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Purification and Characterization of a Novel Cold-Active Lipase from the Yeast *Candida zeylanoides*

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Please review the citation of the figures. Blockade on second page - what do we have to insert?
Several authors were cited in the text but not included in the reference list. Please add the bibliographic information for the following: Callon et al. (2006), Cavicchioli et al. (2002), and de Maria et al. (2005).
In the last paragraph of the Introduction, you mention "Cr. mucoides". Could you provide the full genus name?

Key Words
Lipase · *Candida zeylanoides* · Cold-active enzyme · Cold-active lipase · Lipase purification · Lipase properties

Abstract
Cold-active lipases have attracted attention in recent years due to their potential applications in reactions requiring lower temperatures. Both bacterial and fungal lipases have been investigated, each having distinct advantages for particular applications. Among yeasts, cold-active lipases from the genera *Candida, Yarrowia, Rhodotorula*, and *Pichia* have been reported. In this paper, biosynthesis and properties of a novel cold-active lipase from *Candida zeylanoides* isolated from refrigerated poultry meat are described. Heat-sterilized olive oil was found to be the best lipase biosynthesis inducer, while nonionic detergents were not effective. The enzyme was purified to homogeneity using hydrophobic chromatography and its enzymatic properties were tested. Pure enzyme activity at 7°C was about 60% of the maximal activity at 27°C. The enzyme had rather good activity at higher temperatures, as well. Optimal pH of pure lipase was between 7.3 and 8.2, while the enzyme from the crude extract had an optimum pH of about 9.0. The enzyme was sensitive to high ionic strength and lost most of its activity at high salt concentrations. Due to the described properties, cold-active *C. zeylanoides* lipase has comparative advantages to most similar enzymes with technological applications and may have potential to become an industrially important enzyme.

Introduction

Microbial lipases (acyl glycerol hydrolases, EC 3.1.1.3) constitute a class of enzymes with a broad spectrum of biotechnological applications in food, chemical, pharmaceutical, and other industries [Jaeger and Eggert, 2002; Treichel et al., 2010]. They can hydrolyze ester bonds between glycerol and fatty acids in glycerides, and many of them can also catalyze transesterifications of different esters as well as the synthesis of new esters, which make them

Iva Čanak and Adrienn Berkics contributed to the results presented in this paper equally and should therefore be considered first authors.

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enzymes of choice for many different biotechnological processes. Some lipases are characterized by high substrate specificity, sometimes acting in an enantio-specific manner, which additionally increases the interest of modern biotechnology in this class of enzymes. Lipases are broadly spread among bacteria, yeast, and fungi, and they exhibit different properties, usually reflecting the preferred growth conditions of the producing microorganism. This further broadens the spectrum of their application.

As far as yeast lipases are concerned, the main species found to produce lipases of potential technological importance belong to the genera *Candida, Yarrowia, Rhodotorula*, and *Pichia,* although lipases from many other yeasts have been described as well [Vakhlu and Kour, 2006]. Since most biotechnologically important lipases are secreted by cells, presumably to fulfill their nutritional requirements, the search for lipase-producing yeasts was often focused on different lipid-containing habitats like marine soil in the vicinity of oil extraction platforms [Potumarthi et al., 2008], spoiled olive oil [Ciafardini et al., 2006], etc. Many of these enzymes have subsequently been genetically modified to achieve higher activity, better stability, immobilization, and in the more recent time also surface display [Bielen et al., 2014]. In recent years particular attention has been brought to cold-active enzymes due to their potential application in reactions requiring lower temperatures [for review see Joseph et al., 2008]. A number of bacteria living in cold habitats have been reported to produce lipases, while cold-active lipase-producing yeasts have been reported less frequently as reviewed in Babu et al. [2007]. *Candida antarctica* (teleomorph *Pseudozyma antarctica*) producing two different lipases (CAL-A and CAL-B) was isolated from the Antarctic habitat, although this species has a mesophilic, ubiquitous character and CAL-A is considered as the most thermostable lipase known [Cavicchioli et al., 2002; Kurtzman et al., 2011; Uppenberg et al., 1994]. Screening of basidiomycetous yeasts found in glacial and subglacial waters in Argentina showed that the number of yeasts producing cold-active lipases might be significantly higher [Brizzio et al., 2007].

