ENZYMATIC HYDROLYSIS OF FATTY ACIDS MENTHYL ESTERS IN THE PRESENCE OF LIPASE ENZYME

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Menthol is a major component of the peppermint oil. Out of its 8 stereoisomers, L-menthol is the one widely used as ingredient in a variety of every day used products. Although asymmetric synthesis methods are developed for this products, biosynthetic methods are still appealing due to their characteristics. In this paper, the enzymatic hydrolysis of fatty acids menthyl esters in the presence of lipase enzymes is studied. A variety of lipases, both free and immobilized forms were used to find the most efficient substrate – biocatalyst couple. Furthermore, the performance of the enzymatic hydrolysis was enhanced by addition of a co-solvent.

Keywords: enzymatic hydrolysis, lipase, fatty acids menthyl esters, co-solvent

1. Introduction

L-menthol is a terpenoidic compound, a component of peppermint oil. It is produced at an industrial scale by optical resolution of chemically synthesized *rac*(D/L)-menthol. L-menthol has been largely used as an ingredient in candies, toothpaste, beverages, tobacco products etc. due to its flavor and refreshing properties. Medicinally, it has therapeutic properties such as analgesic, antipruritic and a mild local anesthetic. Also, it is added as an ingredient in cosmetic products improving absorption into the skin [1].

L-menthol can be found as esters with different carboxylic acid, thereby broadening its range of applications. For example, Mansurov et al. reported the mosquitocidal activity exhibited of menthol esters with a variety of acids (e.g. chloroacetic acid, cinnamic acid etc.) [2]. For fatty acid esters of L-menthol, advances and accelerated absorption into the skin is expected compared with L-menthol, especially for long-chain unsaturated fatty acids. Additionally, they (i) offer facile dissolution and emulsification; (ii) increase stability compared to the short chain fatty acid esters; and (iii) protect the skin's surface as significant components of skin fats [3].

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Enzymatic hydrolysis of esters using enzymes with hydrolytic activity such as esterases, proteases and lipases is well documented. Most of the hydrolytic enzymes exhibit enantioselective activity, and thus they can be used to obtain optically active alcohols, very valuable reagents in the manufacture of larger target molecules [4]. Carey and McCann [5] synthesized a buprenorphine pro-drug by means of a regioselective ester hydrolysis using *Candida antarctica* lipase B allowing the cleavage of an alkyl ester bond in the presence of a more labile phenolic ester. In another study, Saito et al. [6] demonstrated the potential application of mammalian pancreatic cholesterol esterases in the degradation of phthalic acid esters. For both examples, the high enantioselectivity characterized the process.

Lipases are enzymes widely distributed throughout plants, animals and microorganisms possessing high industrial potential due to their properties such as high catalytic activity, mild reaction condition, substrate selectivity (enantioselectivity, especially) and very broad substrate range for biotransformation [7]. Being able to catalyze the hydrolysis of esters, their catalytic activity is similar to esterases. However, while the esterases are active towards water-soluble esters, in general, with short chains, the lipases tend to hydrolyze triglycerides, and apolar esters with long chains [8]. Enzymatic hydrolysis of menthyl esters reported previously in the literature are focused on the menthol enantioseparation in the presence of enzymes (e.g. lipase from *Bacillus subtilis* [9], [10] and *Burkholderia cepacia* [11]). Kobayashi et al. [12] investigated the hydrolysis of conjugated linoleic acid (CLA) L-menthyl ester in the presences of the lipase from *Candida rugosa*. Hydrolysis process at temperature under strong alkaline conditions led to the decomposition and isomerization of CLA.

In this context, we investigated the improvement of the performance for a biocatalytic system dedicated to menthyl esters hydrolysis assisted by lipase biocatalyst. Screening of lipase enzymes for different menthyl ester substrates allowed to set up the best couple substrate-biocatalyst. Addition of an organic solvent (co-solvent) in the buffer medium (10 mM PBS, pH=7.4) was expected to improve the solubility of the substrate/products for a homogeneous sample. The catalytic activity of the lipase enzyme (expressed as substrate conversion) was considered in order to evaluate any negative effect of the co-solvent. *Tert*-Butanol, and 2-propanol, for the range of 10-40% concentration, were tested as co-solvent for the lipase hydrolysis of menthyl esters.

