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Evaluation of the potential for use in biocatalysis of a lipase from a wild strain of *Bacillus megaterium*

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Abstract

We evaluated a lipase produced by *Bacillus megaterium* with respect to its potential for use in biocatalysis in organic solvents. After 72 h of fermentation the enzyme was precipitated directly from the culture broth with the addition of ammonium sulphate to 80% of saturation. It was then resuspended and dialysed. The preparation was shown to hydrolyse triacylglycerides, with further determinations of lipolytic activity being done in aqueous systems through the hydrolysis of *p*-nitrophenyl palmitate (*p*NPP) at 37 °C. The temperature for maximum activity, based on product formation over the first minute of reaction, was 55 °C. The lipolytic activity was reasonably stable during incubation at temperatures of 40–70 °C, with a residual activity of 77% after 10 min incubation at 60 °C. Stability in organic solvents was studied by incubating the enzyme at 29 °C in butanol, toluene, hexane, isooctane and heptane and in various mixtures (25, 50, 80 and 100% (v/v)) of acetone, ethanol and isopropanol in water. Not only was it stable in butanol, hexane and toluene but it actually showed activation after being incubated in increasing percentages of isopropanol, ethanol and acetone, which is quite unusual for lipases. For the hydrolysis of *p*NPP at 37 °C in AOT (Dioctyl sodium sulfosuccinate)/heptane reversed micelles, the highest specific activities were obtained with W_0 ($[H_2O]/[AOT]$) values of 5 and 10, being 111 and 104 U mg^{-1}, respectively. The $R_{O/A}$ (ratio of the reaction rates for the same reaction in organic medium and in aqueous medium) was 1.91 for the hydrolysis of *p*NPP, which is notable because $R_{O/A}$ values for lipases are typically much less than 1.0. The lipase of *B. megaterium* shows promise for application in biocatalysis due to its good stability at elevated temperatures and in hydrophobic and hydrophilic organic solvents.

Keywords: Lipase; Biocatalysis; Stability; Reversed micelles

1. Introduction

Lipases are widely used in biocatalysis due to their ability to catalyse not only the hydrolysis of triacylglycerides in aqueous solutions, but also enantioselective synthetic reactions in organic media. Lipases must be reasonably thermostable and maintain their activity in organic solvents if they are to be used in these synthetic reactions. Thermostability is required since many processes use temperatures around 50 °C due to the high melting points of the lipids that are used as substrates [1]. Stability in organic solvents is required since low-water systems, based on organic solvents, are necessary in order to provide conditions that favour the synthetic reaction over the "normal" hydrolytic reaction. Systems based on organic solvents can also offer other advantages, for both

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hydrolytic and synthetic reactions, such as high solubilities of substrates and products, modification of the specificity of the enzyme [2] and thermostabilization of the enzyme [3].

Unfortunately, despite the advantages that biocatalysis in organic-solvent-based systems can bring, the catalytic activities of enzymes in these systems are typically much lower than activities in aqueous solutions [4]. Further, in an apparent paradox, protein stability is lower in water-miscible solvents than in hydrophobic solvents. The poor stability in hydrophilic solvents represents a problem for the use of lipases in reactions involving the esterification of sugars during the production of biosurfactants, given that in these reactions the medium contains polar solvents such as 2-methyl 2-butanol [5].

Currently known microbial lipases do not have the desirable combination of thermostability and stability in both hydrophobic and hydrophilic organic solvents. This has stimulated the search for new lipases. The present work was undertaken in this context. Thirty-six fungal and 11 bacterial isolates were screened with the objective of finding a lipase with both reasonable thermostability and stability in both hydrophobic and hydrophilic organic solvents. Early in the work, the lipolytic activity produced by a newly isolated strain of *Bacillus megaterium* showed highly interesting properties. The present work aimed to evaluate the potential of this enzyme for use in biocatalysis, including stability and activity tests in different solvents and different temperatures, and its activity against several substrates and the hydrolysis reaction in the reversed micellar system.

2. Materials and methods

2.1. Microorganism

A bacterial strain was isolated from a contaminated fungal culture that showed a high lipolytic activity. The initial assay to detect lipolytic activity was done on agar plates containing, per litre of distilled water, 15 g agar, 10 mL olive oil, 0.01 g Rhodamine B and 0.001% (w/v) Tween 80. The lipolytic activity was indicated by a strong fluorescent halo when the plate was irradiated with UV light at 350 nm after it had been incubated for 48 h at 29 °C. It was characterized as *B. megaterium* through biochemical tests and 16sRNA sequence analysis [6,7], by the Oswaldo Cruz Culture Collection (CCOC) located at the National Institute for Quality Control of Health-INCQS, Oswaldo Cruz Foundation-FIOCRUZ, Rio de Janeiro, Brazil and deposited as CCOC-P2637.

