Biochemical characterization of a new thermostable lipase from Bacillus pumilus strain

[Key Words: Bacillus pumilus, lipase, purification, thermostable]

ABSTRACT

Objective: Research and characterization of new thermostable lipases from bacterial strains isolated from tannery waters in the old medina of Fez.

Methods: Gene which encodes the 16S ribosomal RNA for a bacterial species was amplified via PCR and sequenced (Bacillus pumilus HSF544325). The extracellular lipase from B. pumilus is purified by gel filtration (Sephacryl S-200) and cation exchange chromatography (Mono S sepharose cation exchanger). The N-terminal sequences of purified Bacillus pumilus lipase were determined by automated Edman’s degradation, using an Applied Biosystems 470 A protein sequencer equipped with PTH 120A analyser. The activity of lipase was examined within the pH range of 6.0-10.0 and 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.

Results: The results showed that Bacillus pumilus is a strain that produce non-inducible lipase. This enzyme has a molecular weight of 27 kDa and presents a maximal activity at pH 8 and 45°C. The 18 N-terminal amino acid residues showed a high degree of homology with other Bacillus lipase sequences. After treatment in 100°C for 5 min, the thermostable enzyme maintains 60% of its activity, which is greater than that those founded in previous works. The enzyme retained 100% of its activity after 30 min incubation at 70°C.

Conclusion: This newly isolated lipase is thermostable and it has a significant difference which was observed when the biochemical properties of the Bacillus pumilus lipase were compared to others microbial lipases. The Bacillus pumilus lipase can be considered as a good candidature for industrial and biotechnological applications.

Conflict of Interest: The authors have no conflict of interest.
Introduction

Lipases (EC 3.1.1.3) which have been found in many species, plants, bacteria, yeasts and fungi, represent a large group of enzymes in biotechnology [1]. The enzymes of microbial origin are the most interesting, because of their potential applications in different sectors such as food, pharmaceuticals, detergents, biodiesel and cosmetic industries, also the improvement of the biodegradability of effluents from high-fat food industry (dairy, meat and seafood). Lipases used in detergents, amylases and glucose isomerase, used in starch processing and in the bio-processing of raw materials or in the synthesis of organic chemicals, are very efficient [2,3,4]. Most of lipases are serine enzymes and there is a hydrogen-bond network in their active site, consisting of the triad of Ser, Asp (Glu), and His. Most lipase sequences include the conserved region, Gly-Xxx-Ser-Xxx-Gly or Ala-Xxx-Ser-Xxx-Gly, which is the feature of the lipase sequences from Bacillus subtilis [5]. The bacterial lipases receive much attention due the rapid biotechnology development, their substrate specificity and their ability to resist at extremes conditions. The increase in production of microbial lipases requires not only the techniques of molecular biology which contribute to the overexpression of the corresponding genes, but also the understanding of the molecular and biochemical mechanisms that influence the folding and secretion of these enzymes [6,7,8]. Bacillus lipases have attracted much attention because of their biotechnological potential, which has led to the isolation of several lipolytic enzymes of B. subtilis and other species of the genus Bacillus, Geobacillus and Paenibacillus [9]. The lipases, B. subtilis and B. pumilus belong to the subfamily I.4 [10], are smaller than other subfamilies, and characterized by the absence of the structure of the cover. Some lipases such as lipase B. pumilus B26 do not contain the Ca²⁺ binding motif near the catalytic site, its activities and thermostability is independent of Ca²⁺. In 1990 it was determined the first two 3D structures of lipases by X-ray diffraction: it is a part of the structure of a lipase fungus, Rhizomucor miehei lipase [11], also one of the two main digestive enzymes, the HPL [12]. After extensive screening of strains producing lipase, only one bacterial isolated from Fez tannery, give a high lipolytic activity in the solid medium described above. Identification of this strain shows that it is Bacillus Pumilus. The thermostable and thermoactive lipases from Bacillus pumilus are not previously described. Also, the microbial enzymes may be limited in industrial applications due to relatively lower stabilities and catalytic activities under conditions that characterize industrial practical applications like extremes of pH values or non-aqueous solvents and high degrees of temperatures. In the last years, remarkable works in the engineering of enzymes with appropriate characteristics for industrial processes. Thus, screening of microorganisms with lipolytic activities in extreme habitats could aid the discovery of novel lipases specific characteristics. In this paper for the first time we report the production, the purification and the characterization of a thermoactive and thermostable lipase (BPL) from a newly isolated Bacillus pumilus strain. The N-terminal sequence of the BPL was determined and compared to the known bacillus lipases.

