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ORIGINAL PAPER

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Cultivation conditions and properties of extracellular crude lipase from the psychrotrophic fungus *Penicillium chrysogenum* 9′

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Abstract Among 97 fungal strains isolated from soil collected in the arctic tundra (Spitsbergen), *Penicillium chrysogenum* 9′ was found to be the best lipase producer. The maximum lipase activity was 68 units mL^{-1} culture medium on the fifth day of incubation at pH 6.0 and 20°C. Therefore, *P. chrysogenum* 9′ was classified as a psychrotrophic microorganism. The non-specific extracellular lipase showed a maximum activity at 30°C and pH 5.0 for natural oils or at pH 7.0 for synthetic substrates. Tributyrin was found to be the best substrate for lipase, among those tested. The K_m and V_{max} were calculated to be 2.33 mM and 22.1 units mL^{-1}, respectively, with tributyrin as substrate. The enzyme was inhibited more by EDTA than by phenylmethylsulfonyl fluoride and was reactivated by Ca^{2+}. The *P. chrysogenum* 9′ lipase was very stable in the presence of hexane and 1,4-dioxane at a concentration of 50%, whereas it was unstable in presence of xylene.

Keywords Psychrotroph · *Penicillium chrysogenum* · Lipase

Introduction

Lipases (acylglycerol acylhydrolases, E.C.3.1.1.3) are efficient catalysts for lipolytic reactions, initiating the catabolism of fats and oils by hydrolysing the fatty acyl ester bond of acylglycerols [26] to fatty acids and glycerol.

An important characteristic of lipases is their ability to not only hydrolyse the ester bonds, trans-esterify triacylglycerides and resolve racemic mixtures but also to synthesize ester bonds in non-aqueous media [14, 20]. The structures of many triacylglycerol lipases have been determined by X-ray crystallography. All of them have a similar molecular architecture with a central β-sheet and a catalytic triad consisting of serine, histidine and a carboxyl group reminiscent of serine protease [4].

Lipase function depends critically on the reaction medium, which influences the structure of the protein and its interaction with substrate. Knowledge about molecular mechanisms of protein–lipid interaction is essential for our understanding of lipase stereoselectivity. X-ray crystallography studies on lipase–inhibitor complexes provide detailed information about lipase–substrate interactions at submolecular levels [17, 18].

Enzymes from extremophiles are valuable subjects of research for biotechnologists from the point of view of their wide capabilities for technological utilization. Among the enzymes from cold-adapted microorganisms, lipases and proteases have a considerable potential, mainly in the food industry and as additives to detergents. The specificity of lipases facilitates numerous applications, e.g. as flavor-modifying enzymes or stereospecific catalysts [5]. Microorganisms living in extreme environments have adapted to their habitats in such a way that metabolic processes permit them to survive and function [22].

The present paper deals with the screening of lipase-producing psychrotrophic strains isolated from the arctic tundra and the culture conditions for enzyme production by the selected strain. We also characterize the activity and stability of the crude lipolytic extract from *Penicillium chrysogenum* in the presence of various organic solvents, metal ions, detergents and inhibitors.

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Materials and methods

Chemicals

All reagents were of analytical grade and were purchased from POCh (Gliwice, Poland), Fluka (Buchs, Switzerland), Sigma Chemical Co. (St. Louis, Mo., USA) and Difco (Detroit, Mich., USA). Various oil substrates were from the local market.

Microorganisms

Fungi were isolated from soil in the Calypsostrandy, situated in the southern Bellsund region (latitude 77°33′ N, longitude 14°30′ E; Wedel Jarlsberg Land, West Spitsbergen). The composition of Martin medium used for the screening of microorganisms was 1% glucose, 0.5% peptone, 0.1% K_2HPO_4, 0.05% $MgSO_4 \cdot 7\ H_2O$, 0.003% rose bengal (sterilized separately). After sterilization, 0.003% streptomycin was added.

