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Characterization of a new thermostable esterase from the moderate thermophilic bacterium *Bacillus circulans*

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Abstract

The new moderate thermophilic bacterial strain MAS2, previously isolated at 50°C on a mineral medium containing triolein as sole carbon and energy source has been characterized and identified as *Bacillus circulans*. This strain does not produced lipase but an esterase activity which production was not enhanced by addition of Tween 80 or triolein in the culture medium. The reaction rate was maximum at 60°C. After 1 h incubation at 70° and 85°C the remaining activities were 100% and 50%, respectively, showing the high thermostability of this esterase activity. Optimum pH was in the range 8.5–9.5 with an important (60%) activity retained at pH 10.0. The kinetic constants for *p*-nitrophenyl caprylate (pNPC8) hydrolysis were $K_M = 0.24$ mM and $V_m = 4.3$ nmol/min mg. Using fatty acids with different chain lengths, the highest activity was observed on pNPC2 which confirmed the esterase nature of the enzyme. A significant activity remained on mid-chain-length fatty acids such as pNPC6 and pNPC8. Because of its high thermostability, activity at alkaline pH and broad specificity range for fatty acids, this enzyme showed high potential for use in biocatalysis.

Keywords: *Bacillus circulans*; Esterase; Moderate thermophile; Thermostable; Biocatalysis

1. Introduction

Lipases (EC 3.1.1.3) and esterases (EC 3.1.1.1) are enzymes that catalyze the hydrolysis of ester bonds involving a carboxylic acid of variable chain length, i.e., from C2 to C18 or more. In organic media, they also catalyze reactions of esterification, interesterification, alcoholysis or acidolysis [1]. These unique properties have led to a great number of applications mainly at the laboratory level. For instance, lipases are now widely used by organic chemists for the preparation of chiral molecules by optical resolution from racemic mixtures. In contrast, esterases are much less utilized [2]. Only the pig liver esterase (PLE) and the carboxylesterase from *Bacillus coagulans* (CBE) have retained some attention. PLE is mainly used for its broad substrate specificity [3–5] and CBE catalyzes the enantioselective hydrolysis of the racemic ester of 1,2-*O*-isopropylidene glycerol, an important intermediate in the synthesis of active biomolecules [6]. More recently esterases were described for geranyl ester syn-

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thesis [7], for enantioselective hydrolysis of esters [8] and synthesis of naproxen [9]. The relatively low use of esterases seems to be due to their lack of availability in sufficient amounts for organic chemists [2]. Despite their great potential interest as biocatalysts for biotransformations and despite the tremendous amounts of work at the laboratory level, both esterases and lipases are only rarely used in industrial-scale processes. One of the main reasons for their low use is their low stability under operational process conditions [10]. This process stability is generally higher for enzymes with high thermostability. In addition, the stability in organic media, a situation more and more used in processes is related to heat stability [11]. Therefore, there is a real need for new enzymes with high stability under process conditions.

In our laboratory, we have isolated 39 bacterial strains from soil using an enrichment culture at 50°C on triolein as sole carbon source. This approach is an alternative way to the search for thermostable enzymes in thermophilic or hyperthermophilic organisms [12]. We have found that most of these moderate thermophiles produced significant amounts of lipolytic enzymes, i.e., enzymes hydrolyzing *p*-nitrophenyl caprylate (pNPC8) [12]. One of these strains (MAS2) showed the highest activity.

In this work we describe the identification of strain MAS2 and the characterization of a highly thermostable esterase activity.

2. Experimental

2.1. Organism and culture conditions

Strain MAS2 has been previously isolated in our laboratory [12] from a soil sample by using an enrichment culture at 50°C on a mineral medium containing triolein as sole carbon source. Strain identification was done by Dr Kiredjian at the Pasteur Institute (Paris, France).

Enzyme production was studied in two media: Luria–Bertani (LB) medium containing: 10 g/l tryptone (Difco, Detroit, USA), 5 g/l yeast extract (Difco) and 10 g/l NaCl and Nutrient Broth (NB) medium containing: 10 g/l nutrient broth (Difco). In some experiments these media were supplemented as indicated in the text with 10 g/l glucose, 1 g/l Tween 80 (Fluka, Buchs, Suisse) or 1 g/l triolein (Sigma Chemicals, St. Louis, USA). pH was adjusted to 7.5 by addition of 1 N NaOH. Triolein was emulsified by sonication (concentration 10% in water, w/v) and added after sterilization. Media were sterilized by heating for 20 min at 120°C without glucose which was treated separately at 100°C. Growth was done in 250-ml Erlenmeyer flasks containing 25 ml of liquid during 22 h at 50°C under reciprocal agitation (110 strokes per min). The supernatant was recovered by centrifugation ($5500 \times g$, 4°C, 15 min.) and used as the enzyme source.

