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Purification and Characterization of a Novel Thermostable Lipase from Aspergillus Flavus

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Abstract

Aspergillus flavus lipase (AFL) was found as a true lipase. The N-terminal amino acid sequence of the AFL had the same N-terminal sequence like some Aspergillus lipases (AL). Pure AFL was obtained after ammonium sulfate fractionation, Sephacryl S-200 gel filtration and anion exchange chromatography (DEAESepharose) presented as a monomer having a molecular mass of about 55 kDa and its activity was maximal at pH 8.5 and 40 °C. AFL hydrolyses the long chains triacylglycerols more efficiently than the short ones. A specific activity of 4300 U/mg was measured on tributyrin or olive oil emulsion as substrate at 40 °C and at pH 8.5. This enzyme presented the interfacial activation phenomenon when using triolein or olive oil emulsion as substrate. AFL was activated when the enzyme was incubated at 70 °C for 30 min at pH 5. This newly isolated lipase can be considered as a good candidature in synthesis reactions such as esterification, transesterification and interesterification which are used in industrial and biotechnological applications.

Key words: *Aspergillus flavus*/ lipase / purification

Introduction

Lipids are a class of organic molecules (fats or oils) that are soluble in organic solvents and which are distinguished from other components of living matter by their insolubility in water. They are formed by an aliphatic backbone, which are cyclic or polycyclic, that constitute the hydrophobic portion, which can be fixed polar groups and training the hydrophilic portion. Lipids include: fats, oils, waxes and certain substances that are related (sterols, steroids, terpenes, etc...), because they are not electrically charged, like glycerides (acylglycerols), cholesterol and its esters which are called neutral lipids.

Lipases (EC 3.1.1.3) are part of the class of carboxylic ester hydrolases. They catalyze the hydrolysis of carboxylic esters bands of long chain of triglyceride insoluble in water (Ferrato *et al.,* 1997). The optimum temperature of lipases is often between 30 and 40 °C. In general, lipases of plant or animal origin are not heat stable, unlike microbial lipases. Indeed, *Humicola lanuginosa* lipase has an optimal activity at 45 °C (Che Omar *et al.,* 1987). Other lipases are adapted to lower temperatures, such as *Aspergillus Niger*, which has an optimum temperature of 25 °C (Fukomoto *et al.,* 1964). The distribution of

Aspergillus flavus extends to the whole world but preferably in the tropics and subtropics. These species draw particular attention because of its carcinogenic toxins that are found on foods and seeds. However, lipases from *Aspergillus flavus* are the subject of many virtues like the ability to achieve synthesis reactions such as esterification (reaction between an acid and an alcohol), the transesterification (ester and alcohol) and interesterification (ester and ester) and in transfer reactions acetyl group of an ester and other nucleophiles such as amines or thiols. In the last years, remarkable works in the engineering of enzymes had appropriate characteristics for industrial processes. Thus, screening of microorganisms with lipolytic activities in extreme habitats could help the discovery of novel lipases specific characteristics. In this paper we report the production, the purification and the characterization of a thermoactive and thermstable lipase (AFL) from a newly isolated *Aspergillus flavus* strain.

Materials and methods

Chemicals

Tributyrin (99%; puriss) and benzamidine were from Fluka (Buchs, Switzerland); tripropionin (99%, GC) was from Jansen (Pantin, France); phosphatidylcholine, sodium deoxycholic acid (NaDC), yeast extract and ethylene diamine tetraacetic acid (EDTA) were from Sigma Chemical (St. Louis, USA); gum arabic was from Mayaud Baker LTD (Dagenham, United Kingdom); acrylamide and electrophoresis grade were from BDH (Poole, United Kingdom); marker proteins and supports of chromatography used for AFL purification: Sephacryl S-200 and Mono S-Sepharose gels were from Pharmacia (Uppsala, Sweden); PVDF membrane was purchased from Applied Biosystems (Roissy, France); casein peptone was from Merck (Darmstadt, Germany); and pH-stat was from Metrohm (Switzerland).

