the higher catalytic activity of CPO observed with higher overall concentrations of *t*-BHP, could be associated with the reduced availability of this more hydrophobic oxidant on the enzyme microenvironment due to its partitioning in the organic-continuous phase. A similar effect of substrate availability on the CPO catalytic behavior was also recently reported in surfactant-containing emulsions [11]. Based on the above results, *t*-BHP was chosen as oxidizing agent in ternary organic–water systems for various oxidation reactions.

3.3. Epoxidation activity of CPO in ternary systems

The epoxidation of styrene to styrene oxide was used as model reaction. It must be noted that during the enzymatic oxidation of styrene in these media, phenylacetaldehyde, and in some cases phenylacetic acid, were also produced. The enantiomeric excess of styrene oxide ranged from 47 to 49%, which is comparable to that reported in other reaction media [1,31,38].

Fig. 3a and b shows the dependence of the relative reaction rate of the epoxidation of styrene, as well the TTN values obtained for the production of styrene oxide in α-pinene and *n*-hexane-based systems, respectively. As it can be seen, higher catalytic activity of CPO was observed for the epoxidation of styrene in ternary systems with high water content (such as systems H1–H4 and P1–P4). The TTN values obtained for *n*-hexane-based systems were higher at high water content, while in α-pinene-based systems these values remained high even at low water content. In both types of systems, the epoxidation activity of CPO was significantly reduced when the water content was low. This behavior is in accordance to that reported for the biocatalytic modification of various hydrophobic substrates catalyzed by lipases [43], and could be attributed to the partition and therefore the availability of the lipophilic substrates to the enzyme aqueous microenvironment as discussed before. It is interesting to note that when *t*-butanol was used instead of

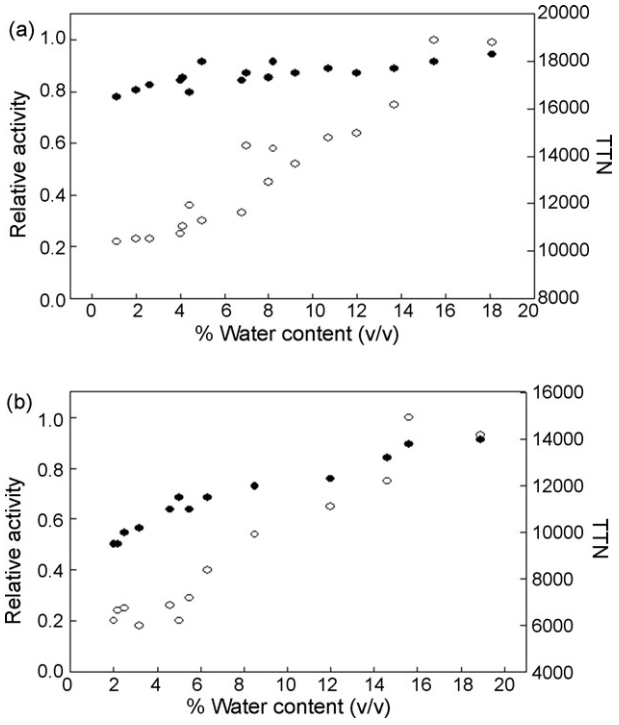


Fig. 3. Effect of the water content on the relative epoxidation activity (◯) of CPO and on TTN values (●) in (a) α-pinene-based and (b) in n-hexane-based systems, respectively. Reaction conditions: CPO 20 U; *t*-BHP: 200 mM; styrene: 200 mM. Relative activity was calculated by comparing the initial reaction rate of each system with the initial reaction rate of H2 and P2 systems respectively.

2-propanol a slight increase (up to 10%) of the initial reaction rates was observed in all ternary systems used (data not shown).

