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Full Length Research Paper

Biochemical characterization of a thermoactive and thermostable lipase from a newly isolated *Trichosporon coremiiforme* strain

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Nonstop demand for greatly thermostable and thermoactive active lipase encourages the research for the new enzyme sources. In this study, a strain of *Trichosporon coremiiforme* was isolated from the traditional tannery in the city of Fez in Morocco, lipase production and their lipolytic activity was studied. Pure *T. coremiiforme* lipase (TCL) was obtained after ammonium sulfate fractionation, G-75 gel filtration and cation exchanger chromatography (Mono-S), having a molecular weight of 67 kDa. TCL presents a maximal activity at pH 8 and 50°C. After a 5 min treatment at 80°C, the enzyme maintained 50% of its activity, which is so far as is known. TCL previously characterized is found to be stable between pH 5 and 10 after 60 min incubation. TCL hydrolyses the long chains triacylglycerols more efficiently than the short ones. A specific activity of 1800 U/mg was measured on tributyrin or olive oil emulsion as substrate. This newly isolated lipase can be considered as a good candidature for industrial and biotechnological applications.

Key words: *Trichosporon coremiiforme*, lipase, purification, thermoactive.

INTRODUCTION

Lipases (EC 3.1.1.3) is a group of enzymes which catalyses the hydrolysis of triacylglycerols (Macrae and Hammond, 1985; Brockerhoff and Jensen, 1974). The lipases can catalyze a range of bioconversion, such as inter-esterification, esterification and aminolysis. They catalyse the hydrolysis of fatty acid ester bond in the triacylglycerol (TAG) and release free fatty acids (FFa) and glycerol (Sheldon, 1993). The reaction is reversible; the direction of the reaction depends upon the water content available in the reaction.

Microbial lipases are able to catalyze not only hydrolysis, but also the synthesis of long-chain triglycerides in low-hydrated medium. On the other hand, they can accommodate a large number of substrates, which

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Abbreviations: TCL, *Trichosporon coremiiforme* lipase; **BSA,** bovine serum albumin; **EDTA,** ethylene diamine tetraacetic acid; **NaDC**, sodium deoxycholate; **SDS/PAGE,** sodium dodecyl sulfate/polyacrylamide gel electrophoresis; **FFa,** free fatty acids; **TAG,** triacylglycerol; **TC_2,** triacetin; **TC_3,** tripropionin; **TC_4,** tributyrin; **GA,** Gum Arabic

combines their important applications in biocatalysis (Gupta et al., 2004). Yeast lipase production is not well developed despite the fact that *Candida rugosais* is the most frequently used organism for lipase synthesis. Indeed, the extreme environments as source of isolation and selection of useful microorganisms have been highlighted (Kademi et al., 2003) and the progress in this area has been be possible with the isolation of large number of microorganisms from different ecological zones of the earth and subsequent extraction of useful enzymes from them (Sangeetha et al., 2010). Tanning is a chemical process, which aims to transform hides into leather by making them more durable and more flexible. In tanning, the skins are cleaned of an outer coating hair and dirt is removed; on their inner surface, the inner skin is removed. The hides are then soaked in tanks containing tannin for a certain period, and then they are removed, washed and dried. In our knowledge, no works have been made on lipase-producing strains isolated from tannery. The aim of this study is to isolate and screen the lipolytic microorganisms in the extreme source of the medina of Fez Morocco tannins. The lipase extracted was purified to homogeneity and biochemically characterized.

MATERIALS AND METHODS

Chemicals

Tributyrin (99%; puriss) and benzamidine were from Fluka (Buchs, Switzerland); yeast extract and ethylene diamine tetraacetic acid (EDTA) were from Sigma Chemical (St. Louis, USA); acrylamide and electrophoresis grade were from British Drug Houses (BDH) (Poole, United King-doom); casein peptone was from Merck (Darmstadt, Germany).

Screening of lipolytic microorganisms

Visualization of lipolytic activity on solid media was determined by using dye Victoria blue B (Shelley et al., 1987). Initial screening of lipolytic microorganisms from Tanner of Fez in Morocco was carried out using a plate assay in a medium containing triacylglycerol. The solid medium contained 5% olive oil, 1% nutrient broth, 1.5 g agar and 0.01% Victoria blue B. The Petri dish was incubated at 30°C. Lipolysis is observed directly by changes in the appearance of the substrate. Lipase production is indicated by the formation of clear blue halos around the colonies grown on agar plates containing triacylglycerol.