Enzymes and proteins

Aspergillus flavus lipase (AFL) was purified in (Laboratoire de Biochimie et de Génie Enzymatique des Lipases, ENIS, BPW 3038 Sfax, Tunisia) (Sayari *et al.,* 2001).

Screening of lipolytic Activity

An initial screening of 74 strains from various Moroccain biotopes was carried out. This screening was realized on a solid medium containing 1% olive oil, 1% nutrient broth, 1% NaCl, 1.5% agar and 1% rhodamin B (pH 7). The culture plates were incubated at 30 °C, and colonies giving rise to widespread clearing around them were regarded as putative lipase producers. Among the 10 strains retained.

Culture conditions

The culture medium was precultured during 12 h at 28 °C and 150 rpm in 250 ml shaking flasks with 50 ml of medium A (17 g/l casein peptone, 5 g/l yeast extract (Difco), 2.5 g/l glucose, 0,5 g/l $MgSO_4$, 2 g/l $CaCl_2$, 1,5 g/l Sodium citrate, 2 g/l $KHPO_4$, pH (5.5). *Aspergillus flavus* cultures used as inocula (2%) were cultivated in 1-l shaking flasks with 250 ml of medium A. The culture was incubated aerobically for during 72 h on a rotary shaker set at 150 rpm at a temperature of 28 °C.

Lipase activity determination

The lipase activity was measured titrimetrically at pH 8.5 and 40 °C with a pH-stat under standard conditions using tributyrin (0.25 ml) in 30 ml of 2.5 mM Tris–HCl pH 8.2, 2 mM $CaCl_2$, 2 mM NaDC or olive oil emulsion (10 ml in 20 ml of 2.5 mM Tris–HCl pH 8.2, 2 mM $CaCl_2$, 4 mM NaDC) (Sarda and Desnuelle, 1958) as substrate. Lipase activity was also measured at pH 7 and 37 °C using TC_2 (0.25 ml) or TC_3 (0.25 ml) in 30 ml of 2.5 mM phosphate buffer pH 7, 3 mM $CaCl_2$ as substrate. Some lipase assays were performed in the presence of bile salts. Assays were carried out in 30 ml of 2.5 mM Tris–HCl buffer pH 7.0 containing 0.1 M NaCl. Standard conditions for measuring enzyme activity at increasing esters concentrations have been described previously (Stöcklein *et al.,* 1993)**.**When measuring AFL activity in the absence of $CaCl_2$, we added EDTA or EGTA to the lipolytic system. Lipolytic activity was expressed as units. One unit corresponds to 1 µmol of fatty acid released per minute.

Determination of protein concentration

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

Procedure of AFL purification

1000 ml of culture medium, obtained after 72 h of cultivation, were centrifuged for 15 min at 8500 rpm and filtered to remove the cells. The supernatant containing extracellular lipase was used as the crude enzyme preparation. Ammonium sulphate precipitation: The cell-free culture supernatant was precipitated using solid ammonium sulphate to 70 % saturation. The pellet obtained after centrifugation (30 min at 8500 rpm) was dissolved in 10ml of buffer A (20 mM sodium acetate pH 5. 4, 20 mM NaCl, and 2 mM benzamidine). Insoluble material was removed by centrifugation at 13,000 rpm during 5 min. Heat treatment: The supernatant obtained (10 ml) was incubated at 70 °C during 30 min. Insoluble material was removed by centrifugation at 13,000 rpm during 5 min. Filtration on Sephadex G-100: The enzyme solution (10 ml) was applied to a Sephadex G-100 column (3 cm × 100 cm) previously equilibrated in buffer A at a rate of 30 ml/h. The fractions containing the lipase activity (eluted at a void volume) were pooled. Cation exchange chromatography: Active fractions eluted from Sephadex G-100 column were poured into a mono-S sepharose cation exchanger equilibrated in buffer A. The column (2 cm × 30 cm) was rinsed with 400 ml of the same buffer. No lipase activity was detected in the washing flow. Adsorbed material was eluted with a linear NaCl gradient (300 ml of 20‑500 mM in buffer A) at a rate of 45 ml/h. AFL activity was eluted between 170 and 220 mM NaCl. The fractions containing the lipase activity were pooled and concentrated.