2.2. Enzyme assay

Lipolytic activity was assayed by measuring the rate of hydrolysis of *p*-nitrophenyl caprylate (pNPC8) (Sigma) at 37°C according to Kordel et al. [13]. One volume of 16.5 mM pNPC8 in 2-propanol was mixed with 9 volumes of 50 mM Tris–HCl pH 8.0, containing 0.4% (w/v) Triton X-100 (Fluka) and 0.1% (w/v) arabic gum (Sigma). To 1.35 ml of this mixture equilibrated at 37°C (a suitable temperature for routine experiments) in a 3-ml spectrophotometer (Shimadzu UV-160A, Roucaire, Courtaboeuf, France) cuvette was added 0.15 ml of the enzyme solution. The absorbance at 410 nm was read against a blank without enzyme and monitored continuously. The concentration of liberated *p*-nitrophenol (pNP) was calculated using an extinction coefficient of 12.75×10^6 cm^2/mol. This value was determined using standard solutions of pNP. For each assay, three different enzyme quantities were tested. Activity was calculated from the slope of the curve of absorbance variation per minute against enzyme amount. One enzyme unit was the amount of enzyme liberating one micromole of pNP per

minute in the conditions used. For the determination of substrate specificity the same conditions were used apart from changing the substrate chain length from C2 to C16. Standard deviations on enzyme assays were less than 10%.

2.3. Effect of pH and temperature on activity

For testing pH effect, a 10 mM Tris–HCl buffer was used and pH was adjusted at the desired value by using 1 N HCl or 1 N NaOH additions. For acidic and alkaline values the final reaction pH was measured. The molar extinction coefficient of pNP was determined at each pH tested: 6.52×10^6, 11.8×10^6, 12.75×10^6, 13.9×10^6, 14.3×10^6, 14.6×10^6 and 14.6×10^6 cm^2 mol^{-1} at pH 7, 7.5, 8, 8.5, 9, 9.6 and 10.2, respectively. The effect of temperature on activity was assayed using a reduced substrate concentration of 0.2 mM to avoid substrate precipitation observed in the standard conditions at temperatures higher than 55°C (see Results).

2.4. Effect of pH and temperature on stability

The enzyme extract was incubated at pH ranging from 6 to 11 in 10 mM Tris–HCl buffers for 1 h at 37°C. The residual activity was assayed in standard conditions. To check stability with temperature the culture supernatant was concentrated by ultrafiltration (Minitan from Millipore, Bedford, USA) with a cut-off of 30 kDa. The concentrate was dialyzed against 10 mM Tris–HCl pH 8.0. This extract (7.9 mg/l, 0.2 U/ml) was incubated during 1 h (pH 8) at temperatures ranging from 30° to 90°C and the residual activity assayed in standard conditions.

2.5. Polyacrylamide gel electrophoresis

Polyacrylamide gel electrophoresis in presence of SDS was run according to Laemmli [14] using a Mini Protean II from Biorad. Enzyme extracts were diluted in 0.0625 M Tris–HCl buffer pH 6.8, containing 10% (w/v) glycerol, 5% (w/v) β-mercaptoethanol and 2% (w/v) sodium dodecyl sulfate (SDS). Samples were not heated at 100°C to allow activity staining after migration. Calibration for molecular mass was made using the following standard proteins: phosphorylase (94 kDa); bovine serum albumin (67 kDa); ovalbumin (43 kDa); carbonic anhydrase (30 kDa); soya trypsin inhibitor (20.1 kDa) and lysozyme (18.5 kDa). Gels were run in duplicate, one being stained for protein and the other for activity. Protein staining was done in a 20% (v/v) ethanol and 10% (v/v) acetic acid mixture containing 0.025% (w/v) Coomasie Blue. Excess blue was eliminated by washing with a 10% (v/v) ethanol and 7% (v/v) acetic acid mixture.

2.6. Detection of esterase activity on gels

After electrophoresis, SDS was eliminated according to Kupke et al. [15] by washing first with 20 mM Tris–HCl pH 6.4 containing 0.08% (w/v) NaOH, 0.025% (w/v) maleic acid and 25% (v/v) isopropanol (30 min) and second by the same solution without isopropanol (15 min). Then, the gel was laid on a Petri dish containing 1.5% (w/v) bacto-agar (Difco laboratories, Detroit, USA) in 10 mM Tris–HCl pH 7.6 and 10% (v/v) pNPC8 in isopropanol. Activity was detected by appearance of clear halos after 1 or 2 h of incubation at 37°C.