Culture conditions

The inocula was pre-cultured during 24 h at 30°C and 200 rpm in 250 ml shaking flasks with 50 ml of medium A (20 g/l casein peptone, 5 g/l yeast extract (Difco), 20 g/l glucose, pH 7.4). Overnight, *Trichosporon coremiiforme* cultures used as inocula were cultivated in 1 L shaking flasks with 100 ml of medium A. The initial absorbance (OD) measured at 600 nm was adjusted to an approximate 0.2 value. The culture was incubated aerobically for 96 h on a rotary shaker set at 200 rpm at a temperature of 30°C. Growth was followed by measuring the OD of the cultures at 600 nm.

Lipase activity determination

The lipase activity was measured titrimetrically at pH 8.0 and 55°C with a pH-stat under standard conditions using tributyrin (0.25 ml) in 30 ml of 2.5 mM Tris-HCl pH 8.5, 2 mM $CaCl_2$, 2 mM sodium deoxycholate (NaDC) or olive oil emulsion (10 ml in 20 ml of 9‰ NaCl pH 8.5, 2 mM $CaCl_2$, 2 mM NaDC) (Rathelot et al., 1975) as substrate. Lipase activity was also measured at pH 7 and 37°C using TC_3 as substrate (0.25 ml TC_3) in 30 ml of 2.5 mM phosphate buffer pH 7, 2 mM $CaCl_2$. The olive oil emulsion was obtained by mixing (3×30 s in a Waring blender) 10 ml of olive oil in 90 ml of 10% GA.

Determination of protein concentration

Protein concentration was determined as described by Bradford (1976) using bovine serum albumin (BSA) as standard.

Procedure of lipase purification

The medium culture, obtained after 24 h with an inoculum size of 2 × 10^7 cells/ml, was centrifuged for 30 min at 8000 rpm to remove the microbial cells. The supernatant containing extracellular lipase was used as the crude enzyme preparation. The crude enzyme solution (1-L), containing 17 000 units, was brought to 70% saturation with solid ammonium sulphate (472 g) under stirring conditions at 4°C. After centrifugation (30 min at 10 000 rpm), the precipitate was resuspended in 15 mL of buffer A (20 mM sodium acetate, pH 5.4, 20 mMNaCl, 2 mMbenzamidine). Insoluble material was removed by centrifugation 10 min at 10 000 rpm. The supernatant (15 mL) was loaded on a column (3 × 100 cm) of gel filtration G-75 equilibrated with buffer A. Elution of lipase was performed with the same buffer at a rate of 40 ml/h. The fractions containing the lipase activity (eluted at one void volume) were pooled.

Analytical methods

The lipase activity was tested after dialysis (Shelley et al., 1987), and also tested after denaturation by heating the dialyzed lipase 5 min at 80°C (Shelley et al., 1987). Analytical polyacrylamide gel electrophoresis of proteins in the presence of sodium dodecyl sulfate/polyacrylamide gel electrophoresis (SDS-PAGE) was performed following the method of Laemmli (Laemmli, 1970). Samples are electroblotted according to Bergman and Jornvall (1987). Protein transfer was performed during 1 h at $1mA/cm^2$ at room temperature. The lipase was run on native PAGE (15%, without SDS) for zymography. Gel was transferred on a 2% agar plate containing 5% olive oil, 1% nutrient broth, 1.5 g agar and 0.01% Victoria blue B for lipase activity detection. After incubation for 3 h at 60°C, lipase activity was visualized as a band of clearance on the olive oil plate (Shelley et al., 1987).

Kinetic study

Lipase activities were measured as a function of various substrate (TC_4, TC_8 or TC_{18}) concentrations (0 to 40 mM). The Michaelis-Menten constant (Km) and the maximum velocity (Vmax) for the reaction with TC_4, TC_8 or TC_{18} as substrate were calculated by Lineweaver-Burk plot.

