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Geotrichum candidum 4013: Extracellular lipase *versus* cell-bound lipase from the single strain

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ABSTRACT

Two types of lipases (extracellular and cell-bound) were produced by *Geotrichum candidum* 4013 in liquid medium and were used as biocatalysts in blackcurrant oil hydrolysis. Reaction products were analysed for the degree of conversion from which enzyme activity was evaluated, and the composition of free fatty acids was compared to the composition of oil substrate. The enzyme activity was measured also before and after the reaction in SC-CO_2. The fatty acid composition of the acids liberated from oil by hydrolysis suggests a specificity of the cell-bound and extracellular enzymes from *Geotrichum candidum* 4013. The extracellular lipase displays low selectivity to the polyunsaturated fatty acids, and the cell-bound lipase possesses selectivity to the saturated fatty acids. Enantioselectivity of the tested processes achieved with both induced enzymes was high (from 43 to 242). The activity of all enzymes has markedly increased after their exposure to SC-CO_2. The treatment of enzymes by SC-CO_2 could be easy-to-use approaches to improve the efficiency of enzymatic reactions.

1. Introduction

Increasing interest in lipases (triacylglycerol ester hydrolases, EC 3.1.1.3.) has been greatly developed at the end of the last century, due to their potential application, in (bio)degradation as well as in (bio)synthesis of glycerides. The advantages of the enzymatic hydrolysis over the chemical process consist in less energy requirements and higher quality of the obtained products. Beside these facts, they are also efficient in various reactions such as esterification, transesterification and aminolysis in organic solvents. Examples in the literature are numerous concerning their use in different fields, such as resolution of racemic mixtures, synthesis of new surfactants and pharmaceuticals, bioconversion of oils and fats and detergency applications [1].

Lipase activity has been found in different moulds [2,3], yeasts [4] and bacteria [5]. Numerous papers have been published on selection of lipase producers and on fermentation process [2,3,6,7]. This kind of information is important in order to identify optimal operation conditions for enzyme production.

Previous works on the physiology of lipase production showed that the mechanisms regulating biosynthesis vary widely in different microorganisms. Results obtained with *Calvatia* [8], *Rhizopus* [9], *Aspergillus* [10], and *Rhodotorula* [11], showed that lipase production seems to be constitutive and independent of the addition of lipid substrates to the culture medium, although their presence enhanced the level of produced lipase activity. On the other hand, it is well known that, in other microorganisms, such as *G. candidum* [12], lipid substrates are necessary for lipase production, and also, carbohydrates can act as repressors of its biosynthesis in *Fusarium* [13].

Supercritical carbon dioxide (SC-CO_2) is a suitable reaction medium for non-polar substrates like vegetable oils. Compared to conventional liquid solvents, the rate of heterogeneous reactions is higher due to the good transport properties of supercritical fluids, CO_2 can be easily separated from the reaction mixture by decrease at pressure, and the products are not contaminated by toxic substances. Different enzymes, including lipases, have exhibited a sufficient activity and stability in SC-CO_2, under the condition of optimum moisture maintenance. This enables us to utilise the advantages of supercritical fluid and specificity of enzyme-catalysed reactions.

The objectives of the present work were (a) to prepare both lipases (extracellular and cell-bound) from *Geotrichum candidum* 4013, (b) to determine their catalytic features, and evaluate the effect of enzyme exposition to supercritical carbon dioxide on their activity, and (c) to describe lipase characteristics with respect to the optimal temperature and pH, both for its activity and stability.

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2. Experimental

2.1. Microorganism and chemicals

The strain of *Geotrichum candidum* 4013 was obtained from the Culture Collection of the Department of Biochemistry and Microbiology (DBM), Institute of Chemical Technology, Prague. Chemicals were purchased from Fluka. Lipase Substrate® was purchased from SIGMA.