Analytical methods

Analytical polyacrylamide gel electrophoresis of proteins in the presence of sodium dodecyl sulfate (0.3 M) and β-mercaptoethanol (0.25 M) or DTT (0.5 M) (SDS/PAGE) was performed by the method of Laemmli (Laemmli, 1970). Samples for sequencing were electroblotted according to Bergman and

Jornvall (1987). Protein transfer was performed during 1 h at 1 mA/cm^2 at room temperature.

Amino acid sequencing

The N-terminal sequence of purified AFL (present study) was determined by automated Edman_s degradation, using an Applied Biosystems 470 a protein sequencer equipped with PTH 120A analyser (Hewick *et al.,* 1981). The sequence was kindly defined by Dr. Hafedh MEJDOUB (Faculty of sciences, Sfax).

Effect of pH and temperature on AFL activity and stability

AFL activity was tested in various buffers at different pH (5‑10.5) at 40 °C. The pH stability of the lipase was measured by incubating the enzyme at different pH (3‑12) for 24 h at room temperature. The residual activity was determined, after centrifugation, under standard assay method. The optimum temperature for the AFL activity was determined by carrying out the enzyme assay at different temperatures (25‑60 °C) at pH 8.5 The effect of temperature on lipase stability was determined by incubating the enzyme solution at different temperatures (30‑80 °C) for 30 min. The residual activity was determined, after centrifugation, under standard assay method.

Results

Production of lipase

The maximal production of AF lipase with inoculum size of 3 × 10^7 cells/ml is 16 U/mL, which was obtained by incubating 1 ml of the enzyme with olive oil emulsion (10 ml in 20 ml of 2.5 mM Tris-HCl pH 8.2, 2 mM $CaCl_2$, 4 mM NaDC) as substrate, The lipase activity Was Measured titrimetrically at pH 8.5 and 40 °C with a pH-stat. The time course of lipase production was followed at 28 °C with cell growth (Figure. 1). The lipase activity was observed to start after incubation and reached the maximum (16 U/mL) at the end of the exponential phase corresponding to 72 h of cultivation.

Purification of AFL

The AFL was purified according to the procedure described. The protein elution profile which was obtained at the final step of the purification was shown in (Figure. 2A).The purified lipase was homogenous when tested by the coomassie blue staining in SDS-PAGE (Figure. 2, line 3). This figure shows that one band was revealed for AFL with a molecular mass of 55 kDa. The purification flow sheet is shown in (Table. 1). The specific activity of the pure lipase reached 4300 U/mg using olive oil emulsified with gum Arabic as substrate at pH 8.5 and 40 °C. Under the same conditions, a specific activity of 3400 U/mg was obtained when using TC4 as substrate.

N-terminal sequence of AFL

The AFL NH_2-terminal sequencing allowed the identification of 18 residues, Y-L-H-G-P-Y-T-L-Y-T-S-C-Y-P -S-T-F (D). This N-terminal sequence exhibits a high degree of homology with the lipases of the same genus previously characterised [*Aspergillus flavus NRRL3357, Aspergillus oryzae RIB40*], indeed AFL has no

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homology with [*Aspergillus flavus* (accession AF404489.1)] (Table. 2).

Interfacial activation of AFL

The hydrolysis rate of different concentration of TC_3 emulsified in 0.33 % GA and 0.15 M NaCl by AFL shows a normal Michaelis‑Menten dependency of the activity on the substrate concentration (Figure. 3). The interfacial activation cannot be taken as the unique criterion required to distinguish lipases from esterases (Ferrato *et al.,* 1997). Lipases are defined as a family of enzymes that are able to hydrolyse long-chain triacylglycerols independently of the presence, or the absence, of the interfacial activation phenomenon. Here, we can say that AFL, which hydrolyses efficiently olive oil, is a true lipase.