Effect of pH and temperature on the activity and stability of *Trichosporon coremiiforme* lipase (TCL)

The activity of lipase was examined within the pH range of 6.0 to

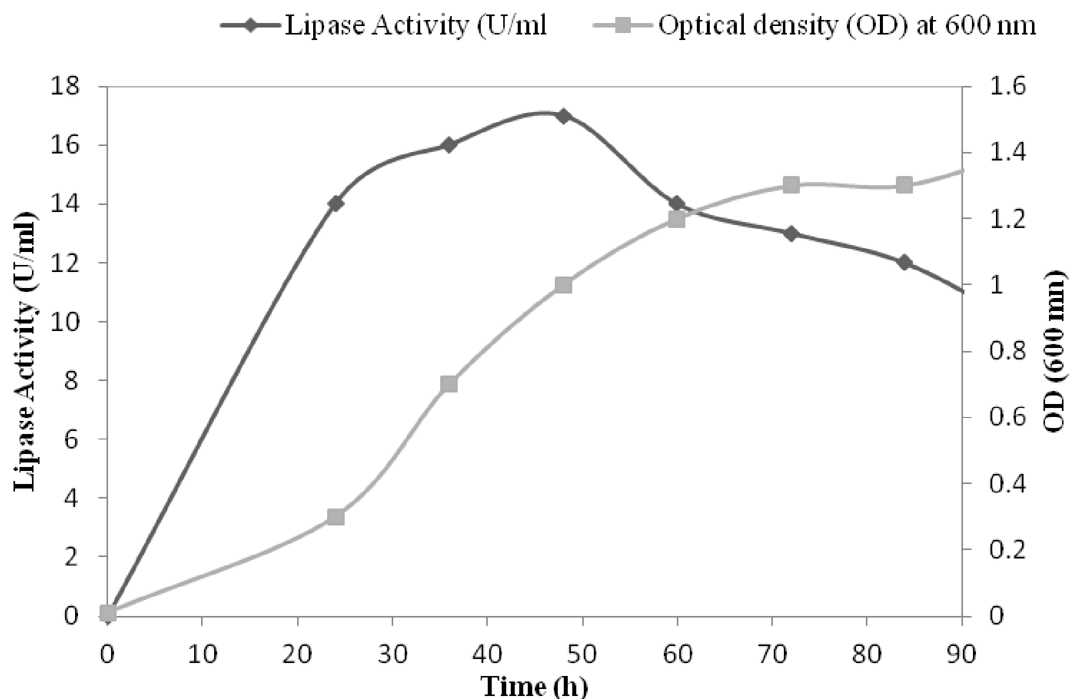


Figure 1. The time courses of lipase production. The culture was carried out at 30°C in shaking at 200 rpm in the presence or in the absence of triacylglycerols or esters.

10.0. The lipase activity was measured titrimetrically at pH 8.0 and 37°C with a pH-stat under standard conditions using tributyrin (0.25 mL) in 30 mL of 2.5 mMTris-HCl, pH 8.5, 3 mM $CaCl_2$ or olive oil (10%) emulsion (10 mL in 20 mL of 2.5 mM Tris-HCl, pH 8.5, 3 mM $CaCl_2$) (Rathelot et al., 1975) as substrate. The effect of pH on lipase stability was determined by incubating the lipase fraction in various buffer solutions ranging from 3.0 to 10.0 for 24 h at room temperature. After the incubation period, the residual activity was determined, after centrifugation, under standard assay method (Rathelot et al., 1975). The optimum temperature for the *T. coremiiforme* lipase activity was determined by carrying out the enzyme assay at different temperatures (20 to 90°C) at pH 8.0. The effect of temperature on lipase stability was determined by incubating the enzyme solution at different temperatures (20 to 90°C) for 60 min. The residual activity was determined, after centrifugation, under standard assay method (Rathelot et al., 1975).