Blackcurrant oil was obtained by extraction with SC-CO_2 of blackcurrant seeds (*Ribes nigrum*), which represent a waste by-product in the blackcurrant juice production in Chelčice, CR, where the plant was grown. Only fraction containing triacylglycerols was used as starting material in the hydrolytic reaction [5]. Carbon dioxide (>99.9%) was purchased from Linde Technoplyn, CR.

2.2. Preparation of inoculum and activation of lipases

2.2.1. Preparation of inoculum [14]

For solid cultures, strains of *Geotrichum* were inoculated from culture slant. Growing medium was used with following composition per liter: glucose (30 g), corn steep (10 g), $MgSO_4 \cdot 7H_2O$ (0.5 g), KH_2PO_4 (1 g), K_2HPO_4 (1 g), $NaNO_3$ (2 g), KCl (0.5 g), $FeSO_4 \cdot 7H_2O$ (0.02 g) and agar (15 g). The tubes were incubated at 25 °C for 3 days and conserved at 4 °C. Liquid culture was prepared with medium consisting of glucose (30 g), corn steep (10 g), $MgSO_4 \cdot 7H_2O$ (0.5 g), KH_2PO_4 (1 g), K_2HPO_4 (1 g), $NaNO_3$ (2 g), KCl (0.5 g), $FeSO_4 \cdot 7H_2O$ (0.02 g) in a simple conical flask (250 ml) containing 100 ml of the medium and closed with sterile stopcocks. The medium was sterilised at 121 °C for 20 min. The medium was inoculated with the cells growing on culture slants and incubated with shaking at 30 °C for 24 h.

2.2.2. Production of lipases [14]

For the activation of the lipase activity (extracellular and cell-bound) the medium was used containing per liter: peptone (50 g), glucose (10 g), $MgSO_4 \cdot 7H_2O$ (1 g), $NaNO_3$ (1 g), olive oil (10 g). Medium (90 ml) was inoculated with 10 ml of prepared inoculum.

2.2.3. Localization of lipase activity in Geotrichum candidum 4013

To determine the location of lipase activity in *Geotrichum candidum* 4013, lipase activity was measured in whole broth samples, supernatant and pellet samples, and whole cells that had been subjects of freezing by liquid nitrogen. The frozen cells were mechanically broken and centrifuged at 5000 × *g*. The lipolytic activity of both supernatant and pellet sample was determined as described bellow.

2.3. Preparation of enzymes

2.3.1. Extracellular lipase

Inoculated medium (5 ml) was filtered through a 0.2 μm filter to obtain a filtrate displaying extracellular lipase activity. The filtrate was lyophilized and used as biocatalyst.

2.3.2. Cell-bound lipase—acetone powder

Cells were harvested by filtration of 100 ml cell suspension (inoculated medium) through a 0.2 μm filter, the remaining retentate was washed in distilled water (100 ml) and ice-chilled acetone (100 ml). Finally, acetone from the pellet was evaporated under atmospheric pressure and ambient temperature. The crude enzyme (acetone powder) was stored in refrigerator under nitrogen.

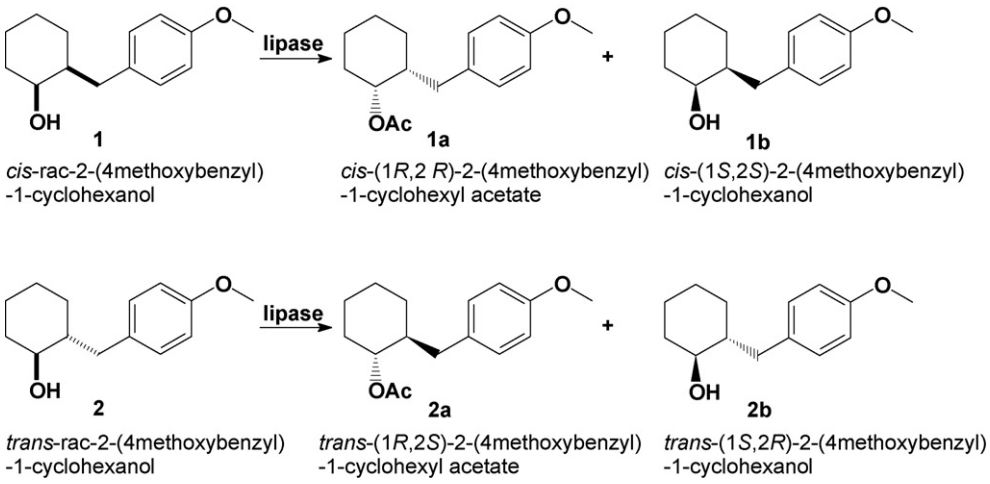
2.3.3. Cell-bound lipase—immobilized cells