The strain was freeze-dried in glass ampoules and maintained at −20 °C. Backup cultures were maintained in Nutrient Broth medium (DIFCO 0003) with 20% (w/v) glycerol at −70 °C. In our laboratory *B. megaterium* CCOC-P2637 was maintained in Luria Bertani (LB) medium with 50% (w/v) glycerol at −18 °C.

2.2. Production of the crude lipolytic extract

B. megaterium CCOC-P2637 was inoculated into 50 mL of LB broth in a 125 mL Erlenmeyer and incubated for 8 h at 29 °C and 120 rpm, at which time it was in mid-exponential phase. One mL of this culture was then inoculated into each of several 500 mL Erlenmeyers, each containing 150 mL of a medium containing (g L^{-1}) KNO_3 3.54; K_2HPO_4 1.0; $MgSO_4{\cdot}7H_2O$ 0.5; NaCl 0.38; $FeSO_4{\cdot}7H_2O$ 0.01, yeast extract 5.0 and 1% (v/v) olive oil and incubated at 120 rpm and 29 °C for 72 h. Growth was followed by total cell counts in an improved Neubauer chamber.

2.3. Preparation of the crude extract

After 72 h of culture, when the lipolytic activity was maximal, the contents of the flask were centrifuged at 10,000 × *g* for 20 min at 4 °C. Ammonium sulphate was added to the supernatant to 80% of saturation, with mild agitation. The extract was then maintained under gentle stirring at 4 °C for 12 h, after which it was centrifuged at 12,000 × *g* for 10 min. The supernatant was removed and the precipitate re-suspended in a minimal volume of 0.02 M phosphate buffer, pH 7.0. This suspension was dialyzed against the same buffer, at 4 °C. The molecular weight cut-off of the dialysis membrane was 14,000 Da. The dialyzed fractions retained within the bag were stored at 4 °C, with the addition of 0.02% (w/v) sodium azide.

To produce sufficient enzyme for the characterization studies, re-suspended precipitates from several flasks, each treated as described above, were pooled. This concentrated crude extract had a volume of 100 mL (after dialysis), a protein content of 1.36 mg mL^{-1} and a volumetric activity of 80 U mL^{-1}.

2.4. Lipase assay in aqueous solution

The main method used for the kinetic characterization and stability assays was the *p*-nitrophenyl palmitate (*p*NPP) method [8]. Activity tests were also done with other esters of *p*NP (*p*-nitrophenol), namely, *p*-nitrophenyl acetate (*p*NPA), *p*-nitrophenyl butyrate (*p*NPB), *p*-nitrophenyl caproate (*p*NPC) and *p*-nitrophenyl caprate (*p*NPCA) (Sigma). The coefficient of extinction of (*p*NP) at pH 7.0 ($\varepsilon = 8.0 \times 10^3$ L mol^{-1} cm^{-1}) was determined from the absorbance at 410 nm of standard solutions of *p*NP. One unit of activity (U) was defined as the production of 1 μmol of *p*-nitrophenol per minute at 37 °C, under the conditions of the assay.

Lipolytic activity was confirmed by the titrimetric method, using triolein, tricaprylin and tributyrin (Sigma) as substrates [9]. An aqueous emulsion of each substrate was prepared with 20% (w/v) of substrate, 6% (w/v) of triethanolamine and 74% (v/v) of 0.05 M phosphate buffer pH 7.0, which was strongly agitated for 30 min. One mL of enzyme sample was added to 5 mL of this emulsion and then incubated at 37 °C

for 20 min under mild agitation (300 rpm) with a magnetic stirrer. Sixteen milliliters of a 1:1 (v/v) solution of ethanol and acetone was then added, and this solution titrated with 0.05 M NaOH. For this method, one unit of lipolytic activity was defined as the production of 1 μmol of free fatty acids per minute, at 37 °C.

2.5. Studies in aqueous media

2.5.1. Effect of pH on activity and stability

Activity assays were done using the *p*NPP method at 37 °C in assay mixtures buffered to various pH values (6.0–10). Molar extinction coefficients of *p*NP at different pH values were used to calculate the activity of the enzyme, as described in Lima et al. [10].

The method needed to be adapted in acid pH values since *p*NP does not absorb at pH values below 6.0. In assays carried out between pH 2.6 and 7.0, the reaction was carried out in a test tube with 4.5 mL of reaction medium pre-equilibrated at 37 °C. After the addition of 0.5 mL of a 100-fold dilution of the concentrated crude extract, aliquots of 0.1 mL were removed and added to 0.9 mL of 0.2 M phosphate buffer pH 8, in an ice bath. The absorbance was measured at 410 nm and the activity calculated using the molar extinction coefficient for pH 8.0 ($\varepsilon = 1.50 \times 10^4$ L mol^{-1} cm^{-1}).