Effects of pH and temperature on AFL activity and stability

The activity of AFL was investigated in different pH using olive oil emulsion as substrate. Unlike some previously described *Aspergillus* lipases (Gwen *et al.,* 2006; Isabel *et al.,* 2000), our results show that AFL remains active at a pH range of 6‑10 (Figure. 4A). Under the same experimental conditions, the ANL or ANsL activity was maximal at pH 6.5 or 6.0, respectively (Gwen *et al.,* 2006; Isabel *et al.,* 2000). In the pH stability study, the lipase is stable at a range of pH values between pH 5‑12 after 24 h incubating (Figure. 4B). The lipase activity was also determined at different temperatures under standard assay conditions (Figure. 4C). The AFL activity increased significantly with increasing the temperature to reach its maximum value at 40 °C. The thermostability of AFL was also determined by measuring the residual activity after incubation of the pure enzyme at various temperatures, the enzyme exhibited maximum stability at 70 °C (Figure. 4D) In contrast to others microbial lipases, which are produced by mesophilic organisms, most lipases showed maximum stability at temperatures in the range of 45 to 55 °C (Laachari *et al.,* 2013, Crisalejandra *et al.,* 2011; Prodipta *et al.,* 2012).

Discussion

In contrast to most microbial lipases, the production of AFL is not induced by the presence of triacylglycerols (like TC_4 or olive oil) or esters (Tween-20). . The specific activity measured was two-fold higher than the one of *Aspergillus niger* (Gwen *et al.,* 2006). These results show that, unlike of AFL which is not selective for short-chain substrates, AFL hydrolyses triacylglycerol with a chain length at high rates. No phospholipase activity was detected when using egg PC as substrate (Simons *et al.,* 1996). On the contrary for SAL wich hydrolyses both triacylglycerols and phospholipids irrespective of their chain length (Simons *et al.,* 1996). Some microbial lipases like *Rhizopus oryzae* lipase (Ben Salah *et al.,* 2001) may lack enzymatic activity when TC_4 is used as substrate in the absence of amphipathic reagent. The high energy existing at the tributyrin/water interface is responsible of the irreversible denaturation (Sayari *et al.,* 2001). 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. Lipases active and stable in alkaline media are very attracting, for example, lipase produced by *Acinetobacter radioresistens* has an optimum pH of 10 and it was stable over a pH range of 6–10; this enzyme has a great potential for application in the detergent industry (Chen *et al.,* 1998). Under the experimental conditions described in section (Effect of pH and temperature on AFL activity and stability), the ANL or ANsL found to be stable between pH 4 and 7 or pH 4 and 9.0, active at 40 °C, respectively (Gwen *et al.,* 2006; Isabel *et al.,* 2000). In contrast to ANL or ANsL which is inactivated after a few minutes when incubated at 60 °C (Gwen *et al.,* 2006; Isabel *et al.,* 2000). AFL retained 100 % of its activity after 30min incubation at 60 or 70 °C, respectively. Thermostable and alkaline lipases are therefore highly attractive to the synthesis of biopolymers and biodiesel and used for the production of pharmaceuticals, agrochemicals, cosmetics, and flavour (Haki and Rakshit, 2003). Due to the physicochemical properties of *A.flavus* lipase, it can be the most efficient lipases in the industrial field (Gwen *et al.,* 2006; Isabel *et al.,* 2000).

Conclusion

AFL was isolated from the culture medium, the newly lipase from *A. flavus* has been reported for the first time in this paper. AFL demonstrates high activity towards olive oil emulsion and TC_4 and exhibits optimal activity under the condition of 40 °C and pH 8.5 and very stable at 70 °C with a mass molecular of 55 KDa which never been reported before. AFL can be considered as a good candidate for industrial and biotechnological applications.