Cells harvested by filtration of 100 ml cell suspension (inoculated medium) through a 0.2 μm filter were immobilized in calcium alginate by the traditional external gelation method [6]. About 20 ml of sodium alginate (3%, w/v) and 5 ml of cell suspension (5×10^6 cells per ml) were mixed well and this slurry was added drop wise to 0.2 M $CaCl_2 \cdot 2H_2O$ solution at room temperature. The beads (~4 mm) formed were then cured in a refrigerator at 4 °C for 1 h. The beads were washed two to three times with sterile distilled water and used as source of lipase.

2.4. Enzymic methods

2.4.1. Hydrolysis in SC-CO_2 medium

The reaction was conducted in continuous-flow regime using home-made equipment consisting of two or three high-pressure columns in series [15]. SC-CO_2 was pumped to the first column (12 ml, i.d. 8 mm) containing water on glass beads and to the second column of identical geometry with blackcurrant seed oil on glass beads from where a solution of oil and water in SC-CO_2 flowed to the third column (4 ml, i.d. 8 mm) containing lipase (2 U) where the reaction took place. In the case of seeds the first column with water was not necessary as they contain both oil and water. The products and remaining oil precipitated from the solution flowing out from the reactor after its expansion to the ambient pressure in a heated micrometer valve and were collected in a vial. The



Scheme 1. Reaction pathways.

Table 1
Esterification of **1** and **2** catalysed by lipases from *Geotrichum candidum* 4013.

Substrate	Extracellular lipase					Cell-bound lipase				
	ch. y. (%)	ee_p (%)	ee_s (%)	*c*	*E*	ch. y. (%)	ee_p (%)	ee_s (%)	*c*	*E*
1	22.4	>99	25.0	0.202	205	10.7	95.1	8.5	0.082	43
2	23.2	>99	26.3	0.210	242	18.3	97.3	16.4	0.145	70

ch.y.—chemical yield.
ee_p—enantiomeric purity of the product **1a** or **2a** after chemical hydrolysis.
ee_s—enantiomeric purity of the product **1b** or **2b**.
c—conversion $c = ee_s/(ee_s + ee_p)$.
E—enantiomeric ratio calculated according to the formula.
$E = \ln[1 - c(1 + ee_p)]/\ln[1 - c(1 - ee_p)]$.

prevailing reaction temperature was 40 °C, the pressure was 20 MPa for blackcurrant oil hydrolysis.

2.4.2. Transesterification

An enzyme (lyophylized extracellular lipase or cell-bound lipase—acetone powder, prepared according to Section 2.3, see above) was added to a solvent of the substrate, racemic *cis*-2-(4-methoxybenzyl)-1-cyclohexanol or *trans*-2-(4-methoxybenzyl)-1-cyclohexanol, (**1** or **2**; 20 mg; 0.092 mmol) in vinyl acetate (2 ml). The reaction was performed in flasks under stirring at 25 °C for more than 6 days. The progress of the reaction was monitored by TLC analysis. Final work-up consisted of filtration off of the enzyme, evaporation of the solvent, and chromatographic separation of the residue. The products were characterised as given by the formulae **1a** or **1b**, **2a** or **2b** in Scheme 1.

Chemical yields of the products are summarised in Table 1.