In order to further investigate the effect of the composition of the ternary systems on the catalytic behavior of CPO, the apparent catalytic constants, k_{cat}^{app} and K_m^{app}, for styrene were determined in three *n*-hexane-based (H1, H4, H9) and three α-pinene-based (P1, P3, P9) ternary systems. In all cases studied the entrapped enzyme showed normal saturation kinetics with regard to styrene concentration. The apparent kinetic constants were determined by fitting the experimental data to the Michaelis–Menten equation (Table 3). As can be seen from Table 3, the values of the kinetic constants as well as the catalytic efficiency of CPO, as depicted by the ratio k_{cat}^{app}/K_m^{app}, depended on the composition of the system as well as on the nature of the organic solvent used. The catalytic activity of CPO (measured by the k_{cat}^{app}), is significantly higher in systems formulated with α-pinene instead of *n*-hexane while the apparent enzyme affinity for styrene (measured by the K_m^{app}) was reduced. It is interesting to note that the k_{cat}^{app} values observed in the present work are either similar or higher than those observed for the oxidation of styrene in *t*-butanol–water mixtures [38]. Moreover, the catalytic efficiency of CPO strongly depended on the water content of ternary systems. The highest catalytic efficiency was observed in ternary systems with the highest water content (systems H1 and P1). As indicated before, this can be plausibly attributed to the fact that in such systems the CPO and substrate molecules are located in close vicinity and there should be no hindrance to their interaction.

Table 3
Kinetic constants estimated for oxidation of styrene by chloroperoxidase from *Caldariomyces fumago* in various ternary systems

Ternary system	V_{max}^{app} (mM min^{-1})	K_m^{app} (mM)	k_{cat}^{app} (min^{-1})	k_{cat}^{app}/K_m^{app} (mM^{-1} min^{-1})	TTN (mol product/mol CPO)	
					Styrene oxide	Phenyl-acetaldehyde
H1	0.328 ± 0.019	324 ± 45	729	2.25	14,000	11,000
H4	0.160 ± 0.041	195 ± 29	354	1.82	12,500	10,000
H9	0.133 ± 0.030	321 ± 76	295	0.92	10,500	8,000
P1	0.455 ± 0.026	458 ± 54	1012	2.21	18,500	14,000
P3	0.629 ± 0.055	1131 ± 157	1398	1.24	17,500	13,000
P9	0.834 ± 0.050	1668 ± 144	1853	0.90	17,500	13,000

Reaction conditions for epoxidation: CPO 20 U (0.45 μM); *t*-BHP in water 200 mM.

As it can be seen in Fig. 3 and Table 3, in α-pinene-based systems, the sum of TTN values obtained for the formation of styrene oxide and phenylacetaldehyde, were up to 32,000, which are more than 10-fold higher than those reported in aqueous media [1,53], and about 30% higher than the value recently reported in surfactant-containing media [11]. The fact that in α-pinene-based systems, TTN values remained high even at low water content, could be attributed to an increased CPO stability in such ternary systems, as discussed in the next section.

3.4. Stability of CPO in ternary systems

The enzyme stability was studied in the absence of substrates at various temperatures (25–50 °C), in ternary systems with water contents varying from 0.4 to 18.9% volume fraction (systems H1a–c and P1a–c), where a constant alcohol/organic solvent ratio was maintained. The progress of CPO inactivation during its incubation at 30 °C in a number of α-pinene and *n*-hexane-based systems varying in their water content is depicted in Fig. 4a and b, respectively. As it can be seen, the residual activity of CPO varied, depending on the water content of the system. The highest stability was observed in the lowest water ternary system formulated with α-pinene (P1a), where the residual activity of the enzyme was about 65% of the initial activity, even after 24 h of incubation, which is significantly higher compared to that reported in other organic-based media [38].

Fig. 5a and b shows the dependence of the deactivation rate constants (log k_d) as a function of the water content of the ternary system used (H1a–c and P1a–c), at various incubation temperatures. As it can be seen, the CPO stability strongly depended on the incubation temperature and on the water content of the systems. At temperatures higher than 30 °C, as well as in systems with higher water content, the enzyme stability significantly decreased. Similar observations for the effect of temperature on CPO stability have been reported for chemically modified [54] or immobilized [55] enzyme in aqueous media.

As the water content increased from 0.4 to 18.9% (v/v), the values of deactivation constants k_d significantly increased (i.e. 80 times at 25 °C), indicating that the enzyme stability is significantly higher in low water ternary systems, especially those formulated with α-pinene. Similar dependence of the deactivation constants by temperature and water content was also observed in ternary systems formulated with different volume ratios of organic solvent to alcohol (data not shown).