Potential habitats for cold-active lipase-producing microorganisms are refrigerated dairy products and meats. Several lipase-producing yeast species like *Yarrowia lipolytica* (syn. *Candida lipolytica*), *Galactomyces geotrichum* (syn. *Geotrichum candidum*), *Candida rugosa*, *Candida zeylanoides,* and *Rhodotorula mucilaginosa* are constant members of the spoiling microbiota in both types of food [Maraz and Kovacs, 2013]. Callon et al. [2006] and Lopandić et al. [2006] found the same species as dominant lipolytic yeasts in association with a broad range of dairy products. Depending on the progress of spoiling, Viljoen et al. [1998] and Ismail et al. [2000] reported *Y. lipolytica* or *C. zeylanoides* predominating in different raw and processed poultry, while molecular genotyping revealed a high degree of polymorphism among the isolates belonging to these two species [Deak et al., 2000].

Later, a much broader spectrum of lipolytic yeasts was recovered from refrigerated poultry including *C. zeylanoides*, *Cryptococcus curvatus*, *Cr[illegible]ucoides*, *Debaryomyces hansenii*, *Metschnikowia pulcherrima*, *Rhodotorula glutinis*, *Rhodotorula minuta*, *R. mucilaginosa*, *Trichosporon asahii,* and *Trichosporon montevideense* [Belák et al., 2011]. Since one of the best lipase producers was *C. zeylanoides*, which has not been studied from this respect, investigation and optimization of the enzyme production, as well as protein purification and molecular characterization of the secreted lipase were the aims of this work.

Results

Screening for the Best Producer Strain and Optimization of Lipase Production

Pre[illegible]us experiments [Belák et al., 2011; Ismail et al., 2003; [illegible]ben et al., 1998] indicated that *C. zeylanoides* is one of the predominating lipolytic yeast species associated with refrigerated poultry meat. In a screen for good lipase producers, six strains were investigated for lipase secretion and compared with the type strain CBS 619. All six strains secreted lipase, but in different amounts. Lipase activity was determined using suspension of grown cells together with the culture medium, and then the sample was centrifuged and the cells were dried. The best producer was the strain YM-7, which had a ratio of activity to dry cell mass of 2.2 U/mg, almost two times higher than the type strain and about four times higher than the worst producer strain, YM-2. Thus, all further work was done with the strain YM-7.

To optimize the cultivation conditions in order to achieve the best lipase production, several parameters were tested. Since the expression of most microbial lipases is induced by the presence of lipids in the growth medium [de Maria et al., 2005; Fickers et al., 2004; Sharma et al., 2001], several potential inducers were tested. Figure 1a shows that by far the best results were obtained using heat-sterilized olive oil. The obtained activity per mg yeast was 7- to 8-fold higher than when using filtered olive oil (using nitrocellulose filter of 0.45 μm pore size),