Stability assays were done by incubating crude extract at 29 °C for 1 h in buffers of pH values 3.0–8.5 (citrate-phosphate pH 3.0–7.0; phosphate pH 7.0 and 8.0; Tris–HCl pH 8.0 and 8.5, all at 0.05 M). In each assay, 10 μL of crude extract was added to 990 μL of buffer solution. The lipase activity of each sample was then measured at 37 °C, using 0.05 M phosphate buffer, pH 8.0, by the *p*NPP method.

2.5.2. Effect of temperature on activity and stability

Activity assays were done using the *p*NPP method with 0.05 M phosphate buffer pH 8.0, at temperatures between 20 and 70 °C. The reaction was followed during 5 min and activities were calculated on the basis of the product concentration at 1 min.

Stability assays were done by incubating concentrated crude extract at 28, 37, 45 and 50 °C, in the absence of substrate. Samples were removed at different time intervals and assayed for lipolytic activity by the *p*NPP standard method, at 37 °C.

2.6. Stability in organic solvents

A diluted preparation of the concentrated crude extract (20 μL containing 1.6 U of activity) was adsorbed onto a small disc of filter paper (3 mm) and incubated at 29 °C in the presence of 1 mL of various water-immiscible organic solvents (Table 3), as described by Sztajer et al. [11]. After 1 h, the filters were taken out, the solvents were evaporated and 1 mL of phosphate buffer 0.05 M pH 7.0 was added. For water-soluble solvents, the enzyme was incubated directly (10 μL of crude extract in 990 μL of solvent). The residual activity was measured using the standard *p*NPP assay system.

Table 1
Lipolytic activity of the crude extract obtained from *Bacillus megaterium* with different substrates in aqueous solutions

Substrate	Specific activity (U mg^{-1})
p-Nitrophenyl acetate (*p*NPA) C:2	3.5 ± 0.2
p-Nitrophenyl butyrate (*p*NPB) C:4	23.3 ± 0.4
p-Nitrophenyl caproate (*p*NPC) C:6	43.6 ± 0.4
p-Nitrophenyl palmitate (*p*NPP) C:16	58.0 ± 1.1
Tributyrin C:4	41.6 ± 3.7
Tricaprylin C:8	25.3 ± 2.5
Triolein C18:1 (Δ^9)	10.0 ± 0.5

2.7. Lipase assay in reversed micelles

The hydrolysis of *p*NPP in the micellar medium was done with 5 mL of a solution of 0.1 M AOT in heptane, containing 0.01 M *p*NPP. The assays were done with values of W_0 ([H_2O]/[AOT]) from 5 to 20 through the addition of different volumes of enzyme solution, containing a fixed quantity of protein (45 μL for a W_0 of 5; 90 μL for a W_0 of 10; 135 μL for a W_0 of 15; 180 μL for a W_0 of 20). The mixture was strongly agitated in a vortex to give a clear micellar solution. Aliquots of 0.1 mL were removed every 60 s and added to 2 mL isooctane and 2 mL 0.1 M NaOH. The absorbance at 410 nm of the aqueous phase was measured. The molar absorption coefficient of *p*NP in this sodium hydroxide solution was determined as 3.5×10^3 L mol^{-1} cm^{-1}.

2.8. Gel eletrophoresis and activity detection

SDS-polyacrylamide gel electrophoresis (SDS-PAGE) was done according to Laemli [12]. The crude extract was dialyzed and diluted in 0.5 M Tris–HCl buffer pH 6.8 containing 10% (w/v) of SDS, 10% (w/v) glycerol, 5% (v/v) 2-mercaptoethanol and 0.05% (w/v) bromophenol blue. In order to allow the detection of activity after the electrophoresis, the samples were not heated to 100 °C. The following molecular weight markers were used: phosphorylase (94 kDa), bovine serum albumin (67 kDa), ovalbumin (43 kDa), carbonic anhydrase (30 kDa), trypsin inhibitor (20.1 kDa) and α-lactalbumin (14.4 kDa). After the electrophoresis, the method of Prim et al. [13] was used to detect lipolytic activity, as follows: The gel was washed for 30 min in 2.5% (w/v) Triton X-100 and then washed rapidly with 0.05 M phosphate buffer pH 7.0 and covered with a 100 μM solution of methylumbeliferyl butyrate (MUF-butyrate). This solution had been previously prepared by dissolving 2.46 mg of MUF-butyrate in 1 mL of ethyleneglycol monomethylether, to which was then added 100 mL of 0.05 M phosphate buffer pH 7.0. The blue fluorescent bands that indicate activity were visualized under UV light at 365 nm. The same gel was then treated with silver nitrate according to the method of Blum et al. [14] to reveal protein bands.