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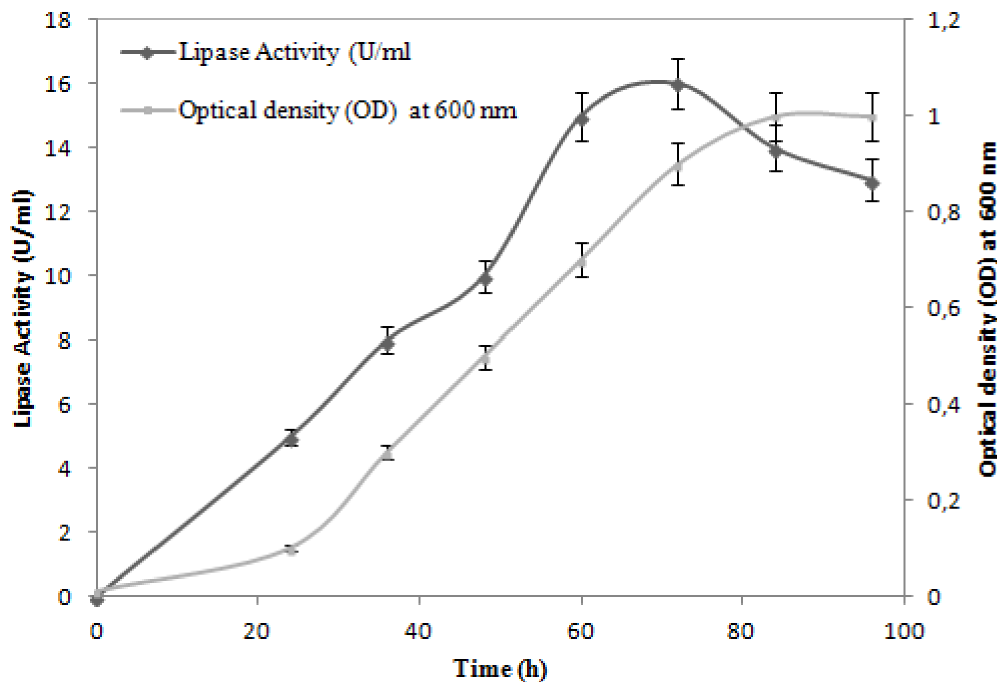

Figure 1. The time courses of lipase production. The culture was carried out at 28 °C in shaking at 150 rpm. Bars correspond to standard deviation