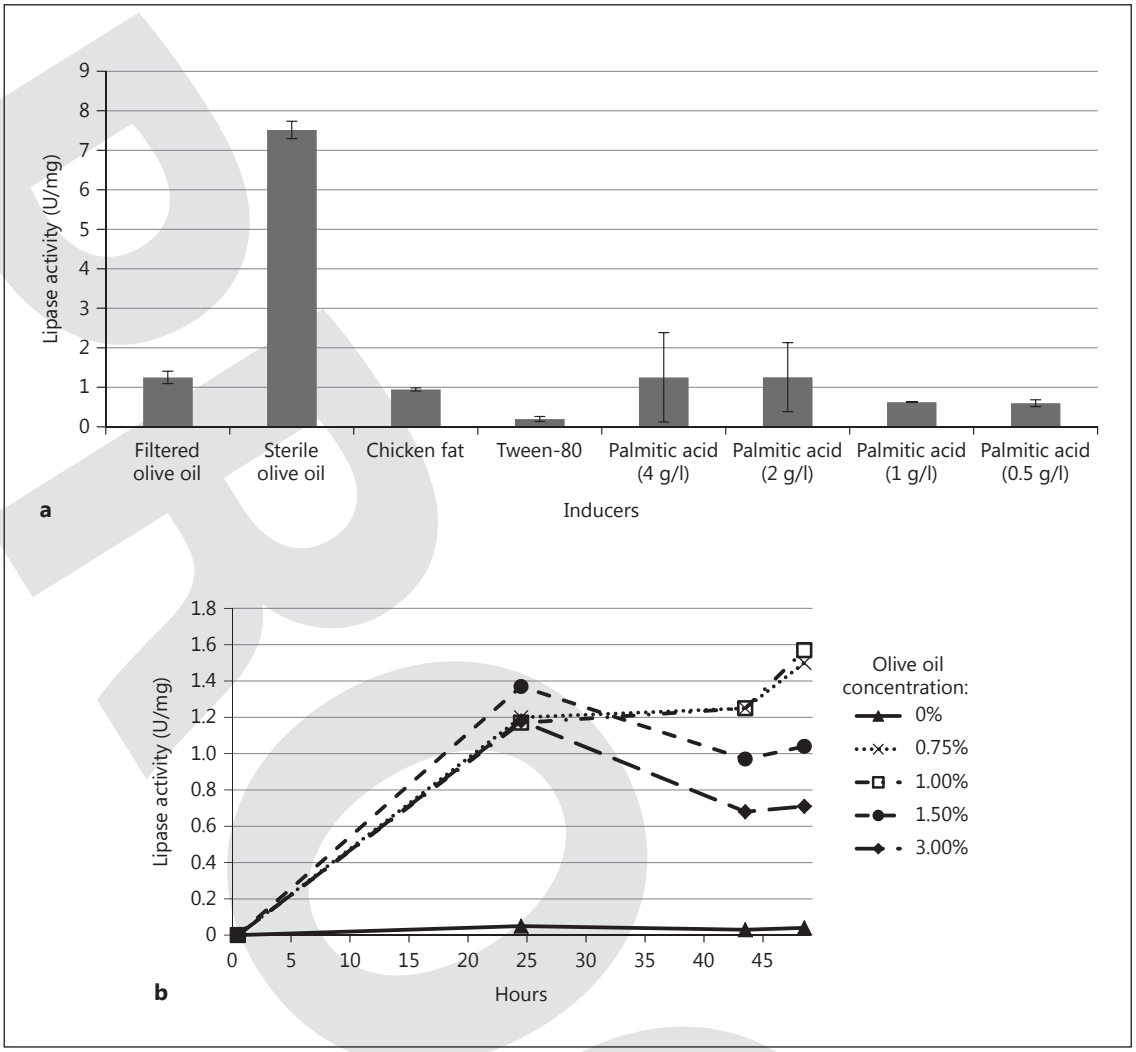


Fig. 1. Lipase activity secreted by *C. zeylanoides* YM7 expressed per mg of dry yeast mass of cells grown in YPD medium containing different potential inducers (1% filtered olive oil; 1% heat-sterilized olive oil; 1% chicken fat; 0.1% Tween 80; 0.5–4 g/l palmitic acid, respectively) (**a**); YNB medium supplemented with different concentrations of heat-sterilized olive oil (**b**).

heat-sterilized chicken fat, or palmitic acid as inducers. Nonionic detergents like Tween-80 could not induce the enzyme production at all.

To establish the optimal inducing concentration of heat-sterilized olive oil, the strain YM-7 was cultivated in YNB media supplemented with olive oil in concentrations from 0 to 3%. Figure 1b shows that in the presence of 1% of inducer, the activity of secreted lipase per mg yeast biomass reached the maximum. Therefore, this olive oil concentration was used in further experiments.

To establish if the types of N-sources in the culture media influence lipase synthesis, yeast growth and lipase production on YP medium were compared to those in YNB medium, both supplemented with 0.1% glucose and 1% sterilized olive oil. In both, media cells reached the stationary phase after about 24 h and the number of cells in YNB medium was about half of that in YPD. Additionally, lipase activity per mg biomass was about 9-fold higher in YPD, suggesting that besides olive oil, the organic N-sources that are missing in YNB medium are required for optimal lipase synthesis. Moreover, in supplemented YPD, the activity per mg continued to increase for another 24 h, reaching a value about 12-fold higher than that in the supplemented YNB medium (data not shown).