3. Results

3.1. Strain identification and production of lipolytic enzymes

MAS2 cells were rods of 0.9 μm diameter and formed spores. Catalase test and gram coloration were positive indicating a *Bacillus* genus. From the other characters tested (data not shown) the strain was identified as a *B. circulans*. Other *B. circulans* strains have been

previously isolated from soil and some of their enzymatic activities have been characterized such as levansucrase [16], chitinase [17] or esterase/lipase [18]. However, this is, to our knowledge, the first report that this kind of strain can grow at 50°C and, consequently, that it may contain thermostable enzymes.

Growth and extracellular lipolytic activity of *B. circulans* MAS2 on rich media (LB and NB) with different additives were tested (Table 1). *p*-Nitrophenyl caprylate (pNPC8) was used since it is a good substrate for both lipolytic activities: esterases and lipases. Well-known lipolytic enzyme inducers [19,20] such as triolein or Tween 80 showed no stimulation of the level of pNPC8 activity (Table 1). In contrast, an enhancement of esterase production in *Sulfolobus shibatae* has been reported [21]. As for the *B. subtilis* esterase [22] glucose showed no negative effect such as the catabolite repression described by Gowland et al. [20]. Moreover, the highest pNPC8 activity was observed when glucose was added to LB medium similar to that reported by Elwan et al. [18]. Altogether, these results strongly suggested that the production of pNPC8 activity was constitutive on the media tested.

3.2. Gel electrophoresis and activity detection

The crude culture medium was used as enzyme source. It contained only one active band on pNPC8 as detected after native- or SDS-PAGE (see Experimental for experimental conditions). The Mr of the protein was estimated at 94 kDa from SDS gels. These results strongly suggested the presence of only one enzyme responsible for pNPC8 activity in the enzyme extract, which validates the use of the crude medium as enzyme source. As it will be demonstrated below in this paper (see Substrate specificity section), this enzyme is an esterase rather than a lipase.

Table 1
Effect of medium composition on enzyme production
The basal media contained either Luria–Bertani (LB) or nutrient broth (NB). Additions were as indicated with: glucose, 10 g/l; Tween 80, 1 g/l; triolein, 1 g/l. Cultures were run in triplicate in 25 ml liquid medium for 22 h at 50°C. Activity of the culture supernatant was assayed on pNPC8. Results are means of three independent cultures.

Medium	U/l	Standard deviation
LB	4.60	0.60
LB + glucose	18.90	1.26
LB + Tween 80	7.45	1.65
LB + triolein	3.08	1.10
NB	3.20	0.70
NB + glucose	3.50	0.92
NB + Tween 80	1.88	0.62
NB + triolein	3.92	1.25

3.3. Effect of temperature on activity and stability

The initial rate of hydrolysis of pNPC8 by culture supernatant was tested at temperatures ranging from 25° to 75°C. In standard assays conditions, the substrate precipitated at temperatures higher than 55°C. Therefore, the pNPC8 concentration was reduced from 1.485 to 0.2 mM. At this value no precipitation was observed in the temperature range tested. This concentration was in the same range as K_M (see later) for pNPC8 that may result in nonlinear kinetics during the assay. However, this was not the case since activity was assayed at substrate conversions less than 1%. This was made possible by the high molar absorbance coefficient of pNP. Thus, the reaction rate was constant during the time (2–5 min) of the activity determination. Results using 0.2 mM pNC8 on the whole temperature range are shown in Fig. 1. The reaction rate increased slightly from 25° to 60°C and then it decreased sharply.

The curve is remarkable in two points. First, the increase of activity from 25° to 60°C was relatively small: only 1.5-fold. Second, a significant amount of activity (30%) was still retained at the highest temperature tested (75°C), i.e., 15°C above the maximum. This latter result suggested a high thermal stability of the enzyme. The temperature for maximal activity was identical to that of the lipase from *Bacillus* sp. [23] and higher than that (55°C) of the lipase

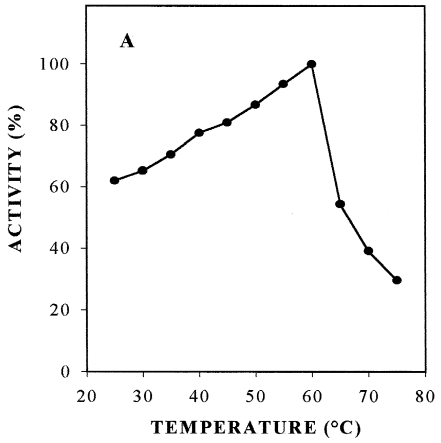

Fig. 1. Effect of temperature on pNPC8 activity of the esterase from *B. circulans* MAS2. (A) Effect on initial hydrolysis rate. Values are means of three independent determinations; standard deviations were less than 5%.

from the thermophilic fungus IPV/434 [24]. In addition, the variation of activity with temperature was very low. This property is quite interesting for the development of processes since any temperature from 20° to 60°C can be used without affecting too much the esterase activity.