3. Results

3.1. Enzyme production profile and electrophoresis of the culture broth

B. megaterium CCOC-P2637 grown in LB broth showed two exponential growth phases. During the first exponential phase, from 0 to 30 h, it grew with a specific growth rate of 0.15 h^{-1}, while during the second exponential phase, from 30 to 108 h, it grew with a specific growth rate of 0.05 h^{-1}. There was almost no lipase production during the first 20 h. The lipolytic activity then increased linearly, reaching 33 U mL^{-1} at 70 h, and then levelled off (Fig. 1).

These results suggest that during early growth the bacterium utilizes various components of the yeast extract that both enable a high specific growth rate and cause repression of lipase synthesis. However, as these components are exhausted, at around 20 to 30 h, the production of lipase is induced by the olive oil, and the use of the liberated fatty acids as substrates supports a lower specific growth rate.

After SDS-PAGE of the crude culture medium, there was only one band with activity, suggesting that *B. megaterium* CCOC-P2637 secretes only one enzyme with activity against MUF-butyrate into the culture medium. The molecular weight of the protein with activity was estimated as 40 kDa (Fig. 2). Nevertheless, one cannot exclude the possibility that other hydrolases (esterases or lipases) were in fact produced but were not renaturated after SDS-PAGE.

3.2. Activity with different substrates

Use of MUF-butyrate to reveal the gel after SDS-PAGE shows only that an esterase is present and is not sufficient to define the enzyme as a lipase, in spite of the fact that Prim et al. [13] used this substrate to screen for lipases. Therefore a characterization of activity against different substrates was done in order to determine whether this enzyme was in fact a lipase.

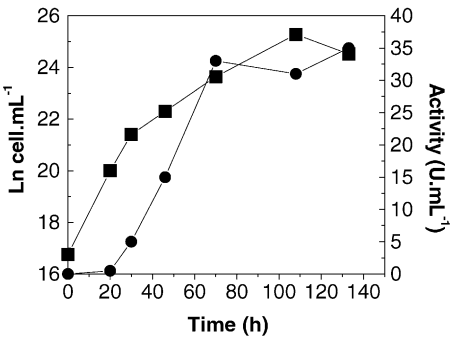

Fig. 1. Growth and lipolytic activity profiles obtained during flask culture of *Bacillus megaterium*. Culture conditions and media: mineral salts (see material and methods), yeast extract 5.0 g L^{-1} and 1% (v/v) of olive oil, Erlenmeyers of 500 mL with 150 mL of media, 120 rpm and 29 °C. (■) natural logarithm of the cell number (●) lipolytic activity.

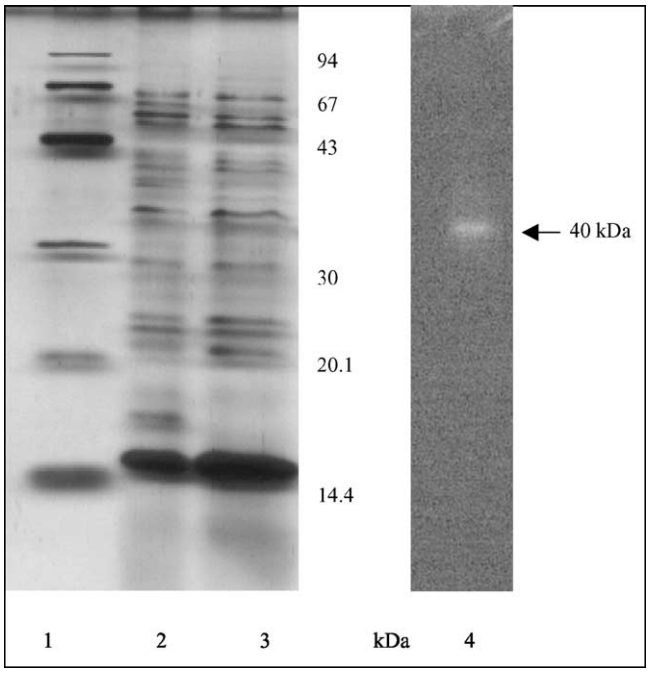

Fig. 2. SDS-PAGE analysis of the crude lypolitic extract produced by *Bacillus megaterium* (1) marker proteins—α lactalbumin 14.4 kDa, tripsin inhibitor, 20.1 kDa, carbonic anhydrase 30 kDa, ovalbumin 43 kDa; bovine serum albumin 67 kDa; phosphorilase B 94 kDa. (2) Fermentation broth, (3) Crude lypolitic extract after precipitation with ammonium sulphate. The gel was stained with silver nitrate. (4) Zymogram assay performed on the crude lypolitic extract shown on lane 3, on SDS-PAGE gel using MUF-butyrate as substrate.

The crude extract of *B. megaterium* showed increasing activity with increases in the length of the acyl chain of esters of *p*-nitrophenol (Table 1). The hydrolysis of *p*-nitrophenol butyrate (*p*NPB, 23 U mg^{-1}) was 6 times more rapid than that of *p*-nitrophenol acetate (*p*NPA, 3.5 U mg^{-1}), and the activity with *p*-nitrophenol palmitate (*p*NPP) was the highest (58 U mg^{-1}) obtained amongst the various *p*-nitrophenol esters tested.