Table 1. Purification of *C. zeylanoides* lipase by hydrophobic chromatography (Octyl-Sepharose CL-4B column)

	Total volume, ml	Activity, U/ml	Total activity, U	Protein concentration, mg/ml	Total proteins, mg	Specific activity, U/mg protein
Lipase-containing culture supernatant	50	1.81	90.78	0.115	5.76	15.75
Column flow-through	15	0.526	7.93	0.070	1.06	7.58
Elution (lipase) fraction	5	2.31	11.55	0.021	0.10	110.96

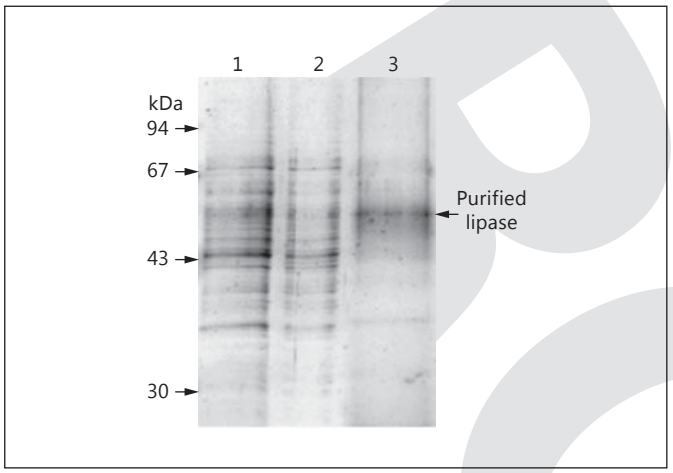


Fig. 2. Purification of *C. zeylanoides* lipase from growth medium using hydrophobic chromatography. Lane 1: culture supernatant containing lipase; lane 2: proteins eluted from the column in the flow through; lane 3: lipase eluted from the column. Positions of the molecular weight standards are indicated at the left; lipase band is marked with the arrow at the right.

When expressed in lipase units per ml of growth medium, the highest yields obtained in different experiments varied between 5.29 and 7.56 U/ml.

Purification and Properties of Secreted Lipase

Lipase secreted by *C. zeylanoides* YM-7 has been purified as described in Experimental Procedures. Initial attempts to purify the enzyme by ammonium sulfate precipitation failed since the method resulted in almost complete loss of enzymatic activity. Lipase could be precipitated at 50% saturation together with most of the proteins present in the medium, which led to a significant decrease of the specific activity. Therefore, this procedure was concluded as inadequate and abandoned. Ion exchange chromatography on DEAE-cellulose was unsuccessful as well since binding of lipase to the exchanger was practically irreversible and also brought about the enzyme inactivation. Purification of *C. zeylanoides* lipase by hydrophobic chromatography (Octyl-Sepharose CL-4B column) as described in Experimental Procedures led to success, as it resulted in a more than 7-fold increase in specific activity (table 1).

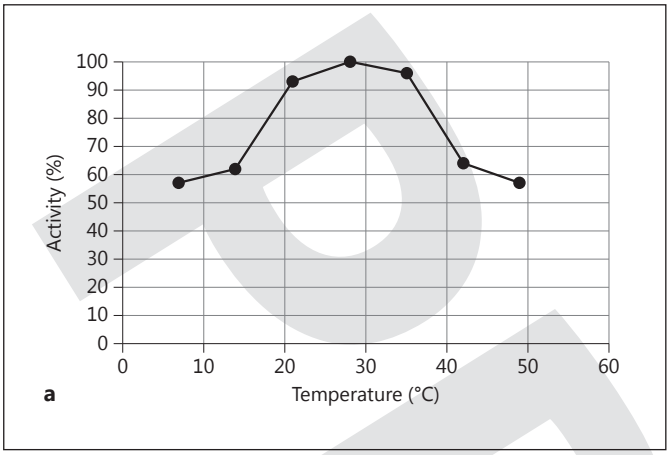
Proteins present in the eluted fraction were separated by SDS-PAGE as described in Experimental Procedures. Figure 2 illustrates that the enzyme was purified to a high degree. The gel was scanned and analyzed using Image J software. Estimated lipase purity was 89%. The purification yield, however, was only about 20%. Nevertheless, it resulted in an apparently pure lipase, enabling the study of its enzymatic properties.