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), Tween 20, 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 BPL purification: Sephacryl S-200 and Mono S-Sepharose gels were from Pharmaecia (Uppsala, Sweden); PVDF membrane was purchased from Applied Biosystems (Roissy, France); trans-blot cell apparatus was from Bio-Rad (Paris, France); vinyl acetate was from Aldrich (Stenheim, Germany); casein peptone was from Merck (Darmstadt, Germany); and pH-stat was from Metrohm (Switzerland).

Enzymes and proteins

Bacillus Pumilus lipase (BPL) was purified in our laboratory, as described by Sayari [13].

Screening of lipolytic microorganisms

An initial screening of 74 strains from various Moroccan 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 37°C, and colonies giving rise to widespread clearing around them were regarded as putative lipase producers. Among the 10 strains retained.

Culture conditions

The bacterium was precultured during 12 h at 37°C and 200 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, pH 7.4). Overnight, B. pumilus 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 during 72 h on a rotary shaker set at 200 rpm at a temperature of 37°C. Growth was followed by measuring the OD of the culture at 600 nm.

Lipase activity determination

The lipase activity was measured titrimetrically at pH 8.2 and 45°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 CaCl₂, 2 mM NaDC or olive oil emulsion (10 ml in
20 ml of 2.5 mM Tris–HCl pH 8.2, 2 mM CaCl₂, 4 mM NaDC as substrate [1]. Lipase activity was also measured at pH 7 and 37°C using TC2 (0.25 ml) or TC3 (0.25 ml) in 30 ml of 2.5 Mm phosphate buffer pH 7, 3 mM CaCl₂ as substrate. Some lipase assays were performed in the presence of bile salts. The enzymatic hydrolysis of solutions and emulsions of esters was followed potentiometrically at 25°C and pH 7.0. 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 [14]. When measuring BPL activity in the absence of CaCl₂, 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 [15] using BSA as standard.

Procedure of BPL purification
Culture medium 1 l of, obtained after 48 h of cultivation, was centrifuged for 20 min at 8000 rpm to remove the microbial cells. The supernatant containing extracellular lipase was used as the crude enzyme preparation.

– Ammonium sulfate precipitation: The crude enzyme solution (1–l), containing 15000 Units, was brought to 60% saturation with solid ammonium sulfate (390 g) under stirring conditions at 4°C. After centrifugation (30 min at 10000 rpm), the precipitate was resuspended in 15 ml of buffer A (20 mM sodium acetate pH 5.4, 20 Mm NaCl, and 1 mM benzamidine). Insoluble material was removed by centrifugation at 10000 rpm during 10 min.

– Heat treatment: the supernatant obtained (15 ml) was incubated at 70°C during 30 min. Insoluble material was removed by centrifugation at 10000 rpm during 10 min.

– Filtration on Sephacryl S-200: The supernatant (15 ml) was loaded on a column (3 cm×100 cm) of gel filtration Sephacryl S-200 equilibrated with buffer A. The elution of lipase was performed with the same buffer at a rate of 45 ml/h. The fractions containing the lipase activity (eluted at 1.3 void volume) were pooled.

– Cation exchange chromatography: The pooled fractions of Sephacryl S-200 column were applied to a Mono S sepharose cation exchanger equilibrated in buffer A. The column (2 cm×30 cm) was rinsed with 300 ml of the same buffer. No lipase activity was detected in the washing flow. Adsorbed material was eluted with a linear NaCl gradient (600 ml of 20 to 500 mM in buffer A) at a rate of 45 ml/h. BPL activity was eluted between 150 and 350 mM NaCl.

– The fractions containing the lipase activity were pooled, concentrated and reloaded on a column (2 cm×160 cm) of gel filtration Sephacryl S-200 equilibrated in buffer A. Elution was performed at a rate of 45 ml/h.

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 as described previously [16]. Sequencing samples were electroblotted according to Bergman and Jornvall [17]. Protein transfer was performed at room temperature during 1 h at 1 mA/cm². The molecular mass of BPL was determined by MALDI-TOF (matrix assisted laser desorption ionisation-time of flight).

Amino acid sequencing
The N-terminal sequence of purified BPL was determined by automated Edman’s degradation, using an Applied Biosystems 470 A protein sequencer equipped with PTH Biosystems 470 A protein sequencer equipped with PTH 120A analyser [18]. The sequence was kindly determined by Dr. Reinbolt (IBMC, UPR 9002, CNRS-Strasbourg, France).

Kinetic study
Lipase activities were measured as a function of various substrate (TC₁, TC₂ or TC₃ₐ) concentrations (0–40 mM). The Michaelis–Menten constant (K_M) and the maximum velocity (V_max) were calculated by Lineweaver-Burk plot.