2. Materials and methods

2.1. Materials

Menthol, *tert*-butanol (*tert*-BuOH), phosphate buffer solution (PBS), and 2-propanol were purchased from Acros Organic. The fatty acids and the free lipases

Candida rugosa, *Pseudomonas cepacia*, *Pseuodomonas fluorescesus*, *Candida antarctica*, *Aspergillus niger* were bought from Sigma-Aldrich. The immobilized lipases Novozyme 435, Tranenzyme, RMIM and TLIM were acquired during a collaboration with professor Ayelet Fishman from the Technion–Israel Institute of Technology, Haifa, Israel.

2.2. Methyl esters synthesis

The synthesis of menthyl esters of fatty acids was achieved using a modified Neises's method [13]. 0.01 mol of fatty acid and 0.001 mol of N,N-dimethylaminopyridine were dissolved in 25 mL of methylene chloride. 0.01 mol of DL-menthol was then added and the solution was cooled to 0 °C. Subsequently 0.01 mol of N,N-dicyclohexylcarbodiimide was introduced and the resulted mixture was stirred at 0 °C for 5 minutes and then at room temperature for 3h. The precipitate was filtered and the supernatant was evaporated under vacuum (10 mmHg) to yield a residue. The residue was then dissolved in a small amount of methylene chloride, and any additional precipitate that formed was further removed by filtration. The solution was washed twice with 25 mL of hydrochloric acid 0.5 N, followed by drying under reduced pressure. The crude product was then purified by column chromatography on silica gel. The elution was carried out using a gradient mixture of EE/EP. The fraction eluted with 1:9 ratio of solvents led upon evaporation of the solvent to an analytically pure compound.

2.3. Menthyl esters characterization

Menthyl esters tested in this study were characterized by FTIR spectroscopy technique using a Bruker VERTEX 70 instrument, equipped with a Harrick MVP2 diamond ATR device. Also, NMR spectra were recorded on a Bruker Advance III 600 MHz spectrometer, corresponding to the resonance frequency of 600.12 MHz for the 1H nucleus, equipped with an indirect detection for nuclei probe head (BBI) and field gradients on Z axis. Samples were analyzed in 5 mm NMR tubes (Willmad). Thin layer chromatography was achieved using as eluent a mixture of EE/EP = 1:1.

Menthyl octanoate (2.08g, Rf=0.72) ^{1}H NMR (600 MHz, Chloroform-*d*) δ 4.66 (td, *J* = 10.9, 4.4 Hz, 1H), 2.25 (td, *J* = 7.3, 1.4 Hz, 2H), 2.00 – 1.93 (m, 1H), 1.90 – 1.80 (m, 1H), 1.71 – 1.55 (m, 4H), 1.54 – 1.42 (m, 1H), 1.39 – 1.31 (m, 1H), 1.31 – 1.17 (m, 8H), 1.08 – 1.00 (m, 1H), 0.97 – 0.90 (m, 1H), 0.90 – 0.82 (m, 10H), 0.74 (d, *J* = 7.0 Hz, 3H). ^{13}C NMR (151 MHz, $CDCl_3$) δ 173.39, 77.22, 77.01, 76.80, 73.81, 47.02, 40.95, 34.73, 34.28, 31.66, 31.35, 29.07, 28.90, 26.22, 25.12, 23.40, 22.56, 21.99, 20.97, 20.72, 16.25, 14.02. FTIR: 3448, 2925, 2862, 1731, 1457, 1374, 1251, 1168, 1104, 915.