2.5. Assignment of the conversion rate and activity of enzyme

The degree of conversion of oil to free fatty acids (FFA) in a sample of reaction mixture in a vial was estimated from the whole amount of the mixture determined gravimetrically, which is approximately equal to the amount of oil substrate yielding from the sample, and the total amount of free fatty acids measured using the colorimetric method according to Kwon and Rhee [16] based on formation of a blue cupric acetate–fatty acid complex. The average enzyme activity was calculated from the amount of FFA in the sample, mass of enzyme in the reactor and the time of sampling. One lipase unit (U) was defined as the amount of enzyme that released 1 μmol FFA per minute.

The activity before and after the enzyme exposure to SC-CO_2 was determined with help of hydrolysis at ambient pressure. The release of yellow *p*-nitrophenol due to hydrolysis of *p*-nitrophenyl palmitate by lipase was measured. A 200-μl reaction mixture containing 0.25 mM *p*-nitrophenyl palmitate (dissolved in ethanol), 50 mM Tris–HCl (pH 7.5), and 5 mg of crude lipase (prepared as mentioned above) was incubated at 25 °C. Since autohydrolysis of substrates produced low but significant background values at 410 nm, the absorbance in each assay was measured against a substrate–buffer mixture. After 10 min of incubation, the reaction was stopped by the addition of 2 ml of ethanol 96%, and the *p*-nitrophenol released was monitored spectrophotometrically at 410 nm. One lipase unit (U) was defined as the amount of enzyme that released 1 μmol *p*-nitrophenol per minute.

2.6. Separation of the products from blackcurrant oil hydrolytic reaction, preparative TLC

The products (free fatty acids, FFA) of the hydrolytic reactions were separated from the final reaction mixture by the preparative TLC, and developed in a solvent mixture containing light petroleum–diethyl ether (4:1) mixture. The separation was made using Polygram Sil G precoated TLC sheeds (Macherey-Nagel, Germany) with silica gel layer (0.2 mm). Lipid bands were identified using a solution of phosphomolybdic acid (10% in methanol). Fractions corresponding to each lipid type were extracted from the plates with freshly distilled and dry diethyl ether, evaporated and weighed to calculate yields.

2.7. Transesterification of lipid fractions

The blackcurrant oil or lipid fractions (FFA) were converted to the fatty acid methyl esters according to an earlier described method [17,18]. The substance (1.5 mg) was dissolved in a chloroform–methanol mixture [2:3 (v/v), 250 μl], and acetyl chloride (29.4 μl) was added. The reaction mixture was heated in a sealed vial at 80 °C for 30 min. After neutralisation with silver carbonate (57.1 μg) and centrifugation of the reaction mixture, the products (fatty acid methyl esters—FAMEs) were analysed by GC.

2.8. Gas chromatography (GC) analysis of FAMEs

The GC analyses were performed using a HP 5890 A (Hewlett-Packard, USA) gas chromatograph equipped with a HP 3393A integrator, a flame ionisation detector (FID), a split–splitless injector (split ratio 1:49) and a DB-WAX column (30 m × 0.25 mm × 0.25 μm; J and W Scientific, USA). Hydrogen was used as a carrier gas at a flow rate of 40 cm s^{-1}. The injector and detector temperatures were 240 and 250 °C, respectively. Oven temperature was 200 °C. The peaks of FAMEs were identified using the equivalent chain length values [17,18].

2.9. Effect of temperature and pH on lipase activity

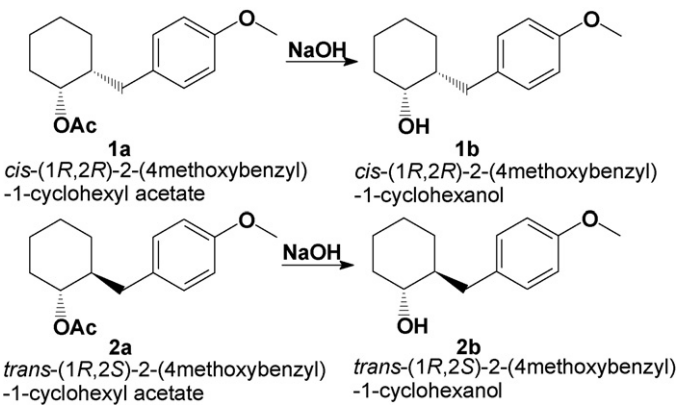
Optimal temperature of induced lipases was tested by incubation of the reaction mixture (15 ml 0.02 M phosphate buffer pH 8.0, 5 ml Lipase Substrate®) (4.5 mM triolein with 1 M NaCl) and enzyme (prepared as described below) at temperatures ranging from 20 to 65 °C for up to 30 min. The pH optimum was tested by using phosphate buffer in the lipase assay at wide range of pH values (3.0–10.0).