On the other hand, the hydrolytic activity with triacylglycerols increased with decreasing length of the acyl chain (Table 1). The highest activity occurred with tributyrin, the activity with this substrate being four times higher (41 U mg^{-1}) than that with triolein (10 U mg^{-1}). Since the enzyme is capable of hydrolysing triolein at a lipid–water interface, it can be classified as a lipase. Hereafter, the enzyme will therefore be referred to as "the lipase" and the extract as the "crude lipolytic extract".

Table 2
Half-lives for thermal denaturation of the lipase from *Bacillus megaterium*

Temperature (°C)	Half-life (min)
30	495
40	123
50	42
60	23
70	4

3.3. Characteristics of the crude lipolytic extract in aqueous solution

The factors affecting the activity and stability of the lipase in aqueous solution were investigated in order to allow later comparison of its behaviour in aqueous solution and in organic solvents.

3.3.1. Effect of pH on activity and on stability of the lipase in aqueous systems

Lipolytic activity was maximal at pH 6.0 (Fig. 3) and high over the range of pH 5.0–7.0. Even at more acidic pH values, reasonable activities were obtained, the values at pH 3.0 and 4.0 being 60 and 73% of the maximum activity, respectively.

The lipase remained stable in the pH range of 5.0–8.0 (Fig. 3) after incubation for 1 h at 29 °C, with the residual activity remaining above 100%. In fact, incubation at pH values in the range of pH 6.0–8.0 led to an increase in the residual activity, which was maximal (160%) at pH 7.5. Even at pH 4.0, 70% residual activity was obtained, but at pH 3.0 the lipase was totally deactivated after incubation for 1 h.

3.3.2. Effect of temperature on activity and stability of the crude lipolytic extract

The lipolytic activity against *p*NPP, measured in terms of the amount of product produced during the first minute of incubation, increased with temperature from 30 up to 55 °C (Fig. 4), with a specific activity of 80 U mg^{-1} at 55 °C. High activities were also found at higher temperatures (70 U mg^{-1} for 65 °C and 60 U mg^{-1} at 70 °C). Activity determinations beyond 70 °C were not done because of the difficulties in rate estimation caused by the spontaneous hydrolysis of *p*NPP, which becomes significant above 70 °C.

To determine the thermal stability of the lipase, it was incubated in the range from 30 to 70 °C, with the residual activity being determined by the *p*NPP method. The lipase of *B. megaterium* was quite stable in the range of 30–60 °C,

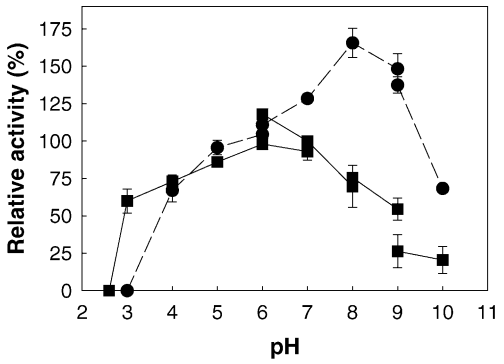

Fig. 3. Effect of pH on activity (■) and stability (●) of the crude lipolytic extract of *Bacillus megaterium*. For stability studies, residual activities were measured after 1 h incubation at 29 °C Buffers: pH 2.6–7.0 citrate-phosphate; pH 6.0–8.0 phosphate; pH 8.0 and 9.0 Tris–HCl; pH 9.0 and 10 glycine-NaOH, all buffers at 0.05 M. Assay conditions: 37 °C, 3.8 μg of protein per assay, 0.03 mg mL^{-1} of *p*NPP. The activities were compared to the activity determined in phosphate buffer, pH 7.0, without incubation.

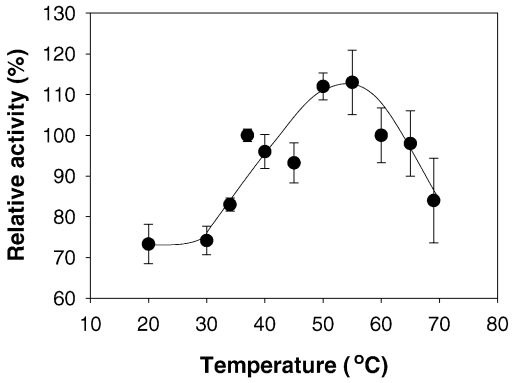

Fig. 4. Effect of temperature on activity of the crude lipolytic extract of *Bacillus megaterium*. Assay conditions: 20–70 °C, 0.05 M phosphate buffer pH 7.0, 3.8 μg of protein per assay, 0.03 mg mL^{-1} of *p*NPP. The activities were compared to the activity determined in 37 °C.

with half-lives of 42 and 23 min for 50 and 60 °C, respectively (Table 2).