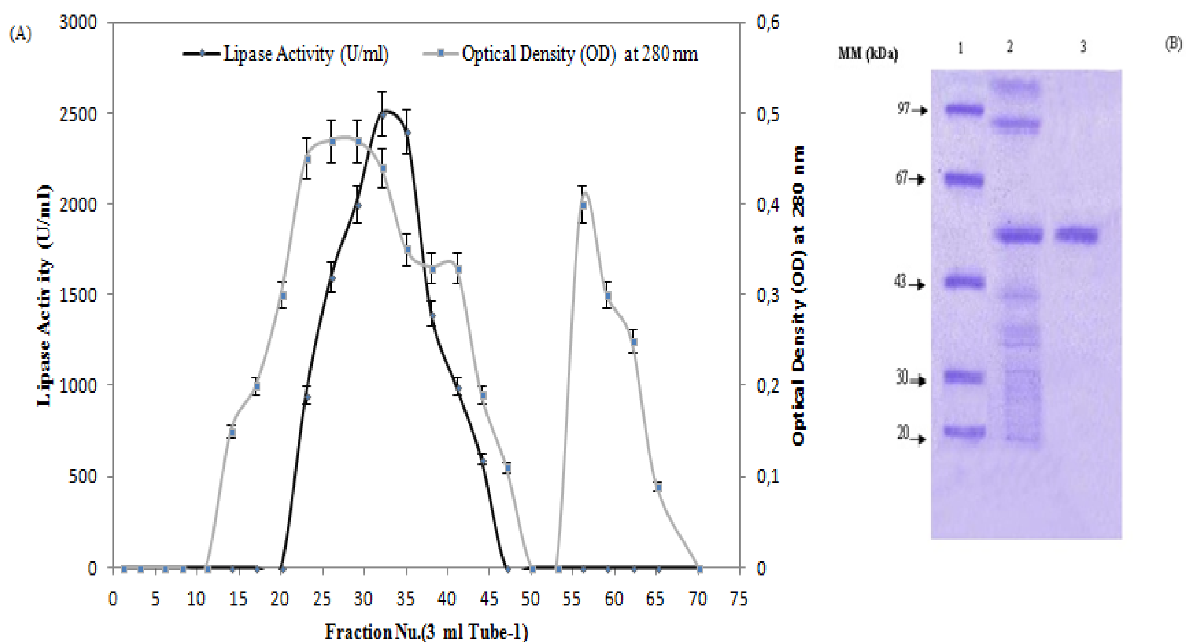

Figure 2. (A) Chromatography of AFL on Sephacryl S-200. The colomn (3 × 100 cm) was equilibrated with buffer A (20 mM sodium acetate pH 5.4, 20 mM NaCl, 1 mM benzamidine). The elution of lipase was performed with the same buffer at a

rate of 26 ml/h. Lipolytic activity was measured under standard conditions at pH 8.5 and 40 °C using a pH-stat. (B) SDS/PAGE (15 %). Lane 1, molecular mass markers (Pharmacia); lane 2, characterisation of the AFL obtained after Mono-S chromatography chromatography. lane 3, 20 µg of purified AFL. Bars correspond to standard deviation

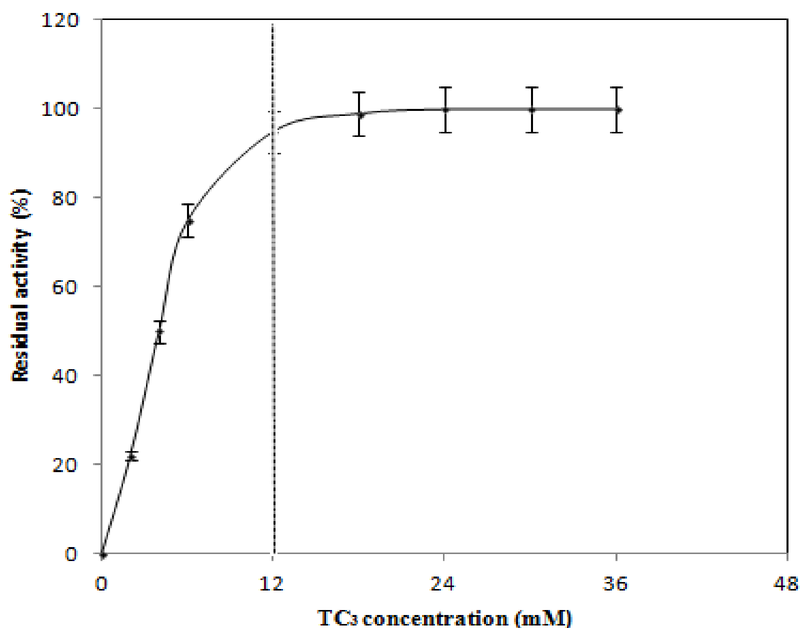

Figure 3. Hydrolysis rate of TC_3 by AFL as function of substrate concentration. The TC_3 solutions were systematically prepared by mixing (3 × 30 s in a warring blender) a given amount of TC_3 in 30 ml of 0.33 % GA and 0.15 M NaCl. The release of propionic acid was recorded continuously at pH 8, 5 and 40 °C using a pH-stat. The CMC of TC_3 (12 mM) is indicated by vertical dotted lines. Bars correspond to standard deviation