Buffers (0.02 M) of different pH (phosphate–citrate for pH 3.0–6.0, phosphate for pH 7.0–8.0, and glycine–NaOH for pH 8.5–10.0) were used.

2.10. Chemical methods

2.10.1. Alkaline hydrolysis of *1a* and *2a* to chiral isomers *1c* and *2c*

2-(4-Methoxybenzyl)cyclohexyl acetate **1a** or **2a** (5 mg, 0.019 mmol) was dissolved in a 1 M solution of sodium hydroxide in absolute ethanol (2 ml). The solution was stirred for 4 h at 25 °C, and the reaction course was checked by TLC. When the reaction was over, the solvent was evaporated under reduced pressure.



Scheme 2. Alkaline hydrolysis of esters **1a** and **2a**.

Purification of the product by column chromatography afforded the respective chiral isomers **1c** or **2c** (Scheme 2).

2.10.2. 3,3,3-Trifluoro-2-methyl-2-phenylpropanoic acid (MTPA) esters of alcohols *1b*, *1c*, *2b* and *2c*

A general procedure used for the preparation of the MTPA esters in a milligram scale starting from the chloride of MTPA is described in detail [19]. In a typical experiment, either both, a solution of the enantiomers of 3,3,3-trifluoro-2-methyl-2-phenylpropanoyl chloride (0.07 mmol) in benzene (500 μl) and a solution of 4-(dimethylamino)pyridine (0.005 mmol) in pyridine (30 μl) was added to a solution of the chiral alcohol **1b**, **1c**, **2b** and **2c** (0.05 mmol) in benzene (200 μl). The mixture was stirred at laboratory temperature for 3–5 h. Thereafter benzene was evaporated and the residue dissolved in light petroleum ether and purified by column chromatography. The spectral data of the products were in agreement with the earlier published data [20,21].

2.11. Analytical methods used

The ^{1}H NMR spectra were recorded on a Varian UNITY 500 spectrometer (in a FT mode) at the respective 499.8 and 125.7 MHz frequency values either in deuteriochloroform using tetramethylsilane ($\delta = 0$) as internal reference or in hexadeuteroacetone using the central line of the solvent ($\delta = 2.13$) as internal reference. The ^{19}F NMR spectra were recorded at 470.27 MHz frequency in deuteriochloroform using hexafluorobenzene as external reference ($\delta = -162.9$). Column chromatographies were performed on a silica gel 60 (Fluka). TLC was carried out on precoated silica gel TLC plates. A column (250 mm × 4 mm) filled with a Biosphere Si-100 solid phase (5 μm; Watrex, Prague, Czech Republic) was employed for HPLC analysis of the MTPA esters of **1b**, **2b**, **1c** and **2c** using light petroleum/ether (9:1, v/v) as mobile phase at 1 ml min^{-1}. The analyses of the chiral products were performed on a chiral Nucleodex β-OH column (150 mm × 4 mm; Macherey-Nagel, Duren, Germany) using methanol/water (4:1, v/v) as mobile phase at flow rate 0.3 ml min^{-1}. Detection of the compounds during the HPLC analysis was at 220 and 275 nm and the eluate was monitored at wavelengths from 200 to 300 nm using a diode array detector.