Isolation of microorganisms from soil

Isolation was completed by means of a dilution plate method. Portions of 1.0 g of representative soil were placed in a dilution tube containing 9 mL of sterile distilled water and shaken for 60 min. Serial ten-fold dilutions were made inoculated onto the surface of Martin medium plates which were afterwards incubated for 8–10 days at 15°C and 30°C. Fungi were isolated from plates that contained no more than 50 well separated colonies.

Preliminary selection of strains for lipase activity

The first selection of lipase-producing strains was the basis of the magnitude of the diffusion zone of the enzyme (diam., mm) secreted into the agar medium by growing colonies. A similar diffusion technique was alternately used to determine the lipase present in culture supernatants. Agar plates (2%, 20 mL) containing 1% tributyrin in 0.1 M phosphate buffer, pH 7.0, were used and the supernatants (0.3 mL) were dropped into wells (10 mm diam.) previously cut out in the agar medium. During the enzymatic reaction (96 h at 28°C), clear zones were formed.

Growth medium and culture conditions

Growth and lipase production were studied on basal GKM medium containing, per liter: 10 g glucose, 6 g KH_2PO_4, 1 g $MgSO_4 \cdot 7H_2O$, 4 g urea, 10 mg $FeCl_3 \cdot 6H_2O$, 8 μg D-biotin, 200 μg thiamin, 4 μg myo-inositol and 10% olive oil. Initial pH was adjusted to 6.0 by addition of KOH solution before autoclaving. The culture medium, except vitamins, urea and olive oil, was autoclaved at 121°C for 25 min. Olive oil and urea were autoclaved separately at 110°C for 15 min and vitamins were sterilized by UV light for 24 h; subsequently, the urea, olive oil and the vitamins were added to the autoclaved medium. Flasks containing 25 mL of medium were inoculated with 1 mL of a suspension of *P. chrysogenum* 9′ mycelium and cultured for 5 days at 20°C on a rotary shaker at 150 rpm.

Various parameters (carbon, nitrogen and lipids sources, initial culture pH) were varied in order to achieve maximum lipase synthesis.

Analytical procedures

At the end of the cultivation period, the mycelium was removed from the culture by centrifugation at 10,000 *g* for 15 min at 4°C. The supernatant, used as the source of lipolytic activity, was called crude lipase.

Lipase activity was determined by the method of Sokolovska, with some modifications [9]. Controls were performed with boiled enzyme. All assays were carried out in triplicate. One unit of lipase activity was defined as the amount of enzyme releasing 1 μmol min^{-1} free fatty acids.

Protein concentration was estimated by the method of Lowry et al. [19], with bovine serum albumin as a standard.

Biomass was determined by estimation of dry weight. Samples of mycelium were washed twice with 50 mL of distilled water and dried at 105°C for 24 h. The biomass was weighed and expressed as dry weight (g) per 1 L of medium. The results were duplicate determinations.

Effect of pH on lipase activity

The pH optimum was tested using 50 mM Britton and Robinson buffer [3] in the lipase assay at a wide range of pH values (3.0–10.0).

Effect of temperature on lipase activity and stability

The effect of temperature on lipase activity was studied by carrying out the enzyme reaction at different temperatures in the range 10–50°C at pH 7.0, using 50 mM phosphate buffer.

The thermostability of lipase was determined by preincubating the enzyme for up to 60 min at temperatures ranging between 20°C and 70°C and then measuring the remaining activity at 30°C after adding substrate. Residual activity was calculated by taking the activity at 30°C as 100%.

Substrate and positional specificity

The specificity of the crude lipase was determined on various pure triacylglycerols and oils. Hydrolysis was assayed in phosphate buffer (0.05 M, pH 7.0).