RESULTS

Production of lipase

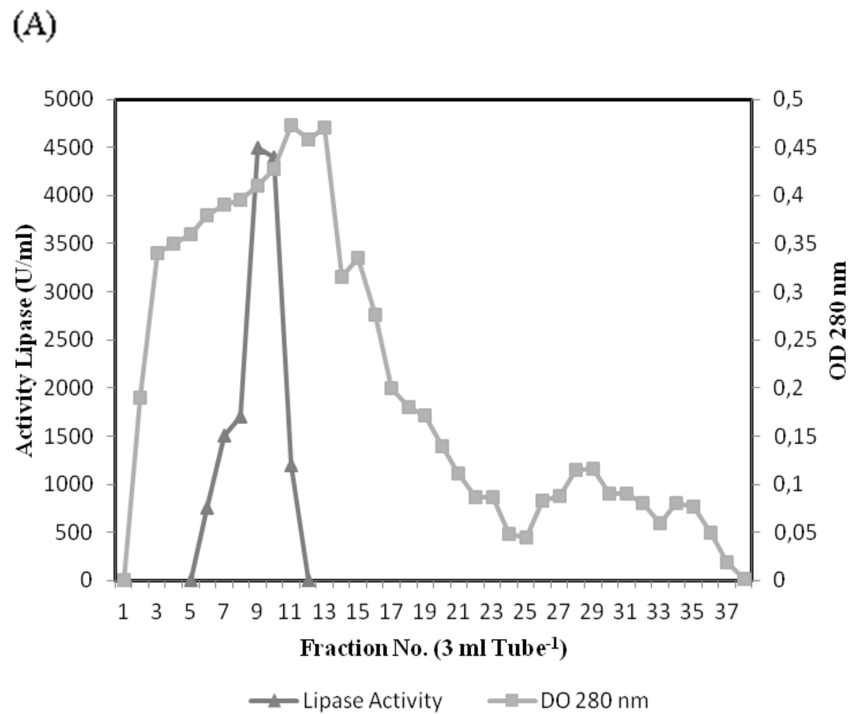
The maximum lipase production for the strain (17 U/ml) was obtained after 48 h incubation (Figure 1). After this time, the lipase activity dramatically decreases. Lipases produced by *Trichosporon* species have been reported in the literature (Chen et al., 1992). *Trichosporon fermentans*WU-C12 isolated from soil showed a maximum lipase production after four days of growth at 30°C. Another lipase produced by a *Trichosporon* species, *Trichosporon asteroid*, was isolated from raw milk (Dharmsthiti and Ammaranond, 1997).

Purification of lipase

The TCL was purified according to the procedure described in the above. The protein elution profile obtained at the final step of the purification is shown in Figure 2A. This figure shows that the lipase was eluted at 1.3 Vo. The results of SDS/PAGE analysis of the pooled fraction of this last step of chromatography are given in Figure 2B. This figure shows that the enzyme exhibited one band corresponding to a molecular mass of about 67 kDa. The purification flow sheet is given in Table 1 which shows that the total activity of 8700 U/ml corresponding to 4.8 mg of proteins with activity recovery of 33.5%, the specific activity of TCL reaching 1800 U/mg was measured at pH 8 and 50°C with gum arabic emulsified olive oil as substrate in the presence of 2 mM $CaCl_2$, and 2 mMNaDC. Under the same conditions, a specific activity of 1700 U/mg was obtained when using TC_4 as substrate. These results show that it is able to hydrolyse triacylglycerols without significant chain length specificity.

Kinetic studies

It has been established that some mammal pancreatic lipases may lack enzymatic activity when TC_4 is used as substrate in the absence of bile salt and colipase. The high energy existing at the tributyrin/water interface is responsible for their irreversible denaturation (Gargouri et al., 1995). Figure 3 shows that TCL is able to hydrolyse