Effect of pH and Temperature on the Activity and Stability of BPL
The activity of lipase was examined within the pH range of 6.0-10.0. 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 mM Tris-HCl, pH 8.5, 3 mM NaCl, or olive oil (10%) emulsion (10 ml in 20 ml of 2.5 mM Tris-HCl, pH 8.5, 3 mM NaCl) [19] 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 [19]. The optimum temperature

![Figure 1. The time courses of lipase production. The culture was carried out at 37°C in shaking at 200 rpm in the presence of or in the absence of triacylglycerols or esters.](image-url)
for the *Bacillus pumilus* lipase activity was determined by carrying out the enzyme assay at different temperatures (20–90°C) at pH 8.0. The effect of temperature on lipase stability was determined by incubating the enzyme solution at different temperatures (20–90°C) for 30 min. The residual activity was determined, after centrifugation, under standard assay method [19].

**Results**

**Production of lipase**

Culture of *B. pumilus* in medium A was realised at 37°C. The maximal production of lipase was obtained after 48 h of incubation. This production attained 15 U/ml, with an inoculum size of 4.10^8 cells/ml (Figure 1). BPL production was not induced by the presence of triacylglycerols (TC4 or olive oil) or esters (Tween-20) (data not shown).

**Purification of BPL**

The BPL was purified according to the procedure described in the Materials and methods section 2.8. 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 V_0. The results of SDS/PAGE analysis of the pooled fraction of this last step of chromatography are given in (Figure 2a). This figure shows that the enzyme exhibited one band corresponding to a molecular mass of about 27 kDa. The purification flow sheet is given in (Table 1), it shows that BPL is able to hydrolyse triacylglycerols without significant chain length specificity; the specific activity of 2100 U/mg was measured at pH 8 and 45°C with olive oil as substrate in the presence of (2 mM CaCl₂ and 2 mM NaDC). BPL was incubated at a temperature of 100°C for 1 to 5 min. The residual activity was determined, after centrifugation, under standard assay method using tributyrin or olive oil (10%) emulsion (Figure 3). BPL was found to be stable at 100°C after 5-min of incubation, maintains about 60% of its activity. Proteins were precipitated by acetone (v/2v) and visualized by SDS-PAGE 15% (Figure 2b), Which is a special case in the world, until now no lipase supports a temperature of 70°C and 100°C [20,21,22].

**N-terminal sequence of BPL**

The BPL NH₂-terminal sequencing allowed the identifica-

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**Table 1. Purification table of BP lipase**

| Purification step               | Total activity (U/ml) | Proteins (mg) | Specific activity (U/mg) | Activity recovery (%) | Purification factor |
|--------------------------------|-----------------------|---------------|--------------------------|-----------------------|--------------------|
| Culture supernatant            | 15000                 | 1500          | 10                       | 100                   | 1                  |
| (NH₄)₂SO₄ precipitation        | 11200                 | 775           | 14                       | 74.5                  | 1.4                |
| Heat treatment (30 min at 70 °C) | 10100                 | 655           | 15.5                     | 67                    | 1.55               |
| Sephacryl S-200                | 8050                  | 90            | 89                       | 53.5                  | 8.9                |
| Mono-S chromatography          | 5530                  | 2.63          | 2100                     | 36                    | 210                |

*1 Unit corresponds to 1 µmol of fatty acid released per minute; *2Proteins were estimated by Bradford.
tion of 18 residues, A-E-K-F-V-G-K-Q-A-A-E-H-N-P-V-V-M-V. This N-terminal sequence exhibits a high degree of homology with lipases of the same genus previously characterised *Bacillus vallismortis* DV1-F-3, *Bacillus pumilus* ATCC 7061 and *Bacillus subtilis* BSn5 (Table 2).

**Activation of BPL by interface**

As it has been shown by Ferrato [17], 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 BPL. The hydrolysis rate of TC_3 emulsified in 0.33% GA and 0.15M NaCl by BPL as a function of substrate concentration shows a normal Michaelis–Menten dependence of the activity on the substrate concentration (Figure 4). The interfacial activation cannot be taken as the unique criterion required to distinguish lipases from esterases “16” as described by Sarda [1]. 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 BPL, which hydrolyses olive oil, is a true lipase.

**Effect of pH and Temperature on the Activity and stability of *B. pumilus* lipase**

The maximal activity of BPL was obtained at pH 8.0 (Figure 5a), and a temperature between 40 and 50°C (Figure 5c). The pH-optimum for BPL activity is the same results as others BL [23,24,25]. BPL was found to be stable at pH 5 and 8 (Figure 5b). In contrast to all Bacillus lipases, this enzyme maintains about 100% of its activity after 30-min incubation at 70°C (Figure 5d).