To check the enzyme thermostability, the enzyme preparation (7.9 mg protein/ml) was incubated for 1 h at temperatures ranging from 30° to 90°C and the residual activity was assayed in standard conditions (Fig. 2). Activity was retained (100%) until 70°C and the temperature for half inactivation was particularly high at 85°C. This pattern fully confirmed the high thermostability of the enzyme from *B. circulans*. This thermostability is higher than that of esterases from thermophilic strains such as those from *B. stearothermophilus* [25] or *Bacillus* sp. [26]. These enzymes retained 65%, 45% and 50% of maximum activity, respectively, when incubated at 70°C in similar conditions. Therefore, the esterase from *B. circulans* was particularly thermostable. This result validates the original assumption of this work that moderate bacterial thermophiles are good sources of thermostable enzymes.

3.4. Effect of pH on activity and stability

The effect of pH on activity was determined by assaying the rate of pNPC8 hydrolysis in standard conditions at the desired pH values. However, several modifications were introduced. First, the chemical hydrolysis was deduced. The highest value was 0.016 ΔDO/min at pH 10 in a 10 mM Tris–HCl buffer. Second, the molar extinction coefficient of pNP was estimated at each pH tested (see Experimental) and used for the calculation of activity. pH values of less than 7 could not be tested because of the low extinction coefficient in acidic conditions.

Activity was maximum at pH 8–9.5 with a significant activity (65%) still remaining at pH 10 (Fig. 3A). No assays could be done at pH higher than 10 due to an important rate of chemical hydrolysis of pNPC8. The optimum pH range on the alkaline side (8.5–9.5) is similar to that of most microbial esterases. For instance, the enzymes from *Bacillus* sp. [26] and from the thermophilic fungus IPV/434 [24] had optimum pH of 8. Interestingly, the activity at pH 10 was still high (65% of maximum) for the new esterase while it was less than 5% for the thermophilic fungus IPV/434.

Also shown in Fig. 3 is the effect of pH on enzyme stability (Fig. 3B). The residual activity

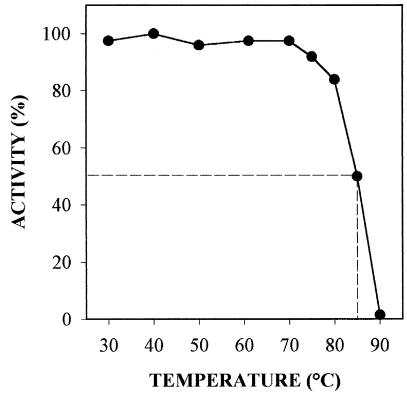

Fig. 2. Effect of temperature on stability of esterase activity from *B. circulans* MAS2. The enzyme preparation was incubated for 1 h at the indicated temperature and the remaining activity was assayed on pNPC8 at 37°C. Values are means of three independent determinations; standard deviations were less than 5%.

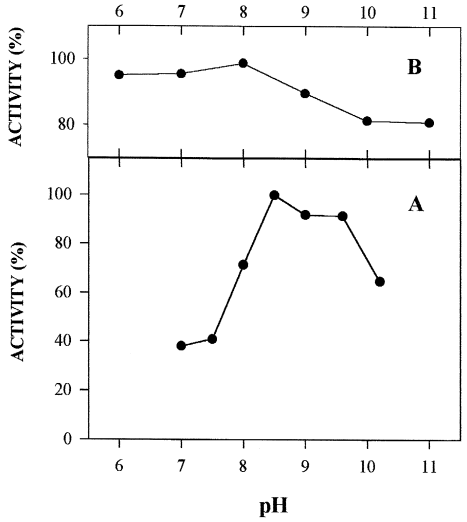

Fig. 3. Effect of pH on pNPC8 activity of the esterase from *B. circulans* MAS2. (A) Activity was assayed at different pH. (B) Effect of pH on stability (remaining activity after a 1-h treatment at the indicated pH). Values are means of three independent determinations; standard deviations were less than 10%.