Positional specificity was explored by thin-layer chromatography (TLC) of the enzymatic reaction products obtained with pure triolein (99% purity; Sigma) as the substrate. The reaction mixture contained 200 µg of lipase, 30 mg of pure triolein and 2 mL of 50 mM phosphate buffer (pH 7.0) and was incubated for 60 min at 37°C. After incubation, 1.5 mL of ethyl ether was added to the reaction mixture to stop the reaction and to extract the reaction products. Aliquots of the ether extract were applied to a silica gel-60 plate (Merck, Darmstadt, Germany) and developed with a 96:4:1 mixture (by vol.) of chloroform/acetone/acetic acid. Pure triolein, 1,3-, 1,2(2,3)-diolein and 1-monoolein (Sigma) were used as reference glycerides. The spots were visualized by iodine vapors and detected with a videocamera (Camag).

Effect of metal ions

For determining the effect of metal ions on lipase activity, enzyme solutions (in 50 mM phosphate buffer, pH 7.0) were preincubated with chemicals (1 mM) containing metal ions such as $ZnCl_2$, $CuCl_2$, $FeCl_2$, $SnCl_2$, $MgCl_2$, $BaCl_2$, $CaCl_2$ and $MnCl_2$ at room temperature for 30 min and then the residual activity was determined as described above.

Effect of detergents and inhibitors on lipase activity

The effect of detergents on lipase activity was determined using ionic [anionic: sodium dodecyl sulfate (SDS), cholic acid, sodium deoxycholate; cationic: hexadecyl trimethyl ammonium bromide (Cetrimide)], non-ionic (Tween 20, Triton X-100) and zwitterionic detergents (3 cholamidopropyldimethyl-ammonio-1-propane sulphonate; CHAPS) at final concentrations of 1 mM and 10 mM. Various inhibitors were added at concentrations of 1 mM, 5 mM and 10 mM. The enzyme/detergent (enzyme/inhibitor) mixtures were incubated for 30 min at room temperature and then subjected to lipase activity assay. The relative activity was expressed as the percentage of activity measured in presence of various detergents or inhibitors, compared with that without detergents (inhibitors).

Determination of the effects of organic solvents and alcohols on lipase activity and stability

The effects of organic solvents and alcohols on lipase activity were analysed by incubation of the enzyme for 30 min at room temperature in 50 mM phosphate buffer, pH 7.0, containing 1, 5, 10, 30 and 50% of the chemicals. The control contained no organic solvent. Lipase activity was measured at the end of preincubation time by the titrimetric assay with tributyrin as substrate. For stability in organic solvents, the enzyme was incubated in the presence of various solvents at a concentration of 50%. After incubation for 24 h at room temperature, the residual lipase activity was measured using the standard assay system.

Determination of Michaelis–Menten constants

Enzyme assays were performed in phosphate buffer, pH 7.0, at 37°C with increasing concentrations of tributyrin from 0.5 mM to 7.0 mM. Lineweaver–Burk curves were plotted to determine K_m and V_{max}.

All samples were analysed in triplicate and they differed by less than 10%. The values given correspond to mean values with a deviation less than 10%.

Results and discussion

Enzyme production

All 97 strains isolated from soil samples in preliminary experiments were tested to determine their lipolytic activity by employing a screening technique on agar plates containing 1% tributyrin as substrate.

For 47 fungal strains, smaller or bigger halo zones (14–27 mm) were observed around circular wells with culture supernatant. Nine strains showed big zones (> 18 mm; data not shown). Out of these nine microorganisms, *P. chrysogenum* strain 9′ was found to produce the highest activity of extracellular lipase. In this case, the clear zone was the largest (> 21 mm diam.). Since the isolate was obtained from representative samples in the environment, we can presume that lipase-producing microorganisms are well distributed in the arctic tundra soil.

The GKM medium was tested to maximize lipase production by the selected strain, *P. chrysogenum* 9′. The initial lipase activity was 31 units mL^{-1} at 72 h in the culture supernatant of the basal medium and lipase production was substantially enhanced by consecutive optimization of the basal medium.