Menthyl stearate (1.44 g, Rf=0.71) ^{1}H NMR (600 MHz, Chloroform-*d*) δ 4.67 (td, *J* = 10.9, 4.4 Hz, 1H), 2.27 (td, *J* = 7.3, 1.3 Hz, 2H), 2.01 – 1.94 (m, 1H),

1.90 – 1.82 (m, 1H), 1.71 – 1.63 (m, 2H), 1.66 – 1.56 (m, 3H), 1.52 – 1.44 (m, 1H), 1.40 – 1.33 (m, 1H), 1.32 – 1.21 (m, 27H), 1.05 (qd, *J* = 13.4, 12.8, 3.8 Hz, 1H), 0.99 – 0.91 (m, 1H), 0.91 – 0.81 (m, 10H), 0.75 (d, *J* = 7.0 Hz, 3H). ^{13}C NMR (100 MHz, Chloroform-d) δ 173.61, 75.14, 46.99, 38.84, 34.25, 34.05, 31.26, 31.05, 29.78, 29.75, 29.71, 29.68, 29.64, 29.63, 29.60, 29.58, 29.31, 29.21, 26.60, 25.24, 23.47, 22.74, 21.48, 18.97, 14.21. FTIR: 3449 2923 2859 1732 1458 1374 1244 1177 1107 981

Menthyl oleate (2.55g, Rf=0.73) ^{1}H-NMR (600 MHz, Chloroform-d) δ 5.34 (qd, *J* = 3.8, 1.8 Hz, 2H), 4.67 (td, J = 10.9, 4.4 Hz, 1H), 2.27 (td, *J* = 7.3, 1.1 Hz, 2H), δ 2.03 – 1.95 (m, 4H), 1.86 (td, J = 7.0, 2.8 Hz, 1H), 1.67 (ddd, *J* = 13.6, 6.5, 2.9 Hz, 2H), 1.63 – 1.58 (m, 2H), 1.48 (ddt, *J* = 8.7, 5.3, 2.8 Hz, 1H), 1.34 (d, *J* = 2.9 Hz, 2H), 1.33 – 1.24 (m, 20H), 1.05 (dd, *J* = 12.8, 3.4 Hz, 1H), 0.98 – 0.91 (m, 1H), 0.91 – 0.86 (m, 10H), 0.75 (d, *J* = 7.0 Hz, 3H). ^{13}C-NMR (151 MHz, Chloroform-d) δ 173.43, 129.98, 129.75, 73.86, 47.03, 40.96, 34.75, 34.29, 31.90, 31.37, 29.76, 29.68, 29.52, 29.32, 29.31, 29.16, 29.12, 29.11, 27.21, 27.17, 26.24, 25.13, 23.42, 22.68, 22.02, 20.76, 16.28, 14.10. FTIR: 3005, 2954, 2923, 2854, 1732, 1457, 1370, 1243, 1176, 1097, 1011, 985.

2.4. The procedure of the menthyl esters enzymatic hydrolysis

The hydrolysis reaction was carried out using a standard procedure. Briefly, in a 1.5 mL Eppendorf™ tube, lipase and *rac*-menthyl ester were mixed and diluted with PBS solution (10 mM, pH=7.4) up to a concentration of 1 mg/mL and 10 mg/mL, respectively. To this mixture, specific volumes of co-solvent (e.g. *tert*-BuOH or 2-propanol) were added to achieve final concentrations between 10% and 40%. The tubes were tightly sealed and vortexed for 10 minutes, followed by homogenization in a Thermoshaker at 1000 rpm and 35 °C for 24 hours. Afterwards, 500 µL of reaction mixture was diluted with 500 µL mixture hexane:isopropanol = 98:2 (v/v) and then centrifuged for ten minutes. The supernatant was filtered and analyzed based on the HPLC approach.

2.5. HPLC analysis

The HPLC analysis was performed using a modular Agilent 1260 system equipped with chiral column (Chiralpak IA (Diacel) with dimensions 250 mm x 4.6 mm and particle size 5 µm) and RID detector. The mobile phase consisted of a mixture hexane:isopropanol = 98:2 (v/v) with a flow rate set at 1 mL/min. The sample injection volume was 10 µL.

The system was calibrated using standard of (L) – menthol and *rac* – menthol. An initial series of injection was done in order to identify the retention time of the two enantiomers (D/L-menthol), leading to 8.497 min for (L) – menthol and 7.891 min for (D) – menthol (Fig. 2).