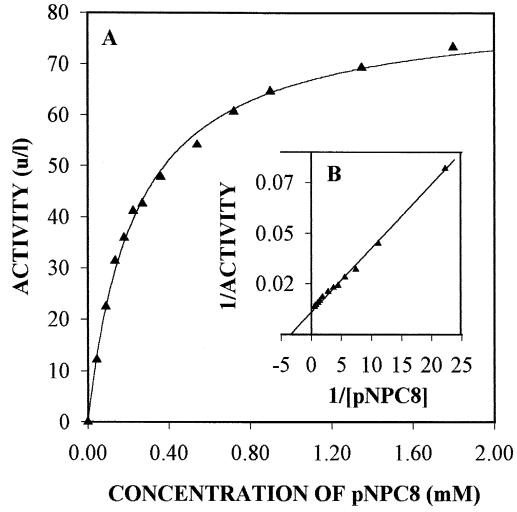

Fig. 4. Effect of pNPC8 concentration on the activity of the esterase from *B. circulans* MAS2. (A) Effect on activity. (B) Lineweaver–Burk plot. Values are means of three independent determinations; standard deviations were less than 5%.

after a 1-h treatment at 37°C is plotted against incubation pH. The activity was found stable from pH 6 to 8 and it decreased slightly at pH higher than 8. This high pH stability makes the new esterase valuable for processes at alkaline pH.

3.5. Effect of substrate concentration

The effect of pNPC8 concentration (range 0.1–1.8 mM) on the hydrolysis rate is shown in Fig. 4. The reaction rate increased with the substrate concentration with some saturation at the highest concentration. The data followed Michaelis–Menten kinetics and the Lineweaver–Burk plot was linear (insert in Fig. 4). Kinetic constants were estimated by nonlinear regression: $K_M = 0.24 \pm 0.001$ mM and $V_m = 4.3 \pm 0.06$ nmol/min mg.

3.6. Substrate specificity

The chain length of the acid moiety of the substrate was varied from acetic acid (pNPC2) to palmitic acid (pNPC16) and the reaction rates assayed under standard conditions. The substrates from pNPC2 to pNPC10 were soluble in the conditions used while those from pNPC12 to pNPC16 were insoluble and formed a stable emulsion. Maximum activity (0.58 u/ml) was found on pNPC2 (Fig. 5). For higher chain lengths of the fatty acid (pNPC4 to pNPC8) the activity decreased rapidly and for carbon chain higher than eight it was very low. This profile is characteristic of an esterase activity, i.e., an

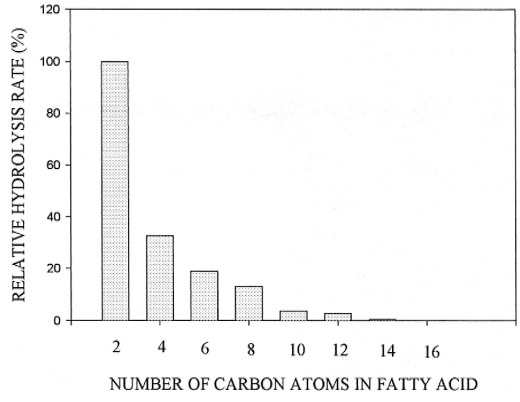

Fig. 5. Effect of fatty acid chain length on specificity of esterase from *B. circulans* MAS2. The initial rate of hydrolysis was assayed on substrate with fatty acid from acetic (pNPC2) to palmitic (pNPC16). The activity on pNPC2 was 0.58 u/ml. Values are means of three independent determinations, standard deviations were less than 5%.

enzyme only active on short-chain fatty acid esters as compared to lipases which are also active on long-chain insoluble fatty acid esters. The esterase nature of the activity was confirmed by lack of detectable activity for olive oil hydrolysis. However, esterases are usually not active on esters of fatty acid of more than six carbon atoms. This was not the case here; a significant activity was observed for mid-chain-length fatty acids: 13% on pNPC8, for instance, which is similar (11%) to that reported for the esterase from *S. acidocaldarius* [27]. This value is quite high since the known esterases usually showed lower activity on pNPC8: 7% for the esterase from *Pseudomonas* sp., [28], 4% for the esterase from *Arthrobacter globiformis* [29], less than 1% for the esterase from *Pseudomonas fluorescens* [30] and from *Pseudomonas* sp. KW1-56, [31]. Therefore, the new esterase is interesting since it can be used not only with short-chain fatty acids but also with medium-chain fatty acids, for instance for flavor ester synthesis in organic solvent.

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