It is interesting to note that the presence of a reducing substrate molecule such as styrene, in the reaction system, had a positive effect on enzyme stability. When CPO was incubated in various ternary systems containing styrene (200 mM), the estimated k_d values were one order of magnitude lower than those obtained when the substrate was not present (data not shown). A similar stabilizing effect of styrene on CPO activity was also observed in *t*-butanol [38]. Moreover, as it can be observed from Figs. 4 and 5, the presence of α-pinene in the reaction system, that can be recognized as substrate by CPO as indicated before,

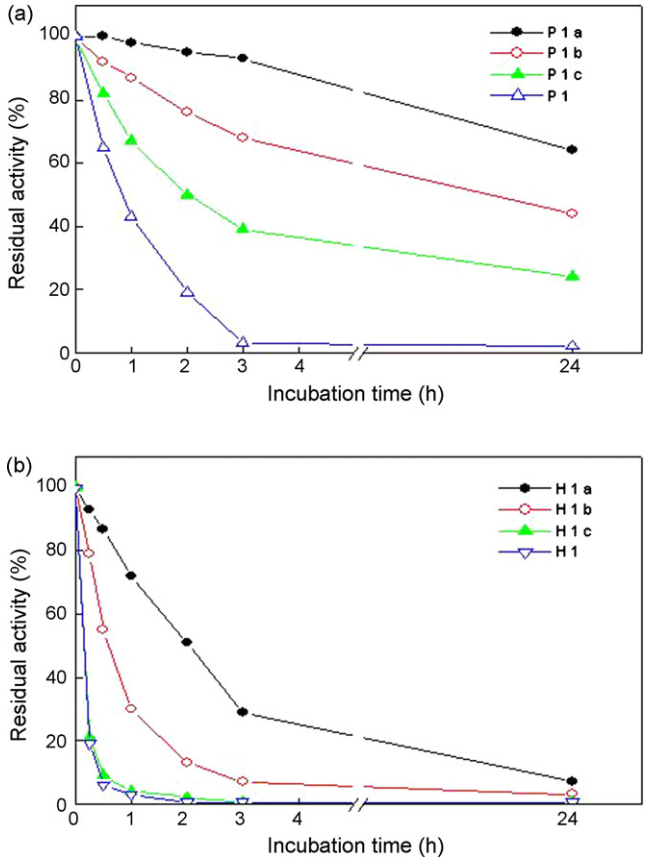


Fig. 4. Time course of residual CPO activity (based on MCD chlorination) following incubation at 30 °C in (a) α-pinene-based systems (P1a–c) and (b) *n*-hexane-based systems (H1a–c) with various water contents. The volume ratio of organic solvent to 2-propanol was 0.242. The volume fractions of aqueous phase were: 0.4% for H1a and P1a; 1.2% for H1b and P1b; 3% for H1c and P1c; and 18.9% for H1 and P1.