3. Results and discussion

3.1. Production of lipases

Geotrichum candidum 4013 was used as the source of lipases. The lipases were activated by olive oil, and production of both extracellular and cell-bound lipases was observed. To identify the localization of lipase produced in microbial cells, washed biomass was frozen and mechanically homogenized. The suspension was centrifuged and both, fraction pellet and supernatant were analysed from the point of lipase activity. The activity was only found in the insoluble fraction including cell walls and membranes while no activity was observed in the supernatant lipase. It was assumed that lipase produced is localized in the vicinity of cell wall and membrane, probably bound.

The kinetics of the cell-bound lipase production reached its maximum when the cell growth exhibited a constant level (stationary phase), i.e. 22 h after induction of lipase activity. The production of extracellular lipase was detected at 26 h of incubation. At this time production of the lipase started to decrease.

Although the substrate (olive oil) was partly utilised, no extracellular lipase was detected up to 10 h. This finding indicates that initially the lipase is intracellular, being released into the culture medium and reaching maximum levels only at the stationary cell growth phase. To determine what the prevalent lipase fraction was, lipolytic activity was measured in fermentation broth after filtration. At the beginning of the cell growth, only cell-bound lipase was detected. Then, after 10 h, lipase release started and continued up to about 26 h when the enzyme was completely exported. Lipase secretion only started when about 50% of the carbon source (olive oil) was consumed. The decay of extracellular and bulk lipase activities was probably caused by proteolysis as previously reported [22].

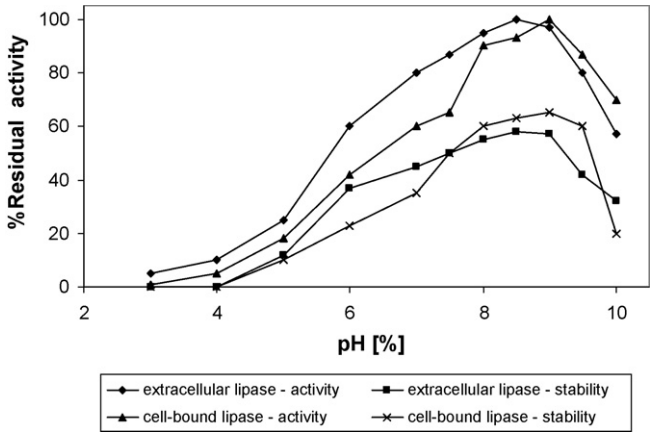
Morphological states of *G. candidum* changed during its cultivation. The inoculum consisted of spores with thick walls. The spores germinated 10 h after inoculation, the hyphae were short and started branching. This is the beginning of the growth exponential phase. After 15 h, long mature hyphae started to fragment themselves, and after 20 h, at the end of the growth phase, the whole culture sporulated. Lipase activity partitions between the biomass and the culture filtrate. Early on in the growth phase most of the activity is associated with biomass, whereas, at the stationary phase of the growth, the activity is found predominantly in the culture filtrate. During sporulation, a proportion of the soluble lipase activity appeared to reassociate with the biomass, before released back into the culture filtrate on prolonged incubation of the arthrospores [23].

The cell-bound lipase presence at the beginning of cell growth was directly assayed using spectrophotometric assay. Our results are in coincidence with finding published by Pereira-Meirelles et al. [24]. Extracellular lipase absence at the beginning of cultivation may be justified as follows: at this stage, olive oil is abundant and cell-bound lipase is able to hydrolyse it at a reaction rate high enough to start cell growth and enzyme induction. As fermentation continues, substrate availability decreases, and the enzyme release is necessary to promote substrate uptake and to ensure cell survival. At this time, enzyme release increases the probability of enzyme–substrate contact and consequently, nutrient assimilation.