The optimum conditions for growth and lipase production were pH 6.0 and 20°C and the lipase activity was 40 units mL^{-1}, with a biomass of 12 g dry weight L^{-1} at 120 h (Table 1). Similar results were reported for *P. citrinum* [21] incubated at 22°C.

As shown in Table 2, among the lipids used in the experiment, corn oil appeared to be the best substrate for lipase production. The activity of lipase synthesized under these conditions was about 55 units mL^{-1}. Still, *P. chrysogenum* 9′ presented a high lipolytic activity when incubated with triolein, olive oil and oleic acid. This is similar to the lipase production reported by *Aspergillus terreus* when corn oil was used as an inducer, but at higher concentration (2% [10]).

Among various carbon sources, sucrose at a concentration of 1% was the best carbohydrate for high lipase production (72.0 units mL^{-1}), but with a low

Table 1 Effect of growth temperature and culture time on lipase activity

Day of incubation	Growth temperature							
	10°C		20°C		25°C		30°C	
	Activity [units mL^{-1}]	Biomass [g L^{-1}]	Activity [units mL^{-1}]	Biomass [g L^{-1}]	Activity [units mL^{-1}]	Biomass [g L^{-1}]	Activity [units mL^{-1}]	Biomass [g L^{-1}]
3rd	4.0 ± 0.33	10.4	2.0	9.6	1.0	10.8	17.0 ± 1.3	2.0
4th	5.0 ± 0.28	11.6	5.0 ± 0.34	11.6	16.5 ± 0.98	10.4	28.0 ± 1.87	1.04
5th	6.0 ± 0.46	10.6	40.0 ± 2.4	12.0	10.0 ± 0.76	8.8	25.0 ± 1.6	0.8
6th	4.0 ± 0.27	11.6	6.0 ± 0.22	9.6	8.0 ± 0.5	8.6	8.0 ± 0.57	0.68
7th	0.0	14.0	4.0 ± 0.12	10.4	0.0	8.0	0.0	0.56

Table 2 Selection of the best lipid substrate for lipase production by *P. chrysogenum* 9′. *Control* Basic medium without inducer

Inducers	Activity [units mL^{-1}]	Biomass [g L^{-1}]	Protein [mg mL^{-1}]
Control	0.0	6.0	0.189
Tributyrin	0.0.	2.0	0.123
Tricaprylin	0.0	1.2	0.342
Triolein	41.0 ± 2.8	4.0	2.457
Soybean oil	21.5 ± 1.4	11.2	1.245
Rape oil	0.0	9.2	1.032
Corn oil	55.0 ± 3.4	6.8	2.80
Olive oil	41.5 ± 2.7	8.0	1.28
Oleic acid	38.0 ± 3.5	10.4	1.64
Caprylic acid	0.00	0.0	0.297

biomass (7.6 g L^{-1}; Table 3). However, the biomass was significantly increased when glucose was added to the medium instead of sucrose. As shown in Table 3, out of various organic nitrogen sources, urea was the best nitrogen source for increasing the lipolytic activity in the fungal culture. Urea has also been used for lipase production from a strain of *Yarrowia lipolytica* [2]. Among inorganic nitrogen sources, sodium nitrate was found to be the most effective compound for lipase production (63.5 units mL^{-1}). Medium without a nitrogen source did not induce lipase production or growth of *P. chrysogenum* 9′.

Effect of pH on lipase activity

The optimal pH for *P. chrysogenum* 9′ lipase activity was found in the range pH 5.0–7.0 and depended mainly on the kind of substrate tested in this experiment. In the presence of synthetic substrates, the maximum activity of lipase was found at pH 7.0 (Fig. 1a). However, in the presence of natural substrates (oils), the maximum lipolytic activity was achieved at pH 5.0 (Fig. 1b). Similar results have not been published to date. Therefore, the mechanism of such reactions is still unknown. Lipase from *P. chrysogenum* [6] showed greatest activity at pH 7.9–8.1.