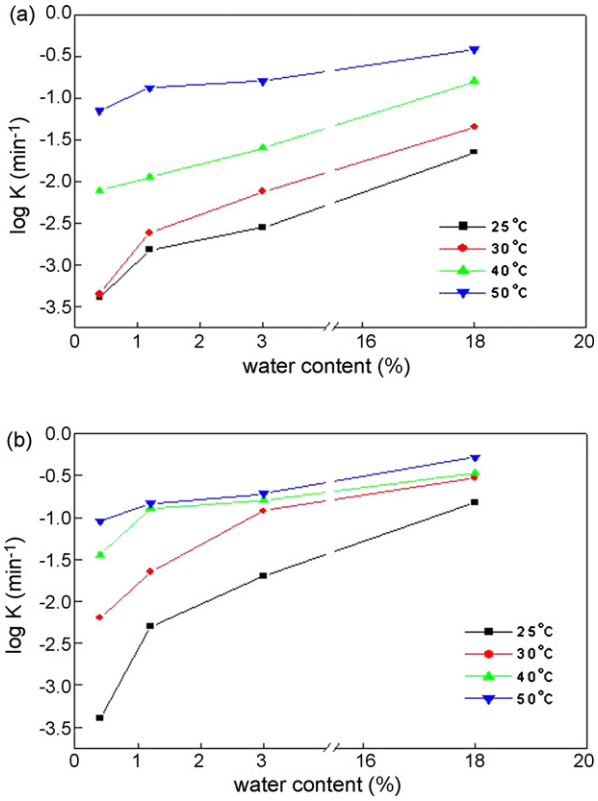


Fig. 5. Dependence of first order deactivation rate constants ($\log k_d$) as a function of water content and incubation temperature of (a) α-pinene-based systems (P1a–c) and (b) *n*-hexane-based systems (H1a–c) ternary systems. The residual activity was measured based on MCD chlorination. The volume ratio of organic solvent to 2-propanol was 0.242 and the volume fractions of aqueous phase were: 0.4% for H1a and P1a; 1.2% for H1b and P1b; 3% for H1c and P1c; and 18.9% for H1 and P1.

had also a positive effect on enzyme stability. Enzyme stabilization by styrene and α-pinene could be possibly attributed to hydrophobic interactions between these hydrophobic molecules and the binding site of CPO. Namely, such molecules stabilizing the enzyme structure are believed to interact with the substrate-binding pocket of CPO, which is characterized by a hydrophobic region located at the bottom of a channel connecting the surface of the enzyme and the heme distal side [18].

3.5. Differential scanning calorimetry study of CPO thermostability

The differential scanning calorimetry (DSC) technique, which registers the heat effect associated with conformational changes when a transition takes place, has been used as a tool to investigate the thermodynamic properties of biomacromolecules and proteins unfolding process [56]. In the present work the denaturation temperature (T_d) of CPO was determined in various *n*-hexane and α-pinene-based ternary systems with different water content. No peaks were observed when rescanning the samples, while no enzymatic activity was detected in samples that had been subjected to thermal analysis, which indicate that all transitions after heating to 80 °C were completely irreversible. In all cases studied an endothermic thermal transition

Table 4
Transition point (T_d) and calorimetric enthalpy change (ΔH_{cal}) determined by DSC for the CPO thermal denaturation various in ternary systems

System	Aqueous buffer (% volume)	T_d (°C)	ΔH_{cal} (kJ g^{-1} of mixture)
P1a	0.4	47.0	0.043
P1c	3.0	45.9	0.162
P1	18.9	43.7	0.178
H1a	0.4	39.5	0.062
H1	18.9	37.0	0.242

The volume ratio of organic solvent (*n*-hexane or α-pinene) to 2-propanol was 0.242 for all ternary systems. The concentration of CPO was 0.1 mg ml^{-1}.

was observed. The T_d can be determined from the minimum of each curve, which corresponds to the temperature point associated with the conformational transition of CPO to the unfolded state in the assayed conditions.

According to the results presented in Table 4, the T_d values increased up to 3.3 °C as the water content of the α-pinene-based systems decreased from 18.9 to 0.4% (v/v). A comparable profile was observed for *n*-hexane-based ternary systems, where a similar decrease in water content resulted in 2.5 °C increase in enzyme's denaturation temperature. The higher stability of CPO in low water ternary systems, observed by DSC technique, is in full accordance with the results described in previous section. A possible explanation of the increased stability exhibited by CPO in low-water ternary systems could be attributed to the formation of dispersed aqueous aggregates or microdroplets in such low-water microheterogeneous media as discussed before. It is expected that in such systems, enzymes should be in their most stable state because they can be completely encapsulated within these confinements, avoiding contact with the organic phase. On the other hand, at high water content the flexibility and therefore the conformational changes of the protein (which accompany the transition of the protein molecule from the folded to the unfolded state), is expected to be more pronounced than in low water ternary systems, which is in accordance to that observed for various enzymes in other low water media [56–59]. As it can be derived from Table 4, the entrapment of CPO in systems formulated with α-pinene increased T_d by about 7 °C compared to the values observed in *n*-hexane-based media, indicating the potential stabilizing effect of α-pinene as described before.

Moreover, the variations in the enthalpy of denaturation (ΔH_{cal}), can also be used to quantify these effects, because the changes represent the total heat absorbed during the irreversible denaturation process [56]. As it can be seen the enthalpy values increased with increasing the water content of the system. Since in high water ternary systems the degree of hydrogen bonding is higher, it is expected that a significant part of the energy that is consumed is used to disrupt inter and intramolecular hydrogen bonds of the protein molecules and hydrogen bonds between protein and water.