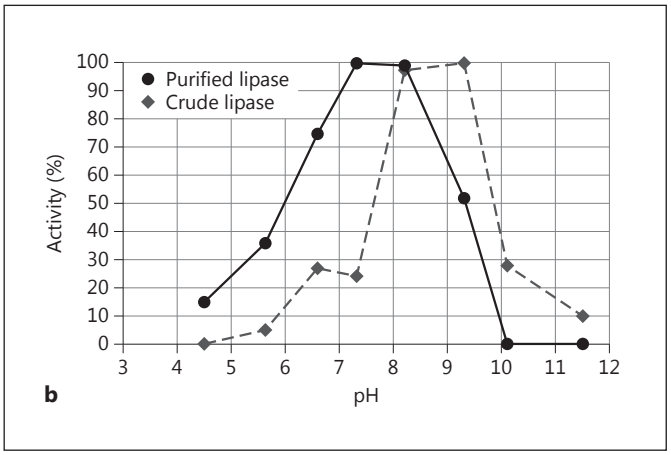
The calculated molecular mass of the protein was about 50 kDa. To estimate the Km value of lipase for p-nitrophenyl-palmitate, the enzyme activity was assayed using substrate concentrations in the range of 0.6–6.0 mM. The estimated Km value was 4.47 mM.

The temperature profile of the enzyme activity was determined in the range of 7–49°C. As can be seen in figure 3a, the optimal temperature for the lipase activity was 28°C. It can also be seen that the enzyme activity at 7°C was as much as 60% of that at the optimal temperature, which makes the *C. zeylanoides* lipase a good candidate for potential application at low temperatures.

The influence of pH on enzyme activity has been determined in the pH range of 3.5–11.5, and the optimal pH for the purified lipase was found to be between 7.5 and 8.0. When the crude preparation of the enzyme (growth medium was concentrated on Amicon Ultra Centrifugal Filters) was tested, the activity profile shifted towards higher pH values with the optimum between 8.2 and 9.3 (fig. 3b).

The influence of ethanol on the lipase activity was checked in the range of 10– 30%. Addition of 20% ethanol increased the enzyme activity by 44%; however, increasing to 30% did not further influence enzyme activity (data not shown).





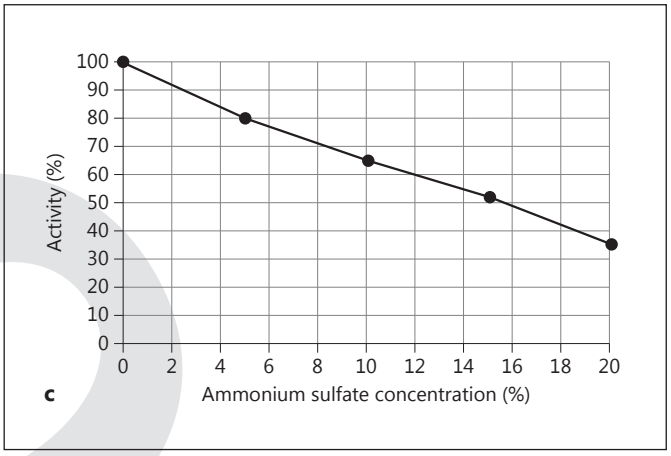


Fig. 3. Activity of purified lipase determined at different temperatures (**a**), different pH values (**b**) (activity of purified enzyme is compared to that of the crude preparation), and different ammonium sulfate concentrations (**c**). The highest activity obtained is designated as 100%.

Stability of C. zeylanoides Lipase

Since the attempts to use ammonium sulfate precipitation as the first purification step failed due to the loss of enzyme activity, stability of lipase at different salt concentrations was investigated. The obtained results indicated that the increase of ionic strength by the addition of ammonium sulfate brought about significant loss of activity (fig. 3c). This, however, does not seem to be due to denaturation since further incubation of the enzyme for 24 h at the same ammonium sulfate concentrations did not lead to any further loss of activity. Similar results were obtained when NaCl was added to the enzyme solution, although the effect was somewhat weaker, as lipase lost about 20% of activity in 0.5 M NaCl (data not shown). Again, further incubation in 0.5 M NaCl did not influence the enzyme activity.