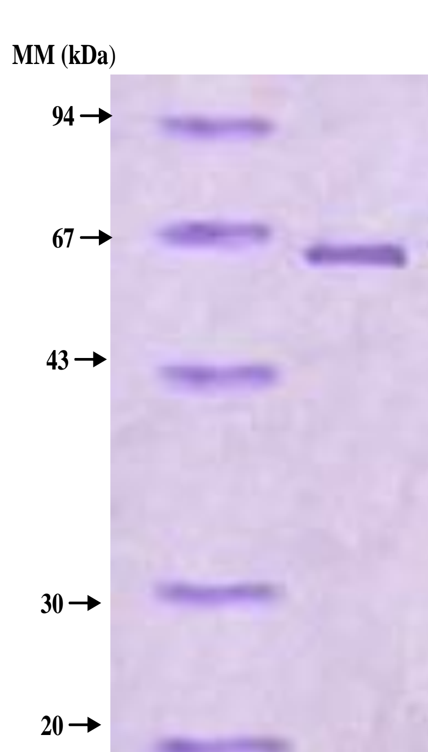


Figure 2. (A) Chromatography of TCL on sephadex G-75. The column(3 × 100 cm) was equilibrated with buffer A (20 mM sodium acetate pH 5.4, 20 Mm NaCl, 1 mMbenzamidine). The elution of lipase was performed with the same buffer at a rate of 30 ml/h. Lipolytic activity was measured under standard conditions at pH 8.00 and 50°C using a pH-stat. **(B)** SDS/PAGE (15%): Lane 1, Molecular mass markers (Pharmacia); lane 2, characterisation of the TCL obtained after sephadex G-75 chromatography.

Table 1. Flow sheet of the strain TC lipase purification.

Purification step	Total activity (U.ml)[a]	Protein (mg)[b]	Specific activity (U/mg)	Activity recovery (%)	Purification factor
Culture supernatant	17000	1700	10	100	1
$(NH_4)_2SO_4$ precipitation	12200	870	14	68.88	1.4
G-75 chromatograghy	11300	710	15.9	41.17	1.59
Mono-S-chromatography	8700	4.8	1800	33.25	180

[a]1 unit corresponds to 1 µmol of fatty acid released per minute; [b]proteins were estimated by Bradford (1976).

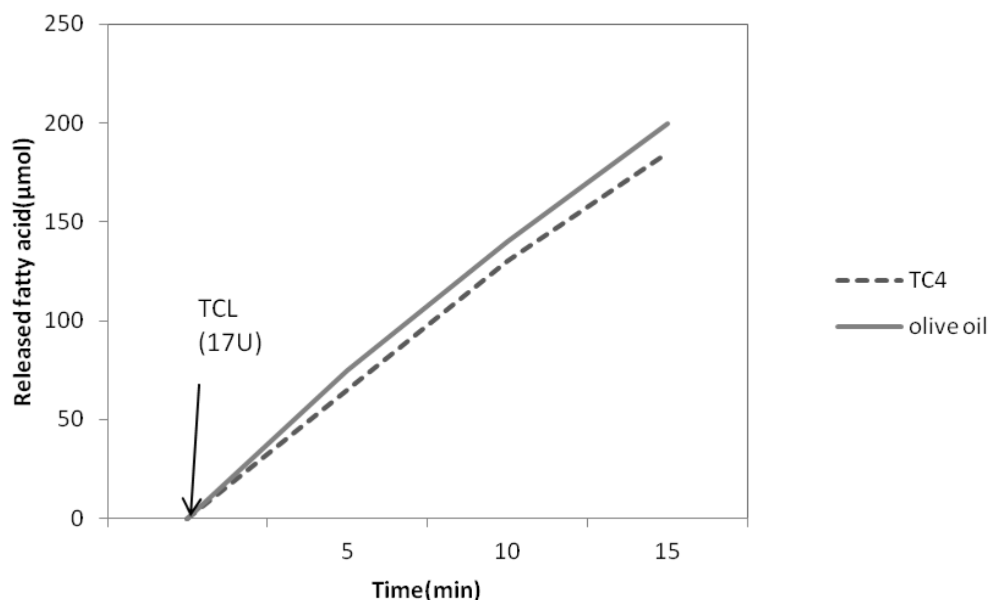


Figure 3. Kinetic of hydrolysis of olive oil or tributyrin emulsions by TCL (17 U).Lipolytic activity was followed at pH 8 and 50°C.

the TC_4 or the olive oil emulsion. The kinetic remains linear for more than 15 min. Accordingly, TCL remains active despite the presence of long chain free fatty acid at the olive-oil/water interface.

Activation of TCL by interface

As it has been shown by Ferrato et al. (1997) among the short chain triacylglycerols tested as substrates (TC_2, TC_3, TC_4), TC_3 is the best system to check the interfacial activation of lipases. In this study, we have selected TC_3 to evaluate the interfacial activation phenomenon of TCL. The hydrolysis rate of TC_3 emulsified in 0.33% GA and 0.15 M NaCl by TCL as a function of substrate concentration shows a normal Michaelis-Menten dependence of the activity on the substrate concentration (Data not shown). The interfacial activation cannot be taken, as described by Sarda and Desnuelle (1958), as the unique criterion required to distinguish lipases from esterases (Ferrato et al., 1997). Lipases are defined as a family of enzymes able to hydrolyse long chain triacylglycerols independently of the presence, or the absence, of an interfacial activation phenomenon. Here, we can say that TCL, which hydrolyses olive oil, is a true lipase.