3.6. EPR spectroscopy study

EPR spectroscopy was used in order to monitor the effect of the entrapment of CPO in different ternary systems on the active

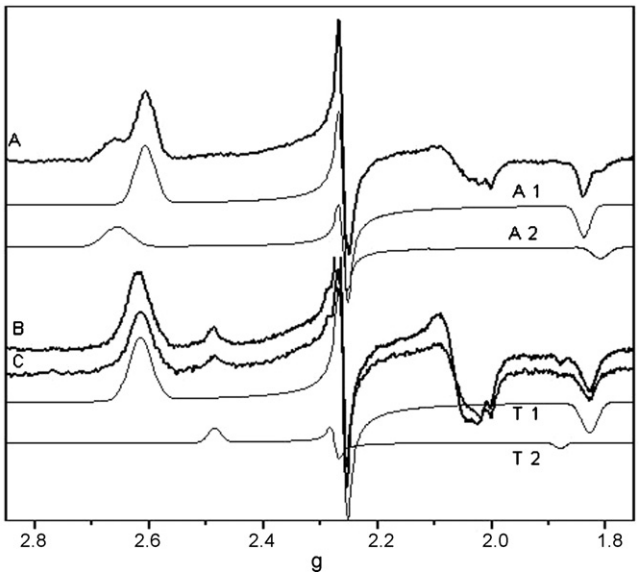


Fig. 6. EPR spectra from CPO in various environments. (A) Aqueous buffer (pH 5.0). (B) α-Pinene-based system; (C) *n*-Hexane-based system. The volume ratio of organic solvent (*n*-hexane or α-pinene) to 2-propanol was 0.242, while the volume fractions of the aqueous phase was 3%. Thin lines correspond to theoretical spectra from $S = 1/2$ sites as described in the text. The arrow indicates a signal from the cavity. *EPR conditions*: $T = 15$ K, modulation amplitude, 10 Gpp; microwave power 2.2 mW; microwave frequency, 9.41 GHz.

center of the enzyme. Representative spectra recorded at 15 K are presented in Fig. 6. Native CPO in an aqueous buffer at pH 5.2 was used to obtain a control spectrum, which was consisted of two rhombic $S = 1/2$ signals, A1 and A2. Theoretical simulations corresponding to each signal are also displayed. Species A1 with $g_1 = 2.607$, $g_2 = 2.260$, and $g_3 = 1.838$ showed parameters almost identical with those reported for native CPO at this pH value [60]. Species A2 was characterized by $g_1 = 2.657$, $g_2 = 2.260$, and $g_3 = 1.810$. These different spectroscopic parameters suggest centers with different coordination properties. For instance a spectrum with similar parameters with A2 was observed in CPO solutions in the presence of Cl^- at pH 5.2 [61]. On the basis of spectral simulations, the ratio between species A1 and A2 was roughly 2. In the same figure (Fig. 6) the CPO spectra in low water content ternary systems H1c and P1c are presented. In both cases almost identical spectra were recorded. They were characterized again by two $S = 1/2$ signals, T1 and T2. The first one was characterized by $g_1 = 2.615$, $g_2 = 2.260$, $g_3 = 1.828$; these parameters were very similar to the majority species A1 observed in the aqueous buffer at pH 5.2. The second species was characterized by $g_1 = 2.485$, $g_2 = 2.275$, $g_3 = 1.880$, while the ratio T1/T2 in such low-water media was estimated at 10. It is interesting to note that in high water content systems (H1 and P1) the T2 low spin signal was enlarged as compared with the T1 form since the ratio between the two species was approximately 1.

As already discussed, the T1 form exhibited parameters similar to those observed in the native active enzyme form. Interestingly, EPR signals with parameters similar to those of T2 have been observed in the ferric form of CPO in alkaline aqueous solutions (denoted as C420) [62]. This species has been associated with a concomitant loss of activity under these conditions. It was argued that this species still retains the thiolate ligation to the iron site and the loss of activity was attributed to other factors which influence the active site. On the basis of the similarities of the signal T2 in the present case with this of C420, it is reasonable to assume that the T2 species arises from ferric sites in a similar coordination environment.