Stability of the enzyme in Triton X-100 was also tested at different concentrations from 0.25 to 1. This detergent had a strong influence on lipase activity as the enzyme lost about 50% of its original activity upon addition of 1% Triton X-100. Investigation of the lipase stability at different ethanol concentrations revealed that it was stable for 24 h in up to 20% ethanol, while in a 30% ethanol solution it lost about 35% of its activity during this period.

C. zeylanoides lipase proved to have relatively low stability at higher temperatures. At 50°C the enzyme lost 90% of its activity in 5 h, while at 60°C it became completely inactive within 4 h (data not shown). Incubation at 28°C, or at 6°C, did not decrease lipase activity in 5 h, and the enzyme was kept in the refrigerator at 4°C. Freezing of the purified enzyme solution at –20°C did not harm lipase, but the activity decreased by about 15% after incubation at this temperature for 5 h.

Regarding the lipase stability at different pH values, the enzyme was stable at a pH around neutral; however, unlike most yeast secreted proteins, it completely lost activity at pH 5 within 5 h.

Discussion

Despite the fact that lipases are present in every living organism, only microbial lipases are of potential biotechnological importance. Reflecting their physiological roles in lipid anabolism, lipases are either located intracellularly, usually in internal storages like lipid particles [Athenstaedt and Daum, 2005], or they are secreted into the medium. Secreted enzymes are of more biotechnological importance. Pronounced versatility of applications of this class of enzymes led to their purification and characterization from a number of bacteria and fungi [Kirk et al., 2002; Sharma et al., 2001]. Often, technologically important producers have been searched for among thermophilic microorganisms synthesizing enzymes active at elevated temperatures. However, cold-active enzymes have also attracted much attention due to their versatile applications in processes requiring lower temperatures. Thus, significant effort has been devoted to characterization of microbial cold-active lipases, either of bacterial or fungal origins. Usually, they were isolated from psychrotrophic microorganisms living either in cold climate parts of the world, or from refrigerated food, respectively [Babu et al., 2007]. So far, the best characterized fungal cold-active lipase is the one secreted by the basidiomycetous yeast *C. antarctica* (now *P. antarctica*) [Anderson et al., 1998]. Additionally, in the last several years, cold-active lipases produced by *Candida albicans* [Lan et al., 2011], *Y. lipolytica* [Fickers et al., 2011; Pignede et al., 2000; Sathish Yadav et al., 2011] and *Aspergillus nidulans* [Mayordomo et al., 2000] have also been described.

Recent investigation of the microbial population isolated from the chilled poultry meat revealed that, together with the most often isolated psychrotrophic bacteria of the genus *Pseudomonas*, lipolytic yeasts like *C. zeylanoides*, *Y. lipolytica*, *M. pulcherrima*, *R. glutinis,* and *R. mucilaginosa* were represented in significant quantities [Belák et al., 2011; Ismail et al., 2000]. Screening of these yeasts for cold-active enzymes turned attention to *C. zeylanoides* as some strains proved to be good lipase producers. In spite of the fact that its frequent occurrence and persistence in spoilage microbiota of dairy products and raw poultry are well documented, conditions for lipase activity and characteristics of the enzymes have not yet been investigated. Therefore, the aim of this work was to purify and characterize extracellular lipase secreted by *C. zeylanoides*, as well as to investigate the growth conditions influencing enzyme biosynthesis. In most microorganisms that secrete lipases, the biosynthesis of these enzymes is induced by lipids in the growth medium [de Maria et al., 2005; Fickers et al., 2004; Sharma et al., 2001]. Similarly, it was proven that *C. zeylanoides* YM-7 did not produce a detectable amount of lipase if lipids were not present in the medium. Different lipids were tested and it was found that sterile olive oil was a better inducer than free fatty acids, while nonionic detergents were quite ineffective. Interestingly, filtered olive oil was not as effective as the heat-sterilized oil indicating that filtration probably removed certain components highly efficient in extracellular lipase induction of *C. zeylanoides*. The most studied lipase-producing yeast, *Y. lipolytica,* possesses 16 paralogs of genes encoding lipase, and the expressed lipases represent different isoenzymes. Lip2p, Lip7p, and Lip8p, which are different in substrate specificity (ranging from medium- to long-chain fatty acids), as well as in the inducers as recently reviewed by Fickers et al. [2011], have probably the most important physiological roles. Interestingly, the biosynthesis of extracellular cold-active lipase from *Y. lipolytica* was not induced by olive oil (although it was an excellent inducer of the cell-bound isoenzyme), but a much higher production was detected if Tween 80 was added to the growth medium [Sathish Yadav et al., 2011]. Fickers et al. [2004] established that the concentration of oleic acid in the medium had little influence on Lip2p lipase production but, at the same time, the biosynthesis is highly dependent on the source of nitrogen. In *C. rugosa,* the nature of the inducer controls the production and ratio of the three lipase isoenzymes. Secretion of Lip2 and Lip3 was induced by all of the tested inducers, while Lip1 was produced only using n-dodecanol or oleic acid [de Maria et al., 2005]. Such results indicate that different factors are involved in the regulation of extracellular lipase production in different yeasts and that our knowledge of the involved mechanisms is still insufficient. Other yeasts producing lipases active at higher temperatures also required lipids as carbon sources for the induction of lipases, but the production regulation was again species dependent [for review see Sharma et al., 2001]. Concerning the amount of lipase secreted by different yeasts, literature data report a whole range of values which can, to some extent, be attributed to different growth conditions, different assay procedures, and different substrates used. Sharma et al. [2001] compared different lipase producers secreting enzymes between 0.5 and 12.5 U/ml. For *A. nidulans,* much lower activities around 0.16 U/ml were reported [Mayordomo et al., 2000], while a higher value of 25.3 U/ml was found for *Pichia*