Effect of temperature on lipolytic activity and stability of the crude extract

Enzyme activity was determined under different reaction conditions, such as temperature, pH and presence of inhibitory compounds. The extracellular lipase of *P. chrysogenum* 9′ was studied regarding the optimum temperature in the presence of different substrates. As shown in Fig. 2a, optimum temperature depended on the substrate. The highest activity of lipase was observed at 30°C with tributyrin used as a substrate. The enzyme was not particularly stable at elevated temperatures (Fig. 2b). At 20°C, the enzyme was stable for 60 min but at 40°C it retained about 50% of the original activity. At higher temperatures (60–70°C), the enzyme was unstable and retained less than 20% of its maximum activity after 15 min of incubation. Similar thermal stability was reported for lipase from *P. expansum*, which exhibited relatively high temperature sensitivity and was unstable above 30°C during 1 h [25].

Table 3 Effect of carbon and nitrogen sources on growth and lipase production by *P. chrysogenum* 9′. *Control A* Basic medium without carbohydrate, *Control B* basic medium without nitrogen source. Urea at a concentration of 0.4%, others at 1%

Carbon source [%]	Activity [units mL^{-1}]	Biomass [g L^{-1}]	Protein [mg mL^{-1}]	Nitrogen source [%]	Activity [units mL^{-1}]	Biomass [g L^{-1}]	Protein [mg mL^{-1}]
Control A	14.0 ± 1.25	0.4	1.64	Control B	0.0	0.04	0.153
Lactose	16.0 ± 1.3	14.4	0.6	Yeast extract	4.0 ± 0.24	11.2	2.02
Maltose	14.0 ± 1.1	14.4	0.321	Peptone	5.0 ± 0.47	14.8	2.43
Starch	20.0 ± 1.9	1.2	1.32	Urea	68.0 ± 4.56	7.2	2.39
Sucrose	72.0 ± 4.3	7.6	3.126	Casein	6.0	10.0	1.64
Xylose	25.0 ± 1.7	15.2	0.504	Asparagine	25.5 ± 2.1	10.0	2.85
Glucose	40.0 ± 2.9	16.0	0.27	$NaNO_3$	63.5 ± 5.2	8.4	1.914
Fructose	6.5 ± 0.35	13.6	0.426	$(NH_4)_2HPO_4$	0.0	16.8	1.233
Galactose	15.0	15.2	0.243	NH_4NO_2	0.0	14.8	0.18

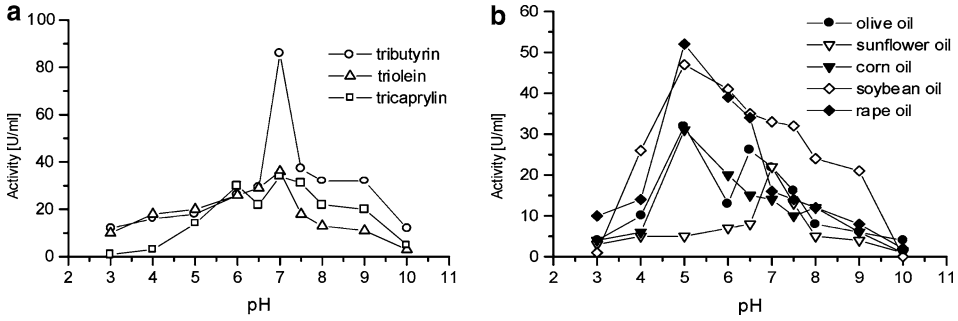

Fig. 1 Activity of lipase from *P. chrysogenum* 9′ against synthetic (**a**) or natural substrates (**b**), depending on pH. Each point is the average of two duplicate tests and the maximum deviation in the data was no more than 10%