In conclusion, the EPR spectrum of CPO in ternary systems was characterized by signal T1, which is pertinent to the active form of the enzyme, as well as a second signal T2, which has been associated with an inactive enzyme form. On the basis of the T1/T2 ratio it can be concluded that in high water content systems T1 species is converted into the 'inactive' T2 species. In accordance with previous results from stability and DSC studies we can assume that in such water-rich media, structural changes take place which affect the ferric environment. However, in the case of CPO entrapped in low water content ternary systems, the majority of the low spin forms derived from the T1 'active' species indicating that under such conditions the ferric environment at the active center of the enzyme does not undergo severe modifications.

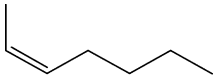
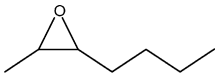
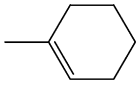
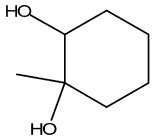
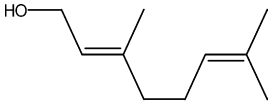
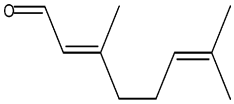
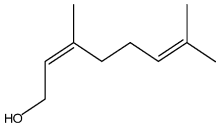
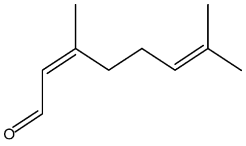
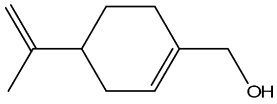
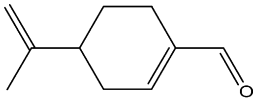
3.7. Oxidation of hydrophobic substrates

In the present work, we explored the ability of CPO-entrapped in surfactant free ternary systems to catalyze the oxidation of various hydrophobic substrates including aliphatic and cyclic olefins such as *cis*-2-heptene and 1-methyl-cyclohexene as well as terpene alcohols including nerol, geraniol and perillyl alcohol. Since most of these compounds are found in essential oils, their transformation catalyzed by CPO was studied in ternary systems formulated with α-pinene (main component of various essential oils), in an attempt to expand the enzyme application for the production of bioactive compounds in a natural-like environment. The fact that in α-pinene-based ternary systems the CPO stability was proven to be higher compared to other reaction media as indicated before, is another benefit of the use of such media for biocatalytic oxidation of various substrates studied in this work.

Fig. 7 shows the reaction progress of the CPO-catalyzed oxidation of geraniol, nerol and perillyl alcohol to corresponding aldehydes in α-pinene-based ternary system (system P1c) formulated with *t*-butanol. It must be noted that no further oxidation of the aldehydes to the corresponding carboxylic acids were observed. TTN values with respect to the CPO-catalyzed oxidation of olefins, terpenols and styrene (200 mM) as determined at 25 °C in α-pinene-based systems using *t*-BHP as oxidizing agent were presented in Table 5. TTN values obtained for the epoxidation of *cis*-2-heptene, was two-fold higher than that reported in other media [63], while for the oxidation of terpenols, TTN was up to 170 times higher than previously reported [14].

The oxidation of *cis*-2-heptene resulted in the formation of a single product (epoxide) with high enantiomeric excess (ee 96%), which is in accordance to that reported for the same CPO-catalyzed reaction in other reaction aqueous media [1,63]. On the other hand, the conversion of a cyclic olefin, such as 1-methyl-cyclohexene, leads to a mixture of four compounds similarly to that observed for the above oxidation in aqueous media [1]. In this case the main product, identified as 1-methyl-

Table 5
Conversion yields (%) after 24 h and TTN (mol product/mol CPO) obtained for CPO-catalyzed oxidations of various hydrophobic substrates (200 mM) in α-pinene-based ternary system (volume ratio of α-pinene to *t*-butanol: 0.242; volume fraction of aqueous phase: 3%)

Compounds	Main product	(%) Conversion	TTN
 *cis*-2-Heptene	 *cis*-2-Heptene oxide	35	22,000
 1-Methyl-cyclohexene	 1-Methyl-1,2-dihydroxycyclohexane	29	n.d[a]
 Geraniol	 Geranial	40	25,500
 Nerol	 Neral	32	20,000
 Perillyl alcohol	 Perillyl aldehyde	24	15,500

Reaction conditions: 140 U of CPO; *t*-BHP 200 mM, incubation temperature 25 °C.
[a] Not determined.