Effect of pH and temperature on the activity and stability of *T. coremiiforme* lipase

As shown in Figure 4A, the activity of the *T. coremiiforme* lipase presents a maximum activity at pH 8.0; beyond this pH value, the activity of the enzyme decreased gradually. Also, our results show that the enzyme activity was found to be stable between pH 5.0 to 10.0 (Figure 4B). This observation is also similar to those found for other *Trichosporons* lipases (Arai et al., 1997; Dharmsthiti and Ammaranond, 1997).

The effect of temperature on the activity of *T.*

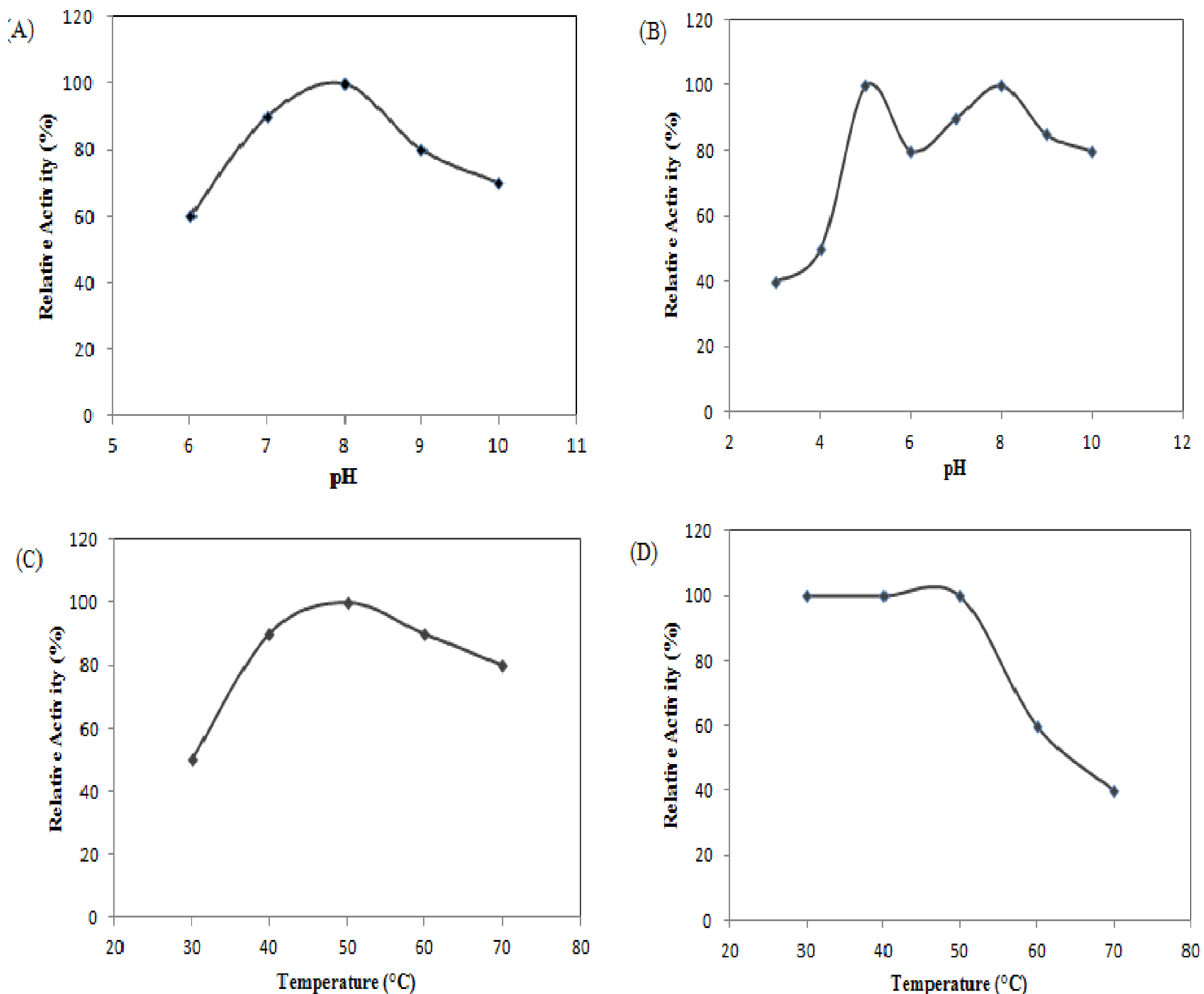


Figure 4. pH effect on enzyme activity (A) and stability (B) of TCL. Optimal pH was determined with tributyrin at 50°C under standard conditions. Stability was analysed after pre-incubating the pure enzyme for 24 h in different buffer solutions at various pH ranging from 3 to 12; C, temperature effect on TCL activity; D, temperature effect on TCL stability. For temperature stability, the pure enzyme was preincubated at different temperatures for 1 h and the remaining activity was measured under the standard conditions.

coremiiforme lipase was also examined in the range of 20 to 90°C. Lipolytic activities of *T. coremiiforme* lipase were found to be profoundly affected by temperature. As shown in Figure 4C, the activity of lipase increased gradually with rise in temperature and the maximum activity was observed around 40 to 60°C. The activity decreased abruptly and the enzyme lost its activity almost completely at 100°C. Furthermore, the thermal stability of lipase was studied from 20 to 90°C. The data shown in