lynferdii [Kim et al., 2010]. In the same study, different *Candida* and *Pichia* species were compared and productivities between 0.22 and 5.67 U/ml were obtained. In our experiments the production of lipase under optimal conditions ranged between 5.29 and 7.56 U/ml which is comparable with other yeast strains reported to be good lipase producers.

The most interesting property of enzymes for potential application at low temperatures is of course the temperature activity profile. As can be seen in figure 3, *C. zeylanoides* lipase has highest activity at about 28°C and, more importantly, at 7°C its activity amounts to about 60% of that at the optimal temperature. Compared to other yeast cold-active lipases, it is similar to the enzyme from *C. albicans* [Lan et al., 2011], somewhat lower than that of the enzyme from *P. lynferdii* Y-7723 [Kim et al., 2010], and significantly higher than the activity of the best characterized cold-active lipase from *C. antarctica,* whose activity measured at 5 or 10°C was below 10% of the maximal activity at 35°C [Adamczak, 2003]. The highest activity at temperatures below 10°C compared to maximal activity was reported for the yeast *Geotrichum* sp. [Cai et al., 2009]. Lipolytic activity of the cold-active lipase from *Y. lipolytica* had a lower optimal temperature (20°C); at 10°C the activity was about 40% of the optimal, but at 5°C almost no activity was detected [Sathish Yadav et al., 2011]. Similar properties were reported for the enzyme from *A. nidulans,* but with somewhat better activity at temperatures below 10°C [Kim et al., 2010]. Such results, at least to some extent, question the application potential of some of the lipases described as cold-active at low temperatures. It has to be mentioned, however, that these data only speak of the level of adaptation of lipases from different species to low temperatures, but do not reflect their actual specific activities, which surely have to be taken into account in choosing the most appropriate producer. One of the potential comparative advantages of lipase described in this paper is its relatively high activity at higher temperatures (over 50% of the activity measured at optimal temperature, see fig. 3), which is usually not the property of cold-active enzymes. *C. zeylanoides* YM-7 grows well at temperatures between 28 and 30°C and, if the appropriate inducer is applied (fig. 2, 3), produces high amounts of enzyme which makes this yeast a promising producer microorganism for this class of enzymes.