3.4. Stability in organic solvents

The crude lipolytic extract was incubated in increasing proportions of the hydrophilic solvents isopropanol, ethanol and acetone and then assayed in aqueous solution by the standard *p*NPP assay method. Residual activities were calculated in relation to a control sample incubated in phosphate buffer.

Residual activity initially increased with solvent concentration, up to 50% (v/v) with isopropanol and up to 80% (v/v) with ethanol and acetone (Table 3). In many cases the enzyme was activated, with residual activities greater than 100%. The lipase maintained its activity in pure isopropanol (97% residual activity), but suffered significant deactivation in pure ethanol and pure acetone.

The lipase was also stable in water-immiscible solvents, showing 121% residual activity with *n*-heptane, and maintaining its activity in butanol, toluene and hexane (Table 3). The lipase was unstable in isooctane, losing a third of its activity during the incubation.

3.5. Hydrolytic activity in reversed micelles

The synthetic substrate *p*NPP was chosen for studies of activity in reverse micelles due to the high activity of the enzyme with this substrate in aqueous medium. The system used was AOT/heptane, given that the enzyme had shown itself to be very stable in heptane. Experiments were done at values of W_0 from 5 to 20, at 37 °C, following the release of *p*-nitrophenol at 410 nm. Specific activity was high for W_0 values of 5 and 10 (Fig. 5), (111 and 104 U mg^{-1}, respectively). The specific activities at W_0 values of 15 and 20 were less than 50% of the activity at a W_0 of 5. Using the activity for a W_0 of 5, the value of $R_{O/A}$, which represents the ratio of the activity in organic medium to that of the same reaction in aqueous solution [4], was 1.91.

Table 3
Stability of the crude lipolytic extract obtained from *Bacillus megaterium* after pre-incubation in various solvents[a]

Organic solvent	Log *P*	% Residual activity after incubation in solvent–water mixture (% v/v)			
		25%	50%	80%	100%
Water miscible solvents					
Isopropanol	−0.28	166 ± 13	176 ± 33	83 ± 8	97 ± 18
Ethanol	−0.24	111 ± 20	130 ± 12	195 ± 23	44 ± 7
Acetone	−0.23	128 ± 2	122 ± 17	127 ± 2	21 ± 2
Pure solvents					
Phosphate buffer pH 8.0					100
Butanol	0.8				102 ± 16
Toluene	2.5				107 ± 8
Hexane	3.5				101 ± 4
n-Heptane	4.0				121 ± 6
Iso-octane	4.51				67 ± 5

[a] The lipolytic extract was incubated in the presence of the solvent for 1 h at 29 °C. Residual activities were determined by the standard assay for hydrolysis of *p*NPP. The activities are expressed as percentages of that obtained for incubation of the extract for 1 h at 29 °C in 0.02 M phosphate buffer, pH 8.0.

4. Discussion

In this paper we have characterized the lipolytic activity of a lipase within a crude extract from *B. megaterium* CCOC-P2637. As discussed below, the lipase has activity and stability properties that justify future efforts to purify the enzyme for a more detailed characterization of its properties.

4.1. Production of esterases and lipases by B. megaterium

The production of hydrolases (lipases and esterases) by *B. megaterium* has recently been reported [15,16]. Ruiz et al. [16] showed, through electrophoresis and zymograms of culture broth and cell extracts, the presence of three enzymes with activity against MUF-butyrate, these enzymes having molecular weights of 19, 58 and 65 kDa. The 19 and 65 kDa enzymes were secreted into the culture broth. They isolated the 19 kDa enzyme and characterized it as an esterase, based on its greater activity against short chain substrates (*p*-nitrophenyl butyrate). Jung et al. [15] detected a fourth enzyme in cell extracts of an isolate of *B. megaterium*. Its molecular weight was 38 kDa, as determined by SDS-PAGE, and it was shown to hydrolyse tributyrin. They extracted the genomic DNA and prepared a library of inserts, which were then cloned into *Escherichia coli* and expressed. The transformed cultures were screened for hydrolysis of tributyrin. This resulted in the identification of a transformant that produced an enzyme of 34 kDa that hydrolysed short-chain esters, but hydrolysis of long-chain esters was not tested. The stability and activity characteristics of this enzyme were different from those found in the present work. Activity was maximal between 25 and 35 °C and at pH 8.0, and it was reasonably stable over the pH range of 6.0–9.0, when incubated at 45 °C.