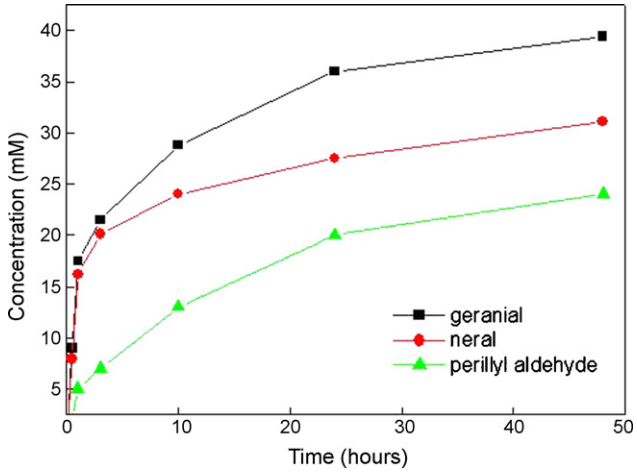


Fig. 7. Reaction progress of the oxidation of various terpenols (200 mM) to the corresponding aldehydes in α-pinene-based ternary systems at 25 °C. The volume ratio of α-pinene to *t*-butanol was 0.242. The volume fraction of aqueous phase was 3%. Reaction conditions: CPO 140 U ml^{-1}; *t*-BHP 200 mM.

1,2-dihydroxycyclohexane, derived from the hydrolysis of the enzymatically formed epoxide. Moreover, CPO is able to catalyze the efficient conversion of natural terpenols such as nerol, geraniol and perillyl alcohol to the corresponding aldehydes using *t*-BHP as oxidizing agent in various ternary systems. It must be noted that the conversion of geraniol or nerol, which is about 20–35% depending on the initial substrate concentration, leads to the formation of both isomers (geranial and neral). The *cis*/*trans*-ratio after 24 h of incubation was 5/95 for the oxidation of geraniol and 85/15 for the oxidation of nerol. Kiljunen and Kanerva [30] showed that for the enzymatic oxidation of simple alcohols, a similar *cis* to *trans* isomerization of aldehydes is not a CPO-catalyzed process but rather a chemical process by some radicals of CPO catalysis or being caused by reaction conditions.

It must be noted that results of the present work represent the first example of the application of CPO for the conversion of large amount of terpenes in a single-step biocatalytic process. The amount of substrates converted in one-step process using α-pinene-based ternary systems, was up to 100 times higher than those reported in other reaction media [1,14,63].

4. Conclusion

In the present work we have shown that surfactant free water–organic solvent ternary systems formulated with n-hexane and more interestingly with α-pinene, can be efficiently used as reaction media for the CPO-catalyzed conversion of various hydrophobic substrates including the oxidation of aromatic or aliphatic substrates as well as the carbonyl oxidation of terpenols. At optimum reaction conditions, the TTN values obtained for the above oxidations were up to two orders of magnitude higher than those reported for other media. Moreover, these media facilitate the solubilization and therefore the bioconversion of large amounts of these hydrophobic compounds (up to 100 times higher than in aqueous-based media) in a single-step biocatalytic process. The biocatalytic activity and stability of the entrapped enzyme can be controlled by the composition and the hydration state of the ternary systems. The enzyme catalytic behavior is affected by the partition and the availability of substrates in the enzyme microenvironment, which illustrates that improvement in CPO biocatalytic behavior can be achieved through a medium engineering approach. The high activity and stability of CPO observed in α-pinene-based ternary systems indicates that these systems could increase CPO potential for the large-scale oxidation of various synthetic and natural hydrophobic substrates expanding the enzyme application for the production of valuable bioactive compounds in an essential-oil simulated environment.

Acknowledgements

This work has been funded by the project PENED 2003. The project is cofinanced 75% of public expenditure through EC—European Social Fund, and 25% of public expenditure through Ministry of Development—General Secretariat of Research and Technology and through private sector, under measure 8.3 of OPERATIONAL PROGRAMME "COMPETITIVENESS" in the 3rd Community Support Programme." The authors wish to thank Dr. T. Giannopoulos for providing excellent assistance and expertise during GC–MS measurements.

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