**Discussion**

In this study, our findings show that *B. pumilus* is able to produce a thermostable lipolytic enzyme. Also, as lipases are generally produced using carbon source such

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**Figure 3.** Hydrolysis rate of TC_3 by BPL 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 TC3 in 30 ml of 0.33% GA and 0.15 M NaCl. The release of propionic acid was recorded continuously at pH 8 and 45°C using a pH-stat. The CMC of TC_3 (12 mM) is indicated by vertical dotted lines.

**Figure 4.** pH effect on enzyme activity (a) and stability (b) of BPL. Optimal pH was determined with tributyrin at 45°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 BPL 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.
as oils, fatty acids, glycerol or tweens in the presence of organic nitrogen source, in this study, the production of B. pumilus is not induced by the presence of triacylglycerols (like TC, or olive oil) or esters (Tween 80). According to SDS-PAGE, we have a protein of 27 KD molecular size which will be the first protein for this type of bacterium with this size (Figure 2b). The importances of alkaline and thermostable lipases for different applications have been growing rapidly. A great deal of research is currently going to develop lipases which will work under alkaline conditions as fat stain removers. Our results show that B. pumilus lipase remains active at a pH range of 6.0 to 10. This result can be very attractive and can have a great potential application in many areas. Our protein was found to be stable at 70°C with considerable activity (100%), and at 100°C with half-lives of 5 min (Figure 5). Many enzymes were produced by bacteria and yeast showed maximum activities at high temperatures, such as Pseudomonas aeruginosa (70°C), a thermophilic Bacillus sp. (60 to 70°C) [26], and the yeast Kurtzmanomyces sp. (75°C) [27]. Just few fungal lipases reported other studies presented as a thermophilic behavior. Among fungi of the genus Penicillium, which are mesophilic organisms, most lipases showed maximum activities at temperatures of 25 to 45°C [28,6]. Lipase of B. pumilus maintains its activity despite the denaturing conditions and treatment at high temperature (70°C and 100°C) (Figure 4d; Figure 5), similar as the lipase from Burkholderia cepacia ATCC 25416 and Bacillus pumilus RK31 appears to be tolerant to temperature, as were reported to be stable in the range temperature between 30°C to 60°C [29,30]. The same findings were described by fairolniza, the lipase from bacillus sp was thermostable in the temperature range of 55 to 75°C; and considerable activity (75%) was retained. Enzyme activity sequentially decreased as the incubation time is increased [31]; other lipase from bacillus subtilis was stable during 273.38, 51.04 and 41.58 min, at 60, 70 and 80°C and has an optimum activity in temperature of 60°C and stable in the pH of 7.0–9.0 and 40–70°C of temperature [32]. Due to the physicochemical properties of B. pumilus lipase, it can be the most efficient lipases and the best candidates in the industrial field [33].

### Conclusion

BPL was isolated from the culture medium, the newly lipase from B. pumilus has been reported for the first time in this paper. BPL demonstrates high activity towards olive oil emulsion and TC4 and exhibits optimal activity under the condition of 45°C and pH 8.0 and very stable at 70°C with a mass molecular of 27 KD. BPL hydrolyses the long chains more efficiently than the short chain triacyl-

**Table 2. N-terminal sequence comparison of BPL with Bacillus vallismortis DV1-F-3, Bacillus pumilus ATCC 7061 and Bacillus subtilis BSn5**

| Bacillus pumilus (27Kda) | AEKFGVQAAEHPVMV | Present study |
|--------------------------|-----------------|--------------|
| Bacillus vallismortis DV1-F-3 | ^30SLFVGSKKAEHPVMV | accession ZP_10511292 |
| Bacillus pumilus ATCC 7061 | ^30SMFVQPSAKEYAEHPVMV | accession ZP_03056417 |
| Bacillus subtilis BSn5 | ^30SLFALQPSAKEYAEHPVMV | accession YP_004206227 |

Figure 5. Temperature (100°C) effect on crude enzyme (a). Residual activity was determined with tributyrin at pH 8.00 and 45°C using a pH-stat. SDS/PAGE (15%) (b), characterization of the PBL obtained after heat treatment in 100°C, Lane 1, heat treatment for 1 min; Lane 2 (2 min); Lane 3 (3 min); Lane 4 (5 min).
glycérols and presents the interfacial activation phenomenon also there is a significant difference observed when the biochemical properties of the BPL were compared to other lipases.

Acknowledgments

We would like to thank “Dr Yousef TALLAL GARGOURI Directeur de Laboratoire de Biochimie et de Génie Enzymatique des Lipases-ENIS”. We thank also Pr. Hafedh MEJDOUB (FSS) for the sequencing of the NH2-terminale. This work is a part of a doctoral thesis by Faouzi LAACHARI. Whose research was supported financially by “Ministère de l’enseignement supérieur, de la recherche scientifique et de la formation des cadres- Maroc”.

Conflict of Interest

There are no conflicts of interest among the authors.

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