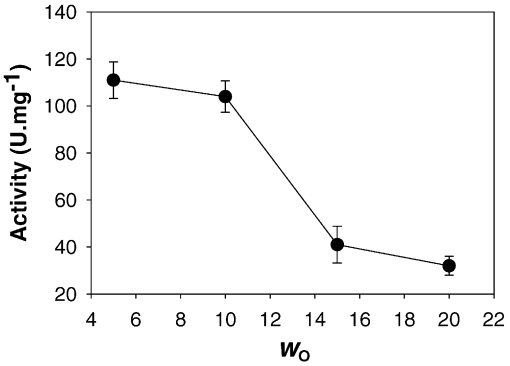

Fig. 5. Activity of the lipase of *Bacillus megaterium* in reverse micelles in different W_0 values. Assay conditions: 37 °C, micellar medium with 0.1 M AOT in heptane, containing 0.01 M de *p*NPP. The assays were done with values of W_0 ([H_2O]/[AOT]) from 5 to 20 through the addition of different volumes of enzyme solution (45 μL for a W_0 of 5; 90 μL for a W_0 of 10; 135 μL for a W_0 of 15; 180 μL for a W_0 of 20).

In the current work we have shown that a crude extract obtained from the culture medium of *B. megaterium* CCOC-P2637 has lipolytic activity, based on the ability of the crude extract to hydrolyze triolein at a lipid–water interface [17] and the fact that the activities obtained with the acyl esters of *p*-nitrophenol increased with increasing chain length of the acyl moiety. We have presumptively ascribed this lipolytic activity to an enzyme with a molecular weight of 40 kDa, which showed activity against MUF-butyrate after SDS-PAGE. However, given the possibility that other esterases or lipases might have been present in the crude extract but not renatured after SDS-PAGE, it is not possible to assign the lipolytic activity conclusively to the 40 kDa enzyme. Further work, involving purified enzyme, is currently being undertaken to clarify this matter.

4.2. Specificity of the lipase of B. megaterium

The lipase from *B. megaterium* CCOC-P2637 was most active with long chain esters of *p*NP (16 C) and showed the opposite behaviour against triacylglycerides. The same pattern for hydrolysis of *p*NP-esters has been reported for other microbial lipases, such as the lipases of *Bacillus subtilis* [18],

Penicillium aurantiogriseum [10], *Pseudomonas aeruginosa* LST-03 [19], *Pseudomonas* sp [20] and *Pseudomonas cepacia* [21].

In the case of the genus *Bacillus*, the substrate preference of lipases is variable with relation to the hydrolysis of both esters of *p*-nitrofenol and triacylglycerols. While in the current work the lipase of *B. megaterium* CCOC-P2637 was more active with *p*NPP (C16) and tributyrin (C4), the preferences were *p*NP-caprate (C10) and tricaprylin (C8) for the lipase of *B. stearothermophilus* P1 [22], *p*NP-caprylin (C8) and trilaurin (C12) for the lipase of *B. stearothermophilus* L1 [23], *p*NP-caproate (C6) and tricaprylin (C8) for the lipase of *B. thermoleovorans* [24] and *p*NP-caprate (C10) and tributyrin (C4) for the lipase of *B. thermocatenulatus* [25].

4.3. Temperature activity and stability in aqueous solution

The activity and stability of the lipase produced by *B. megaterium* CCOC-P2637 at temperatures of 50–60 °C could make it suitable for application in biocatalysis. Activity and stability in this temperature range is not common for lipases of mesophilic *Bacillus* species such as *B. megaterium*. The lipase of the mesophile *B. subtilis*, for example, hydrolyses *p*NPP at 35 °C and is stable only up to 40 °C [26]. In fact, the characteristics observed in the present work for the lipase of *B. megaterium* are similar to those found for thermophilic *Bacillus* species such as *B. stearothermophilus* [27], *B. thermoleovorans* [1] and *B. thermocatenulatus* [28] and for some strains of *Pseudomonas*, such as *P. cepacia* [21], *P. fragi* [29], *P. luteola* [30] and *Pseudomonas* sp. LP7315 [31]. The lipases produced by thermophilic species of *Bacillus* are active between 50 and 80 °C, with good stability between 50 and 65 °C, while the lipases of species of *Pseudomonas* are active between 50 and 70 °C and stable between 55 and 75 °C. Apart from these two genera, there are only few examples of microorganisms reported to produce lipases that are active and stable above 50 °C. The known lipases produced by the genus *Acinetobacter* are active between 40 and 50 °C but are not stable above 45 °C, with the exception of the recombinant lipase of strain RAG-1 [32], which is stable at 70 °C. Lipases of *Staphylococcus aureus* [33], *Staphylococcus simulans* [34] and *Staphylococcus haemolyticus* [35] have their best activities between 28 and 42 °C, although they are stable at temperatures between 50 and 60 °C. Amongst fungi and yeasts, thermostable lipases are even rarer. The lipases of *P. aurantiogriseum* [10], *Aspergillus niger* [36] and *Fusarium heterosporum* [37] show good activity at temperatures around 50 °C while the lipases of *Humicola lanuginosa* [38], *A. niger* [39] and *Aspergillus terreus* [40] have been reported to be reasonably stable up to 65 °C.