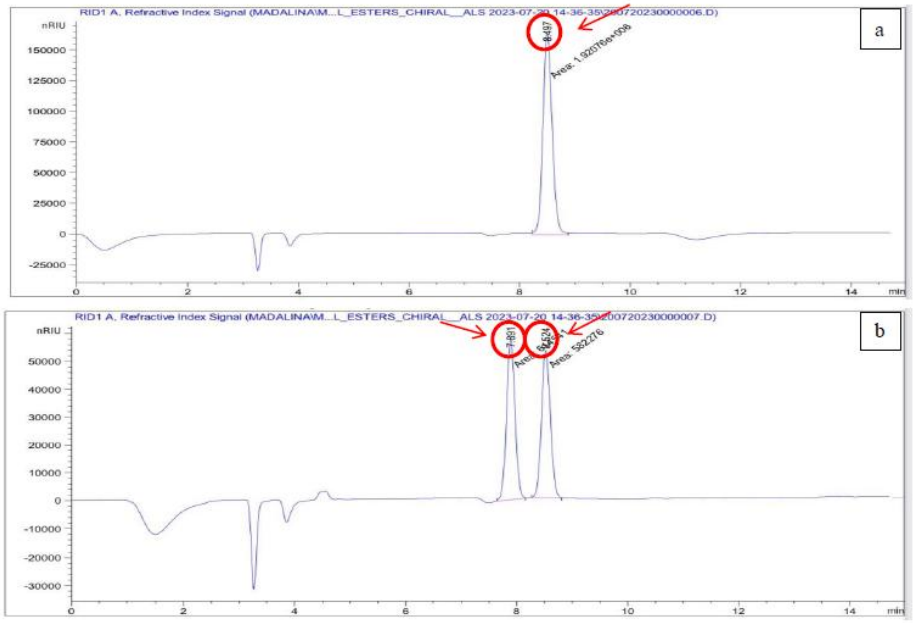

Fig. 1: HPLC chromatograms of: (L) – menthol (**a**) and *rac* – menthol (**b**).

Fig. 2 presents the calibration curves obtained by plotting the RID response against known concentration solutions in the range 0.91 – 7.3 mg/mL.

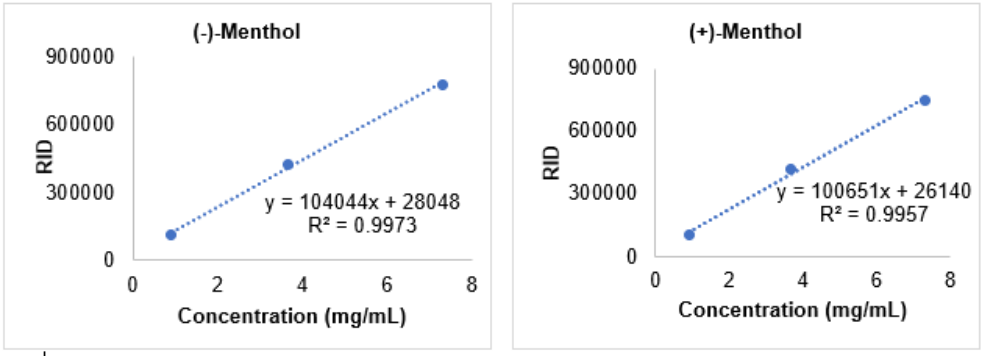

Fig. 2: Calibration curves for (-) – menthol and (+) – menthol

3. Results and discussions

3.1. Screening of the lipase biocatalyst

Screening of lipase enzyme was performed for different menthyl ester substrates. The results are presented in Table 1. Lipases from *Candida rugosa*,

Pseudomonas cepacia, *Pseuodomonas fluorescesus*, *Candida antarctica*, *Aspergillus niger* were tested as free enzyme dispersed in the reaction phase. Also, the biocatalytic system was evaluated for immobilized lipase (e.g. Novozyme 435, Transenzyme, RMIM and TLIM). The performance of the biocatalytic system was determined by calculating the substrate conversion (%) (Table 1).

Table 1:

Lipase screening for enzymatic hydrolysis of menthyl esters.