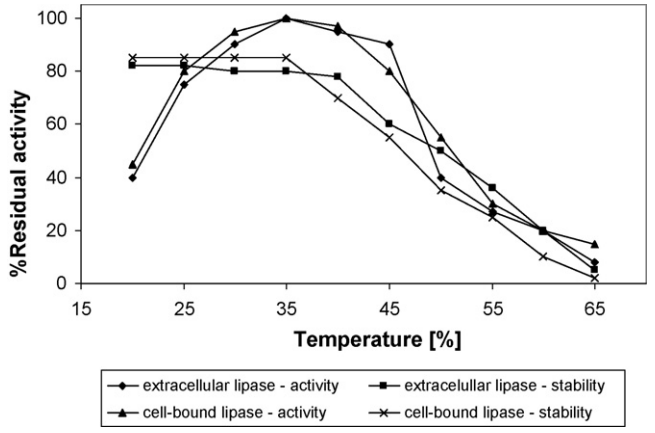
The cell-bound lipase was preponderant at the beginning of the cell growth. When cell-growth stops and substrate is exhausted, the enzyme molecules, still cell-bound, are released, and only the basal level remains inside.

3.2. Effect of pH on lipase activity and stability

The effect of pH on lipase activity of both produced enzymes with triolein as substrate was examined at various pH values at 30 °C (Graph 1). The cell-bound lipase was active in the range of pH 8.0–10.0, and the maximal activity was shown at pH 9.0. It is a characteristic of most microbial lipases that the optimum pH falls on the alkaline side [25]. The pH stability was tested after incubation at 30 °C for 5 h, more than 65% of the original activity was retained at pH 8.0–10.0. This high activity and stability make the lipase applicable at alkaline pH conditions. The optimal values of extracellular lipase pH is 8.5 and its pH stability is slightly lower



Graph 1. pH optima and stability for extracellular and cell-bound lipases from *Geotrichum candidum* 4013.



Graph 2. Temperature optima and stability for extracellular and cell-bound lipases from *Geotrichum candidum* 4013.

in comparison to cell-bound lipase. The results were in accordance with studies on crude lipase from *Geotrichum* sp. [26]. The lipase was most active between pH 7.5 and 9.0.

3.3. Effect of temperature on activity and stability

Temperature and pH optima were detected in the presence of triolein as substrate. The extracellular lipase was active in the temperature range of 30–45 °C with maximal activity at 35 °C. The thermostability of the enzyme was examined by measuring the residual activity for a period of incubation at different temperatures at pH 9.0 (Graph 2). After incubation for 5 h, the enzyme was stable at 30–53 °C with residual greater than 85% of the initial activity. The optimal temperature for the cell-bound lipase was 35 °C and it was found that the thermostability of cell-bound lipase was related to extracellular lipase.

The activity of the cell-bound lipase at optimal conditions (pH_{opt} 9 and t_{opt} 35 °C) was 0.129 U g^{-1}. The activity of extracellular lipase at its optimal conditions reached value 0.089 U g^{-1}. The optimum temperature at optimum pH for both enzyme was the same, 35 °C.

3.4. Blackcurrant oil hydrolysis catalysed by enzymes from *Geotrichum candidum* 4013

As the substrate consisted only of triacylglycerols, neither its solubility in SC-CO_2 nor the degree of conversion was changing during experimental runs. The activity of the three enzymes prepared from *Geotrichum candidum* 4013, evaluated from the degree of conversion of oil to FFA, is listed in Table 2. The best results were obtained for the cell-bound immobilized lipase. A substantial increase in the activity at ambient pressure was observed for all enzymes after their exposure to SC-CO_2.

The composition of free fatty acids obtained by the enzymatic hydrolysis was compared with the initial fatty acid distribution in blackcurrant oil (Table 3). While the extracellular-lyophilized enzyme shows low specificity, as the mutual ratio of major fatty acids remains unchanged within the accuracy of analysis, the class of FFA released by the cell-bound enzymes is rich in saturated acids and oleic acid, while the content of linoleic acid is strongly reduced, and linolenic acids are either present at very low concentrations or absent at all. This could, however, reflect fatty acid degradation, as the total amount of major fatty acids in the samples was also reduced.