4.4. pH activity and stability in aqueous solution

The activity and the stability shown by the lipase of *B. megaterium* CCOC-P2637 at acidic pH values are not common amongst the lipases produced by bacteria, which in general are most stable and active at neutral or alkaline pH values. However, there are some exceptions. The lipases produced by *B. stearothermophilus* and *B. licheniformis* are stable at pH 3.0 [41] and the lipases of *Acinetobacter calcoaceticus* LP009 and *S. simulans* are stable at pH 4.0 [42,34]. The characteristics of the lipases of *B. megaterium* CCOC-P2637 are similar to those of lipases of fungi and yeasts, which generally have activity optima in acid or neutral pH values and show stability over the pH range of 4.0–10.

4.5. Stability in organic solvents

The lipase produced by *B. megaterium* CCOC-P2637 is remarkably stable in both hydrophilic and hydrophobic organic solvents. Stability in organic solvents is desirable if lipases are to be used in synthesis reactions, which are carried out in systems with low water contents, these low water contents being obtained through the use of organic solvents. Hydrophilic solvents ($-2.5 < \log P < 0$), such as acetone and ethers, are generally incompatible with enzymatic activity because they remove the solvation water layer from the surface of the enzyme, thereby destabilizing the protein and causing high denaturation rates [11,43,44]. Interestingly, the lipolytic extract obtained from *B. megaterium* CCOC-P2637 presented an opposite behaviour: it was activated with solvents with low log P values (i.e. around −0.2).

Good stability of bacterial and fungal lipases in hydrophilic organic solvents is rare, and the activation of activity by these solvents is even rarer. In previous work, we tested the stability of the lipases of *P. aurantiogriseum* and *Penicillium corylophilum* in organic solvents, using the same methods and under the same conditions as those used in the current work [10,45]. Both enzymes showed less than 50% residual activity after incubation in 20% (v/v) solutions of the hydrophilic solvents tested in the current work. No residual activity remained after incubation at higher concentrations of these solvents. The lipase from *Pseudomonas mendocina* PK12CS [46] is the only reported example of a microbial lipase that is reasonably stable against a hydrophilic solvent; when incubated for 2.5 h in 100% ethanol, the residual activity was 83%.

According to Sugihara et al. [21], the activation of lipases in the presence of some organic solvents, such as isopropanol, can be explained by the disruption of aggregates formed between the enzyme and lipids of the fermentation medium or between the enzyme molecules themselves. In fact, gel filtration chromatography of the crude extract obtained from *B. megaterium* CCOC-P2637 has shown that the lipolytic activity is produced in a form of a high molecular weight aggregate that is already present in the fermentation broth (data not shown). Disruption of this aggregate by the polar solvents tested could have led to the activations observed.

The activation obtained in this work with the hydrophobic solvent *n*-heptane (a residual activity of 121%) has

previously been reported for lipases. For example, the lipases of both *P. aurantiogriseum* and *P. corylophilum* were activated by incubation in *n*-heptane (113% of residual activity for *P. aurantiogriseum* and 130% for *P. corylophilum*) [10,45]. The activation by hydrophobic solvents could be due to the presence of residues of these solvents, taken back into the assay solution with the enzyme after the pre-incubation. These solvent molecules can interact with hydrophobic amino acid residues present in the lid that covers the catalytic site of the enzyme, thereby maintaining the lipase in its open conformation. Without the lid covering the active site, the activity in the assay is higher [47].

4.6. Activity in the reversed micelle system

The value of 1.91 obtained for $R_{O/A}$, the ratio of the activity in organic medium to that of the same reaction in aqueous solution, is higher than values reported by many other authors for other lipases. In fact, it is more common for the activity in organic solvents to be much lower than that presented in aqueous media (i.e. $R_{O/A}$ values tend to be much smaller than 1). Pencreac'h and Baratti [4] compared the activities of 32 commercial lipases in aqueous media and in direct catalysis in *n*-heptane. Only 6 of these enzymes presented $R_{O/A}$ values higher than 1. Even in reverse micelle systems, $R_{O/A}$ values are typically less than 1. For instance, the lipase of *P. corylophilum*, studied under the same conditions as those in the present work, had an $R_{O/A}$ of only 0.004 [45].

5. Conclusion

In this work, we demonstrated the activity and stability, in both aqueous medium and water-restricted media, of the crude lipolytic extract produced by a newly isolated strain of *B. megaterium*. Besides being produced at high levels in the fermentation broth, the enzyme presented activity and stability at high temperatures in aqueous medium and a remarkable stability towards both hydrophilic and hydrophobic organic solvents. In the AOT/*n*-heptane reversed micellar system, the activity was higher than that in aqueous media. These results, especially the good stability in hydrophilic solvents, which is rare for lipases, support the search for potential applications of the enzyme in biocatalysis in organic media and justify future work on purification of the enzyme.

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