Enzyme	Conversion (%)		
	Menthyl octanoate	Menthyl stearate	Menthyl oleate
Candida rugosa	-	9	11
Pseudomonas cepacia	5	8	**64**
Pseudomonas fluorescesus	6	6	11
Candida antarctica	11	7	5
Aspergillus niger	-	**12**	9
Novozyme 435	-	3	12
Transenzyme	4	9	15
RMIM	-	-	8
TLIM	**17**	3	-

The experimental results demonstrated that the lipase recognized differently each tested substrate. Therefore, TLIM recognized specifically menthyl octanoate (17% conversion), while menthyl stearate was hydrolyzed by lipase from *Aspergillus niger* (12%). Lipase from *Pseudomonas cepacia* exhibited the highest conversion for menthyl oleate (Table 1). It has to be mentioned that free lipase exhibited higher conversion compared with immobilized lipase. This is a common enzyme behavior reported several times in the literature.

3.2. Influence of the organic solvent (co-solvent) on the enzymatic hydrolysis

Biocatalytic system for lipase hydrolysis of menthyl oleate assisted by *Pseudomonas cepacia* lipase was tested after the addition of *tert*-BuOH as co-solvent. The results are presented in Fig. 3. The basic idea was to offer a friendly environment for substrate, enzyme, and product(s), too. Menthyl esters, menthol and fatty acids have hydrophobic property with low solubility in the buffer solution. It is expected that the addition of the organic solvent leads to increase their solubility for higher performance of the biocatalytic system. Based on the experimental data (Fig. 3), this behavior was noticed when the solvent concentration was raised from 10 to 20%. Simultaneous, the conversion was enhanced from 40% to 100%. More solvent addition (40% *tert*-BuOH) did not affect the system. Enantioselectivity of the biotransformation across all concentrations of *tert*-BuOH was 100%. Based on the experimental results, 20% *tert*-BuOH can be considered for further experiments in order to provide an efficient

biocatalytic system specifically for menthyl oleate hydrolysis catalyzed by *Pseudomonas cepacia* lipase.

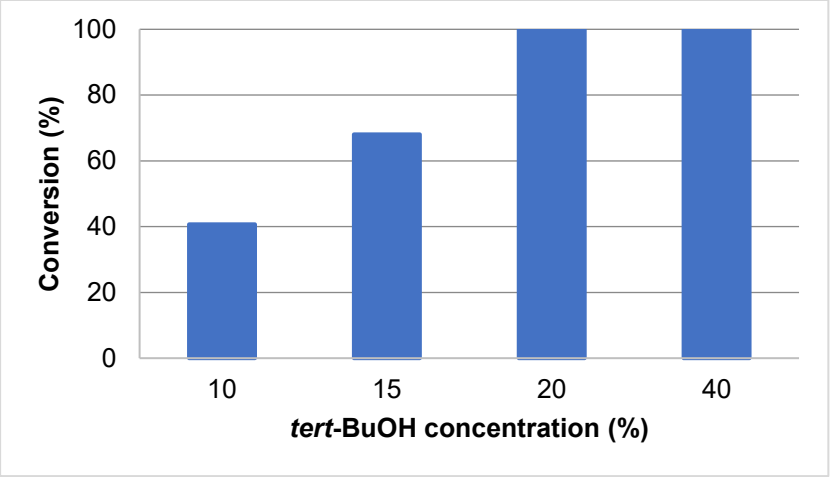

Fig. 3: Influence of *tert*-BuOH concentration on the hydrolysis of menthyl oleate in the presence of *Pseudomonas cepacia* lipase

In a similar way, menthyl octanoate hydrolysis assisted by TLIM enzyme was evaluated. The results are presented in Fig. 4 and the same behavior as previously can be observed. It is clear that the presence of *tert*-BuOH in the reaction mixture promotes the transformation of menthyl octanoate. An over 3-fold increase, from 29% to 100% conversion of ester, was observed when the content of alcohol increases from 10% to 40%. In addition, the hydrolysis using TLIM is strongly enantioselective, leading to only (D)-menthol formation. According to these finding, 40% concentration of *tert*-BuOH was taken into consideration for further experiments, providing an efficient menthyl oleate enzymatic hydrolysis.