Figure 4D, indicates that *T. coremiiforme* lipase has appreciable stability (50 to 90°C) after prolonged incubation of about 60 min. The enzyme exhibited maximum stability at 40 to 50°C but also found to be fairly stable up to 50°C with concomitant loss of activation after 60 min incubation, and it was highly stable at 80°C with half-lives of 5 min.

Effects of bile salts on TCL activity

In order to check if the purified TCL is able to hydrolyse triacylglycerols in the presence of some surface-active agents like bile salts, we measured the hydrolysis rate of TC_4 and emulsified olive oil by TCL in the presence of various NaDC concentrations. We showed that NaDC has no inhibitory effect on the TCL activity even at a large concentration (10 mM) (Figure 5). This result confirms that, in contrast to many lipases described so far from

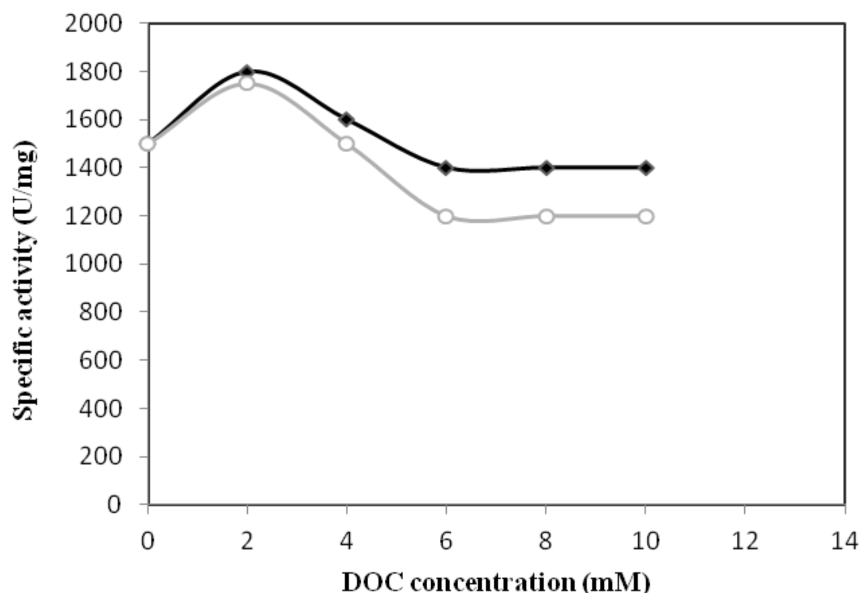


Figure 5. Effect of increasing concentration of NaDC on the rate of hydrolysis of tributyrin (○) and olive oil emulsion (♦) by TCL.lipolytic activity was measured under standard conditions at pH 8 and 50°C using a pH-stat.

different origins (Gargouri et al., 1983; 1984), TCL is able to reach its substrate even in the presence of some surface active agents like bile salts. Comparable results were obtained by Sayari et al. (2001) with SSL. Simons et al. (1997) showed that SHL was not inactivated by anionic detergents such as NaTDC. Hence, it can be deduced that TCL probably presents a penetration power higher than those of pancreatic and some microbial lipases which allows it to hydrolyse TC_4 or olive oil in the presence of bile salts.