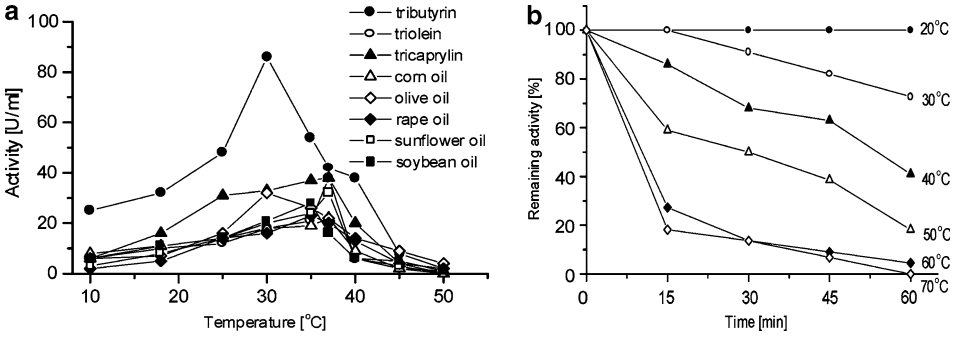

Fig. 2 Effect of the temperature on lipase activity and stability. **a** The effect of temperature on lipase activity was determined at various temperatures, as described in the text. **b** For the stability assay, the crude lipase was assayed after incubation at various temperatures for up to 60 min at pH 7.0. Each point is the average of two duplicate tests and the maximum deviation in the data was no more than 10%

Substrate and positional specificity

A comparison of the lipase activities towards different pure triacylglycerols and oils demonstrates important differences in substrate specificity. Lipase from *P. chrysogenum* 9′ hydrolysed triacylglycerols consisting of short-chain fatty acids more readily than those containing long-chain fatty acids (data not shown). Pure triacylglycerols were the preferred substrates, while oils (mixtures of triacylglycerols) were hydrolysed slowly. Similarly, the extracellular lipase of *Rhizopus rhizopodiformis* showed no activity with triacetin but showed the highest preference for tributyrin followed by tricaprylin and triolein [23].

The positional specificity of the lipase for triolein was examined by TLC. A typical TLC plate of the extracted lipids shows that the enzyme did not discriminate between the 1- and 2-positions of triolein during the hydrolysis of this substrate (Fig. 3). Spontaneous acyl migration was considered negligible because of the short reaction time and low temperature of the assay. These results suggest that *P. chrysogenum* 9′ produces non-specific lipase. This finding in accordance with studies on other *Penicillium* lipases—the lipase from *P. expansum* [25] can attack fatty acid chains regardless of their position.

Effect of different ions and detergents

The stimulatory role of metal ions in enzyme activity has been well documented [1, 16]. The effect of metal ions on lipase activity is shown in Table 4. The catalytic activity was slightly enhanced to 113% and 103% in the presence of Ca^{2+} and Mn^{2+}, respectively. Similar effects were reported for the lipase from *Ophiostoma piceae* [7]. Ca^{2+} and Mn^{2+} also enhanced the lipase activity by over 20%. Sn^{2+}, Zn^{2+} and Cu^{2+} caused moderate inactivation (about 20–30%) of the lipase from *P. chrysogenum* 9′.

The effect of the various detergents on *P. chrysogenum* 9′ lipase activity varied considerably. The non-ionic surface-active agents, cationic and zwitterionic detergents inhibited lipase activity at all concentrations tested (Table 4). These were strong inhibitors, causing total