Enzymatic hydrolysis of menthyl stearate was achieved in a biocatalytic system assisted by *Aspergillus niger* lipase in the presence of different concentrations of *tert*-BuOH (Fig. 5). The general trend is similar to those observed for the other esters. However, the enhancement of the efficiency is much more accentuated, going from 12% to 100% as the *tert*-BuOH concentration increases from 10% to 40%. Another interesting feature is the leap in conversion from 36% to 76% when the *tert*-BuOH concentration changes from 15% to 20%. These results suggest that a concentration of 40% *tert*-BuOH is efficient in enzymatic transformation of menthyl stearate.

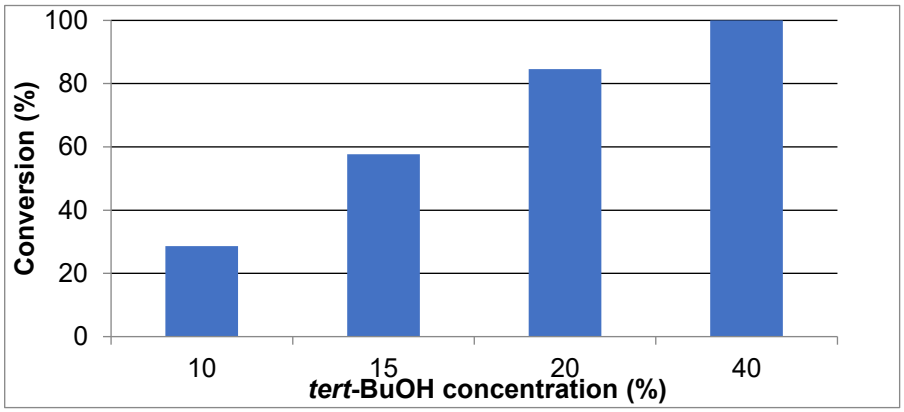

Fig 4: Influence of *tert*-BuOH on menthyl octanoate hydrolysis in the presence of TLIM enzyme.

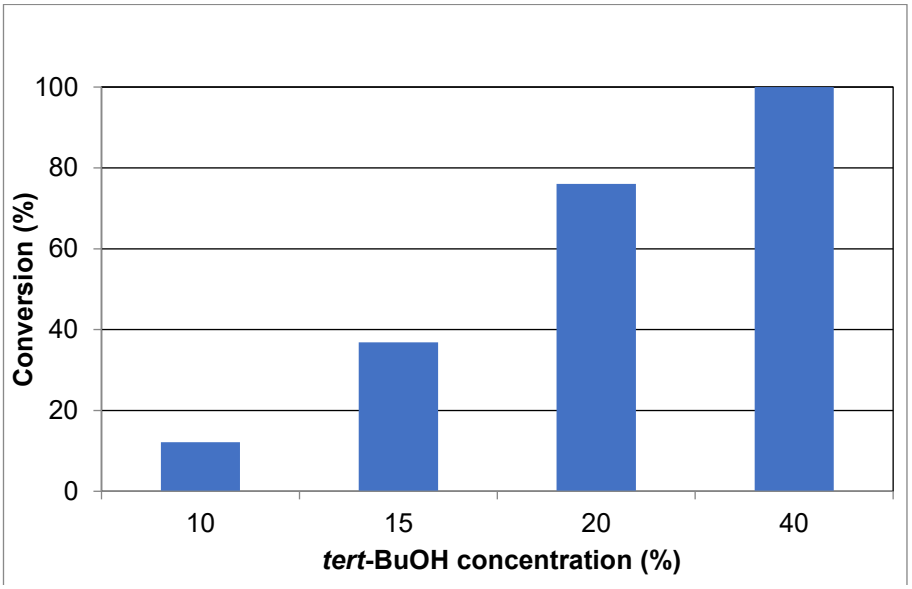

Fig 5: Influence of *tert*-BuOH on menthyl stearate hydrolysis in the presence of *Aspergillus niger* lipase.

The enzymatic hydrolyses of menthyl oleate and menthyl stearate in the presence of *Candida rugosa* lipase, at various concentrations of *tert*-BuOH were further studies (Fig. 6). At high concentration of *tert*-BuOH, the activity of the enzyme is similar for both cases (100% conversion at 40% *tert*-BuOH). However, the activity of the enzyme was considerably different towards the two substrates. For instance, at 10% *tert*-BuOH, the conversion of menthyl oleate was 58%, while the conversion of corresponding saturated ester was only 11%. This is clear

evidence that lipase from *Candida rugosa* recognizes selectively unsaturated residue chain (affinity for menthyl oleate against menthyl stearate).