Regarding the *C. zeylanoides* lipase stability, like most cold-active enzymes it was rather unstable at temperatures above 40°C. A specific property of this enzyme, however, was its low stability at high ionic strengths, which may present a significant limit to its potential application. This also limited the choice of methods for the purification of the enzyme. However, hydrophobic chromatography proved to be an efficient method which brought about purification to about 90% purity in a single step. Similar results have been described for the purification of the enzyme from *A. nidulans* [Mayordomo et al., 2000], *Candida rugosa* [Benjamin and Pandey, 2001], and *Cryptococcus* sp. S-2 [Kamini et al., 2000]. Specific activities of purified lipases ranged between 64 and 865 U/mg; however, low yields were recorded, which points out that there is still significant space for further optimization and new approaches in the purification procedures.

Experimental Procedures

Yeast Strains and Media

C. zeylanoides strains SZ1/9, YC-1, YC-4, YM-2, YM-7, and YS-7 isolated from samples of the refrigerated poultry meat as described in Belák et al. [2011], as well as the type strain CBS 619 were used in this work. The strains are maintained in the culture collection of the Department of Microbiology and Biotechnology, Corvinus University of Budapest, and are publicly available on request. Yeasts were grown in standard YNB (Sigma-Aldrich) or YPD (10 g/l yeast extract, 5 g/l peptone, 10 g/l glucose) media supplemented with different inducers of lipase biosynthesis (as indicated in different experiments) at 28°C on a rotary shaker (170 rpm) for 48 h.

Enzyme Purification

After 48 h of cultivation, the cell-free supernatant supplemented with 0.2 M ammonium sulfate was loaded to an Octyl-Sepharose CL-4B (Pharmacia) column (20 ml) previously equilibrated with binding buffer (50 mM phosphate buffer, pH 7, containing 0.2 M ammonium sulfate). Unbound material was washed out with 90 ml of binding buffer and elution was performed with 20 ml of 10 mM phosphate buffer, pH 7, containing 0.5% Triton X-100. All steps were performed at 5°C.

Lipase Activity Assay

Lipase activity was measured by a slightly modified method of Winkler and Stuckmann [1979]. Substrate solution was prepared by mixing nine parts buffer A (50 mM phosphate buffer, pH 7, containing 1.1 mg/ml gum arabic and 4.4 mg/ml Triton X-100) with one part *p*-nitrophenyl palmitate dissolved in isopropanol (1 mg/ml). 0.4 ml of the enzyme-containing solution was added to 0.1 ml of 50 mM phosphate buffer (pH 7), and after 5 min of incubation at 30°C the reaction was initiated by adding 0.5 ml of substrate solution. The substrate concentration in the final reaction mixture was 0.19 M. Absorbance (A_{410}) of the reaction mixtures was measured immediately (t_0) and after 30 min of incubation (t_{30}) at 30°C. The difference in absorbance between t_{30} and t_0 was used to determine enzyme activity. One unit of lipase activity was defined as the amount of enzyme required to release 1 µmol of p-nitrophenol in 1 min.

Effect of pH and Temperature on Lipase Activity

The optimal pH was determined by the assay described above using different buffers (50 mM) ranging between pH 3.5 and 11.5 (citric acid-sodium phosphate, pH 4.5–6.8; sodium phosphate buffer, pH 7.2–8.2; glycine-NaOH, pH 9.2–10.0; sodium phosphate-NaOH, pH 11.5). Optimal temperature was determined by incubating the enzyme between 7 and 49°C.

Lipase Stability

pH stability of lipase was determined by incubating equal volume of the enzyme solution with 50 mM buffers ranging between pH 5 and 8 at 23°C for 5 h. Residual activities were determined periodically at 1-hour intervals. For thermostability determination, the enzyme was incubated at temperatures ranging between –20 and 60°C for up to 5 h, and the residual activities were determined periodically at 1-hour intervals.

The effect of ammonium sulfate (from 5 to 20%), ethanol (10, 20, and 30%), NaCl (from 0.1 M to 0.5 M), or Triton X-100 (from 0.25 to 1%) on enzyme activity was determined by standard procedure in the presence of annotated reagents in the reaction mixture.

Protein Analysis

Protein concentration was estimated according to Bradford [1976] using bovine serum albumin as the standard.

Protein samples for electrophoresis were prepared by methanol/chloroform (2:1 v/v) precipitation. Precipitated proteins were washed once with methanol and resuspended in Laemmli sample buffer. SDS-PAGE was performed using 12% (w/v) acrylamide gel as described by Laemmli [1970]. Proteins were stained by the silver staining procedure.

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