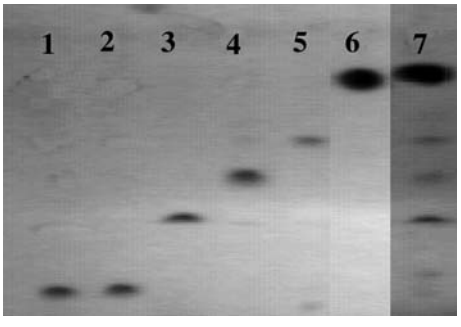

Fig. 3 TLC analysis of hydrolysis products after incubation of *P. chrysogenum* 9′ lipase on triolein as substrate at 37°C for 60 min. *Lane 1* 1-Monoolein, *lane 2* 2-monoolein, *lane 3* oleic acid, *lane 4* 1,2-diolein, *lane 5* 1,3-diolein, *lane 6* triolein, *lane 7* lipase hydrolytic products

Table 4 Effect of metal ions and detergents on lipolytic crude extract from *P. chrysogenum* 9′. *Control* Without the addition of any substance. Standard error for all experiments was less than 10% of the value reported

Metal ion	Concentration	Residual activity [%][a]	Detergent	Concentration	Residual activity [%]
			Triton X-100	1%	0.0
Control		100.0		10%	0.0
$Cacl_2$	1 mM	113.0	Tween 20	1%	0.0
$BaCl_2$	1 mM	90.0		10%	0.0
Mnl_2	1 mM	103.0	Cetrimide	1 mM	0.0
$MgCl_2$	1 mM	91.0		10 mM	0.0
$SnCl_2$	1 mM	82.0	SDS	1 mM	0.0
$ZnCl_2$	1 mM	82.0		10 mM	0.0
$FeCl_2$	1 mM	87.0	Cholic acid	1 mM	127.3
$CuCl_2$	1 mM	72.0		10 mM	118.2
			Sodium deoxycholate	1 mM	72.7
				10 mM	72.7
			CHAPS	1 mM	0.0
				10 mM	0.0

[a] The enzyme was preincubated with various compounds at room temperature for 30 min and the residual activity was assayed

inhibition of enzyme activity. Among the anionic detergents tested, cholic acid stimulated lipase activity at all concentrations tested with a maximum effect at 10 mM (almost 128% relative activity); and sodium deoxycholate caused moderate inhibition. SDS was a very strong inhibitor. These results were in accordance with earlier reports [15].

Effect of inhibitors on lipase activity

The effect of various inhibitors on lipase activity are shown in Table 5. The lipase was strongly inhibited by the addition of 5 mM and 10.0 mM phenylmethylsulfonyl fluoride (PMSF; 69% inhibition in both cases), suggesting that the lipase belongs to the class of serine hydrolases and a serine residue plays a key role in the catalytic mechanism [8]. The lipase showed high sensitivity to the metal-chelating agent EDTA and this suggested that the enzyme was a metalloenzyme. This inhibition was prevented when calcium was added simultaneously with the chelator. A Ca^{2+} concentration double that of EDTA could restore 91% of the activity lost after EDTA treatment of the enzyme. This indicated that *P. chrysogenum* 9′ lipase was a calcium-dependent enzyme. Lack of activity inhibition by dithiothreitol (DTT) and cysteine confirmed the absence of sulphur-containing amino acids in the active site of the lipase. Others inhibitors such *N*-ethylmaleimide (NEM), *o*-phenantroline (zinc-specific chelator) and eserine (esterase inhibitor) did not affect the enzyme activity. The thiol reagent, iodoacetamide, appeared to have no effect on enzyme activity at the concentrations tested.

Table 5 Effect of inhibitors on the crude lipase. The lipase was incubated with various compounds that may inhibit the enzyme and the remaining activity was measured under standard conditions. Standard errors for all experiments were less than 10% of the value reported

Inhibitor	Remaining activity (%)		
	1 mM	5 mM	10 mM
EDTA	36.3	27.3	18.2
DTT	81.8	81.8	100.0
Cysteine	91.0	81.8	81.8
PMSF	36.6	31.8	31.8
NEM	91.0	91.0	91.0
o-phenantroline	100.0	100.0	100.0
Eserine	100.0	100.0	100.0
Jodoacetamide	100.0	100.0	100.0