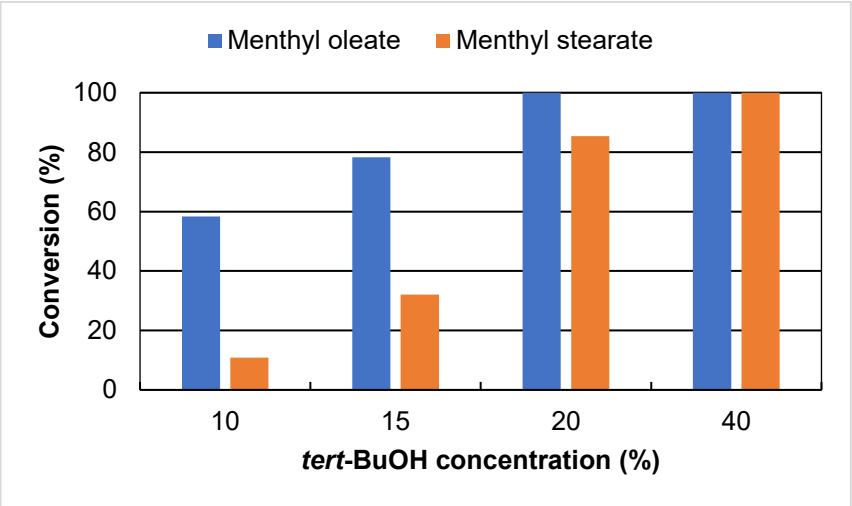

Fig. 6: Influence of *tert*-BuOH concentration on menthyl oleate and menthyl stearate hydrolysis in the presence of *Candida rugosa* lipase

Another important aspect to notice is the process enantioselectivity in the presence of *Candida rugosa* lipase and *tert*-BuOH. According to the results presented in Table 2, higher enantioselectivity was detected for menthyl stearate even at low concentrations of *tert*-BuOH (at least 90% ee), while the enantiomeric excess for (D) – menthol was only 37% ee after the hydrolysis of menthyl oleate at 10% *tert*-BuOH.

Table 2

Enantiomeric excess (ee) in D-menthol for menthyl oleate and menthyl stearate hydrolysis in the presence *Candida rugosa* lipase at different *tert*-BuOH concentrations

tert-BuOH concentration (%)	Enantiomeric excess	
	Methyl oleate	Menthyl stearate
10	37	90
15	91	93
20	97	100
40	100	100

The activity of *Candida rugosa* lipase was also tested under the addition 2-propanol as co-solvent. The influence of the co-solvent addition on the substrate conversion can be observed in Fig. 7. Under these conditions, the lipase was more reactive towards menthyl stearate, leading to conversions up to 89%, while in the case of menthyl oleate the maximum was 30%. Another effect to notice is that, after

a certain concentration (15% for oleate and 20% for stearate), the conversion was dropping. This decrease in the conversion is attributed to the concentration of alcohol that can decrease the activity of the enzyme, and it was also described before in the literature [14].

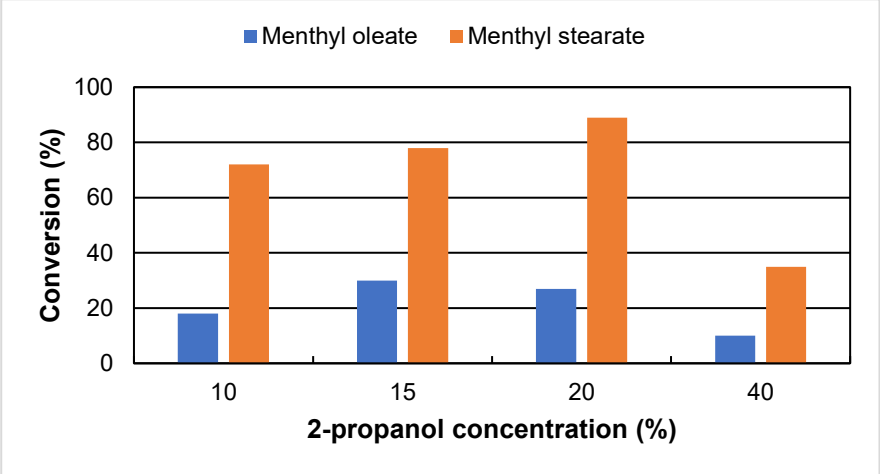

Fig. 7: Influence of 2-propanol concentration on menthyl oleate and menthyl stearate hydrolysis in the presence of *Candida rugosa* lipase