DISCUSSION

Some of the common sources of lipases are reported in the literature. Among microbial lipases, extensive reviews have been written on bacterial lipases (Arpigny and Jaeger, 1999). Despite, yeasts are considered to be easy to handle and grow, in comparison to bacteria (Kademi et al., 2003), and *Candida rugosa*is the most frequently used organism for lipase synthesis, yeast lipases have received a raw deal. Among yeasts, *Candida albicans, Candida antarctica, Candida rugosa, Geotrichum asteroids, Geotrichum candidium, Saccharomycops is lipolytica, Yarrowialipolytica,* among others are reported to produce multiple lipase forms. For the first time in this study, our findings show that *T. coremiiforme* is able to produce a lipolytic enzyme. Also, as lipases are generally produced using carbon source such as oils, fatty acids, glycerol or tweens in the presence of organic nitrogen source, in our case, the production of *T. coremiiforme* is not induced by the presence of triacylglycerols (like TC4 or olive oil) or esters (Tween 80). The same findings were described by several authors (Rahman et al., 2010). According to SDS-PAGE, we have a protein of 67 KD molecular size which will be the first protein for this type of yeast so we can say that it is a new protein (Figure 2B). The importance of alkaline and thermostable lipases for different applications has been growing rapidly. A great deal of research is currently going into developing lipases which will work under alkaline conditions as fat stain removers. Our results show that *T. coremiiforme* lipase remains active at a pH range of 6.0 to 10. This result can be very attractive and could have a great potential application in many areas. For example, lipase produced by *Trichosporon asahii* MSR 54 was active over a pH range of 6-10; this enzyme has a great potential for application in the detergent industry (Kumar et al., 2009). Our protein was found to be stable up to 50°C and stable at 80°C with half-lives of 5 min which is so far as is known. Many enzymes produced by bacteria and yeast showed maximum activities at high temperatures, such as *Pseudomonas aeruginosa* (70°C) (Karadzic et al., 2006), a thermophilic *Bacillus* sp. (60 to 70°C) (Nawani and Kaur, 2006), and the *yeast Kurtzmanomyces* sp. (75°C) (Kakugawa et al., 2002); just few fungal lipases reported in literature presented such thermophilic behavior. Among fungi of the genus *Penicillium*, which are mesophilic organisms, most lipases showed maximum activities at temperatures in the range of 25 to 45°C (Costa and Peralta, 1999; Jesus et al., 1999; Stocklein et al., 1993; Tan et al., 2004). One

exception is *Penicillium aurantiogriseum*, which produces a thermophilic lipase with optimum activity at 60°C and pH 8.0 (Lima et al., 2004). Lipase of *T. coremiiforme* maintains its activity despite the denaturing conditions and treatment at high temperature (80°C). These properties allow us to make a zymogram to detect the lipase band on polyacrylamide gel (data not shown). The crude enzyme solution prepared from 70% ammonium sulphate saturation of *T. coremiiforme* was dialyzed against 200 mMTris-HCI buffer, pH 8.4 for 24 h and then heated 5 min in 80°C. The physicochemical properties of *T. coremiiforme* lipase made it among the most efficient lipases and the best candidates in the industrial field (Vakhlu and Kour, 2006).

Conclusion

The identification and the characterization of the *T. coremiiforme lipase* is an important aspect of knowledge for the discovery of new industrial biocatalysts. The optimum pH and temperature of the lipase were 8.0 and 50°C, respectively and it was highly stable at 80°C with half-lives of 5 min. This enzyme presented several differences regarding its production and properties in comparison with a previous report of a lipase produced in submerged fermentation by the same yeasts species, showing enhanced specific activity, greater thermal stability and higher optimum temperature. Then lipase of *T. coremiiforme* can be considered as a good candidate for industrial and biotechnological applications.

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