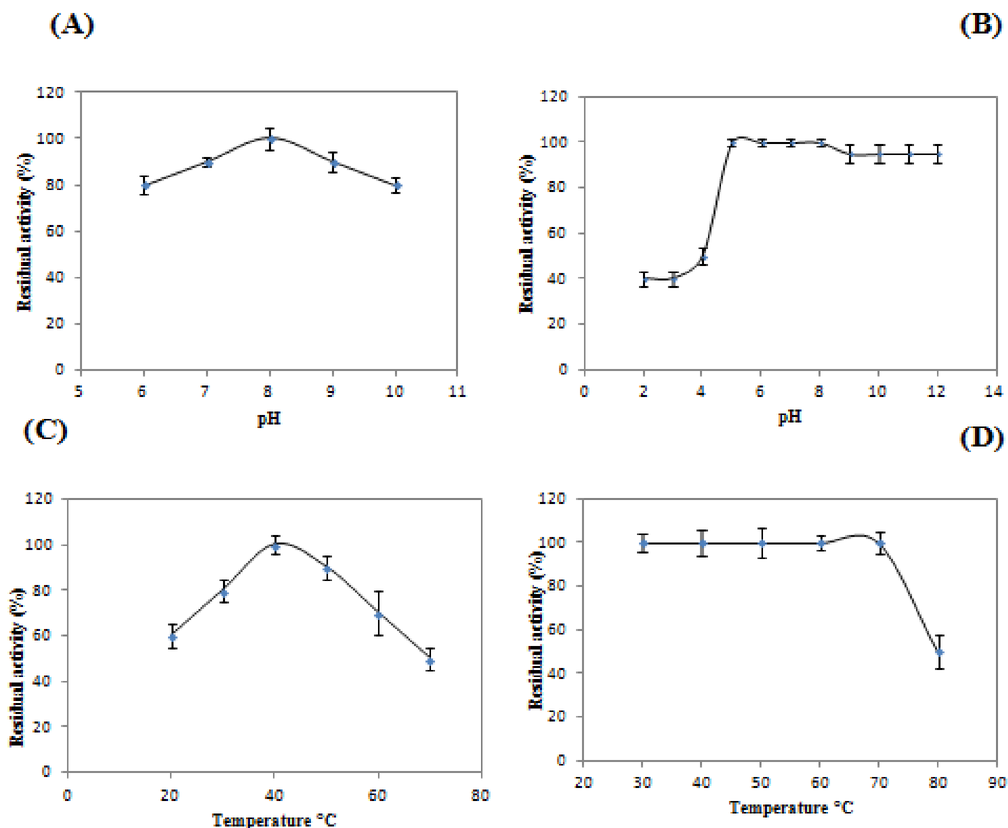

Figure 4. Effect of pH on enzyme activity (A) and stability (B) of AFL. Optimal pH was determined with tributyrin at 40 °C under the standard conditions. Stability was analysed after preincubating the pure enzyme for 24 h in different buffer solutions at various pH ranging from 3 to 12. Temperature effect of on AFL activity (C) and stability (D). For temperature stability the pure enzyme was preincubated at different temperatures for 30 min and the remaining activity was measured under the standard conditions. Bars correspond to standard deviation

Table 1. Flow sheet of the strain AF Lipase purification

Purification step	Total activity (U/mL)[a]	Proteins (mg)[b]	Specefic activity (U/mg)	Activity recovery (%)	Purification factor
Culture supernantant	16000 ± 270	1600 ± 0,5	10	100	1
$(NH_4)_2SO_4$ precipitation	12200 ± 190	1870 ± 0,6	14 ± 0,44	76 ± 0,44	1,4 ± 0,04
Heat treatment (30 min at 70 °C)	11900 ± 175	590 ± 0,9	20 ± 0,11	74 ± 0,44	2 ± 0,11
Sephacryl S 200	9070 ± 80	75 ± 0,8	120 ± 0,11	56 ± 0,33	12 ± 0,11
Mono-S chromatography	6075 ± 65	1,41 ± 0,6	4300 ± 0,16	37 ± 1,66	430 ± 1,88

[a] 1 Unit corresponds to 1 µmol of fatty acid released per minute
[b] Proteins were estimated by Bradford
± Standard deviation

Table 2. N-terminal sequence comparison of AFL with AOL RIB40 and AFL NRRL3357

Lipase	N-terminal sequence	Accession Number
Aspergillus flavus (55 kDa)	YLHGPYTLYTSCYPSTFD	(Present stady)
Aspergillus flavus NRRL3357 (33,5 kDa)	[123]AYLHGPYTLYTSCYPSTFD	XM_002380691.1
Aspergillus oryzae RIB40	[52]AYLHGPYTLYTSCYPSTFDP	XM_001823718.2
Aspergillus flavus	[52]NNYVAKDGE 123FPQTDPGL	AF404489.1