3.3. Influence of the reaction time on the enzymatic hydrolysis of menthyl oleate

The influence of the reaction time on the enzymatic hydrolysis of menthyl oleate was further studied, considering the experimental conditions set up previously (Fig. 8).

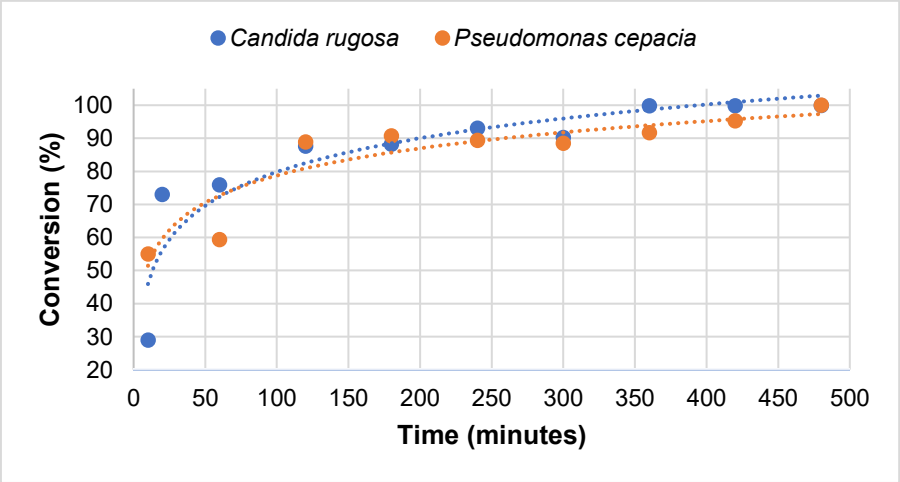

Fig. 8: Influence of reaction time on the enzymatic hydrolysis of menthyl oleate

The enzymatic reaction in the presence of *Candida rugosa* lipase in PBS and 40% *tert*-butanol was produced with high rate in the first 20 minutes, and maximum 73% conversion, followed by a leveling stage where the conversion was increasing very slowly in time. Conversion of 100% was achieved after 6 hours of incubation. The enzymatic hydrolysis carried out in the presence of *Pseudomonas cepacia* lipase and 20% *tert*-BuOH, reveals a slower reaction rate with 100% completion after 8 hours.

6. Conclusions

Enzymatic hydrolysis of fatty acids menthyl esters in the presence of a variety of lipases enzymes, both free and immobilized was studied. The experimental results reveal that different lipases exhibited varying level of specificity towards the hydrolyzed substrate, with *Pseudomonas cepacia* lipase showing the highest conversion for menthyl oleate, TLIM for menthyl octanoate and *Aspergillus niger* for menthyl stearate.

The performance of the biocatalytic systems was significantly enhanced by the presence of *tert*-BuOH, likely due to increase solubility of hydrophobic substrates, enzymes, and products in the reaction mixture. For each substrate, optimum conditions were identified: 20% *tert*-BuOH for *Pseudomonas cepacia* lipase and menthyl oleate, 40% *tert*-BuOH for TLIM and menthyl octanoate, and 40% *tert*-BuOH for either *Aspergillus niger* or *Candida rugosa* lipase and menthyl stearate. According to time-course studies, *Candida rugosa* lipase achieved 100% conversion of menthyl oleate in 6 hours, while *Pseudomonas cepacia* lipase required 8 hours for the same substrate. These findings provide insights for performant menthyl ester hydrolysis based on biocatalytic alternative using lipase enzyme as biocatalyst.

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