3.5. Enantioselectivity of lipases from Geotrichum candidum 4013

To analyse activity and stereoselectivity of lipases from *Geotrichum candidum* 4013 in organic media, the esterification of racemic aromatic secondary alcohols **1** and **2** was performed in vinyl acetate, which was employed as the solvent and the acyl donor at the same time. The transesterication reaction (Scheme 1) catalysed by the lipases was stereoselective as expected. On the basis of the determined absolute configuration of the products, it was concluded that lipases preferred *R*-enantiomer of racemic secondary alcohol substrate, which is in agreement with chiral preferences known for many other lipases [26,27].

As shown in Table 1, the activated lipases produced compounds with high optical purity (ee > 95%), however, the conversion of the substrate into the product was found less satisfactory. The highest enantioselectivity (E = 242) was achieved when the extracellular lipase was used as biocatalyst of the hydrolysis of **2**. The compounds **1** and **2** were also esterified by commercially available lipases. It was found that only lipase from *Candida cylindracea* (CCL) produced **1a** with 85% ee at 18% chemical yield and PPL produced **2a** with 90%

Table 2
Fatty acid composition of free fatty components (%) obtained by hydrolysis of blackcurrant oil with lipases from *Geotrichum candidum* 4013.

Fatty acid	Formula	Content of FFA in blackcurrant oil (%)			
		Before hydrolysis	After hydrolysis with lipase		
			Extracellular-lyophilized	Cell-bound	
				Acetone powder	Immobilized
Palmitic	16:0	6.3	1.2	17.43	10.5
Stearic	18:0	1.9	1.0	4.59	3.5
Oleic	18:1*n*-9	13.7	13.7	30.1	25.0
Linoleic	18:2*n*-6	47.7	50.3	5.36	10.23
γ-Linolenic	18:3*n*-6	13.0	15.2	0	2.5
α-Linolenic	18:3*n*-3	11.9	13.5	0	1.4
Other		5.8	5.1	42.5	46.9

Table 3
Activity of lipases from *Geotrichum candidum* 4013 (U g^{-1}) before and after their hydrolytic reaction in SC-CO_2 and during the reaction.

Lipase	Extracellular-lyophilized	Cell-bound-acetone powder	Cell-bound-immobilized
Before reaction[a]	0.0668	0.0832	0.0065
After reaction[a]	0.4256	0.1233	0.6088
In SC-CO_2[b]	2.56	3.208	6.876

[a] Hydrolysis of *p*-nitrophenyl palmitate.
[b] Hydrolysis of oil.

ee at 30% chemical yield, which means the *E* value of 22.01 [28]. Whereas esterification of **1** catalysed by extracellular lipase activated from *Geotrichum candidum* 4013 yielded **1a** with 99% ee at 22.4% chemical yield (*E* = 205).

The absolute configurations of the products *cis*-(1*R*,2*R*)-2-(4methoxybenzyl)-1-cyclohexyl acetate **1a** and *trans*-(1*R*,2*S*)-2-(4methoxybenzyl)-1-cyclohexyl acetate **2a** were determined after their chemical hydrolysis to **1b** and **2b** (Scheme 2) on the basis of ^{1}H NMR and ^{19}F NMR analysis of their diastereoisomeric Mosher's (MTPA) esters prepared for this purpose in a microscale [29,30]. Based on the known absolute configuration at the C(1) center and on the relative configuration at C(1) and C(2), the absolute configuration at C(2) can be unambiguously established.

4. Conclusion

In general, enantiomeric purity of the products higher than ee = 95% at high conversion and *E* > 100 are considered as synthetically useful enzyme-catalysed reactions [31]. Both lipases prepared from *Geotrichum candidum* 4013 showed high enantioselectivity and can be regarded as potent catalysts for asymmetric transesterification of secondary alcohols under mild reaction conditions. This study contributes to the development of bioprocesses for environmentally sustainable biologically active compound in their chiral forms. The enzymes can be used as tools for isolation of polyunsaturated fatty acids from different types of oils. The extracellular lipase displays low selectivity to polyunsaturated fatty acids. In contrast, the cell-bound lipase possesses selectivity to saturated fatty acids.

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