Activity and stability of enzyme in organic solvents

Lipases are known for their ability to work in both organic solvents and in aqueous solutions. A remarkable stimulation of the enzyme (up to 30%) was observed upon adding dimethylsulphoxide (DMSO) to the assay mixture (Table 6). Similar results were reported for the lipase from *Cryptococcus* sp. S-2 [13]. The activity was also increased in 1,4-dioxane, hexane and methanol at concentrations of 1% and 5%. In contrast, the activity was inhibited in xylene and diethyl ether at all concentrations tested, with complete inhibition at 30% concentration. Methanol, butanol and pentanol (at higher concentrations) were strong inhibitors, causing total inhibition of enzyme activity.

P. chrysogenum 9′ lipase showed more than 90% stability in the presence of hexane, 1,4-dioxane and almost 70% in the presence of cyclohexane. Lipase from *P. chrysogenum* 9′ exhibited full inhibition by xylene, butanol and pentanol. A similar effect was reported for the lipase from *Bacillus stearothermophilus* MC 7 [12].

Kinetic study

Various enzymes from poikilothermal organisms, including thermophilic and psychrophilic microorganisms, show the lowest K_m values for their substrates at the physiological temperatures of the source organisms [11]. In our experiment, the K_m of the crude lipase from *P. chrysogenum* 9′ was also low. The K_m and V_{max} values of the enzyme using tributyrin, as calculated from the

Table 6 Effect of organic solvents on lipase activity and stability. To assess relative activity, *P. chrysogenum* 9′ lipase was prepared in 50 mM phosphate buffer (pH 7.0) and incubated in organic solvent at room temperature for 30 min. The effect of organic solvents on lipase stability was determined by mixing enzyme aliquots with the solvents in a 1:1 ratio for 24 h at room temperature. Activity was determined with tributyrin as described in the Materials and methods and is expressed as a percentage of the activity of the enzyme sample prepared in phosphate buffer in the absence of solvent

Solvent	Relative activity (%) in the presence of different solvent concentrations					Stability (%) after 24 h
	1%	5%	10%	30%	50%	
Acetone	100	91	54.5	27.2	9.1	9.1
Acetonitrile	109	72.3	36.6	9.1	0	9.1
1,4-Dioxane	118.2	109	63.6	27.2	9.1	91.0
DMSO	163.6	154.5	136.6	118.2	45.4	22.7
Diethyl ether	72.3	63.6	45.4	0	0	27.2
Cyklohexane	118.2	81.8	54.5	18.2	0	68.2
Xylene	63.6	36.6	9.1	0	0	0
Hexane	136.6	136.6	81.8	81.8	72.3	109.0
Methanol	145.4	100	59.1	0	0	9.1
Ethanol	91	63.6	27.2	9.1	0	9.1
n-Propanol	104.5	54.5	36.6	27.2	18.2	27.2
Butanol	91	18.2	0	0	0	0
Pentanol	81.8	45.4	0	0	0	0

Lineweaver–Burk plot, were 2.33 mM and 22.1 units mL^{-1}, respectively. Schuepp et al. [24] obtained a K_m value of 9.66 mM for the purified exolipase from *Pseudomonas fragi* CRDA 037, using tributyrin as substrate.

To conclude, the *Penicillium chrysogenum* 9′ lipase has several properties of significant industrial importance, in particular an activity and stability in various organic solvents and a wide substrate specificity. The lipase was stable at low temperatures below 30°C, which could be also useful for possible industrial applications. However, further studies should be done with a purified preparation of the enzyme.

The importance of the data presented here points to the possibility of obtaining an active lipase producer from psychrotrophic microorganisms. *P. chrysogenum* 9′, as the fungus of highest lipolytic activity, will be of great interest in biotechnological research. However, the isolation, growth and production of lipase by this organism on a bench scale, as performed in our experiments, require some additional research on a large scale (bulk cultures).

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