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Production and Purification of a Solvent-Resistant Esterase from *Bacillus licheniformis* S-86

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Abstract New thermophilic and organic-solvent-tolerant *Bacillus licheniformis* S-86 strain is able to produce two active and solvent-stable esterases. Production of type I and II esterases was substantially enhanced when oils and surfactants were supplied as carbon sources. Grape oil (0.1% *v*/*v*) and Tween 20 to 60 (0.1% *v*/*v*) had enhanced enzyme production between 1.6- and 2.2-folds. Type II esterase was purified to homogeneity in a five-step procedure. This esterase was purified 76.7-fold with a specific activity of 135 U mg^{-1}. Molecular mass of the enzyme was estimated to be 38.4 kDa by sodium dodecyl sulfate polyacrylamide gel electrophoresis. Type II esterase was active mostly on esters with short acyl chains, which allowed to classify the enzyme as a carboxylesterase with a K_m of 80.2 mmol l^{-1} and a V_{max} of 256.4 μmol min^{-1} mg^{-1} for *p*-nitrophenyl acetate. Also, *B. licheniformis* S-86 type II esterase displayed activity in presence of water-miscible organic solvents at 50% concentration and stability after 1-h incubation.

Keywords Esterases · *B. licheniformis* · Production · Purification · Enzyme characterization · Detergents · Oils

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Introduction

Esterases (E.C. 3.1.1.1, carboxyl ester hydrolases) catalyze the hydrolysis of esters composed of short-chain fatty acids, ester synthesis, and transesterification reactions [1]. Particularly, these enzymes have attracted considerable attention due to their ability to synthesize new compounds of high commercial value, which can be used in food, pharmaceutical, and chemical industries. Among useful reactions performed by esterases, we can highlight the resolution of racemic mixtures by transesterification or enantioselective hydrolysis of esters for obtaining optically pure compounds [2–4]. The majority of these synthesis reactions are performed in organic media, where enzymes show unique and attractive properties, such as changes in their enantioselectivity and substrate specificity, and have the ability to conduct reactions that are suppressed in water environments [5]. In addition, biotransformations in organic solvents offer other industrially attractive advantages, such as the suppression of water-dependent side reactions, resistance to bacterial contamination, and, in some cases, enhanced enzyme thermostability. However, enzymes are generally not stable in the presence of organic solvents and are easily denatured [6]. For this reason, biocatalysts that remain stable in the presence of organic solvents could be very useful industrially.

Numerous studies have demonstrated that solvent-tolerant bacteria could produce solvent-tolerant enzymes [7–10]. According to this, we had previously isolated a wild-type organic solvent-tolerant bacillus strain, *Bacillus licheniformis* S-86, which produced organic solvent-stable esterases [11]. *B. licheniformis* S-86 produced two different molecular weight esterases detected by gel electrophoresis. One esterase activity was inhibited by phenylmethylsulphonyl fluoride (PMSF) and named type I and could be classified a serine-type enzyme. Contrariwise, PMSF has no effect on the other enzyme activity (type II). Despite most organic solvents being toxic to microorganisms because of their effects on cellular membranes [12], when this strain was cultivated in the presence of some hydroxylic organic solvents, esterase production was higher than in the absence of an organic solvent [13].

In this paper, we are describing some nutritional factors which affect the production of the organic solvent-tolerant esterases produced by *B. licheniformis* S-86 and we are reporting the results of the purification and partial characterization of type II esterase, a non-sensitive PMSF enzyme.

Materials and Methods

Reagents

All reagent used in this work were of analytical or microbiological grade from Sigma (MO, USA) or Merck (Darmstad, Germany).

Bacterial Strain and Culture Conditions

Growth and enzyme production experiments of *B. licheniformis* S-86 were carried out in a synthetic medium (SM) containing (g l^{-1}): $NaNO_3$, 1.2; KH_2PO_4, 3.0; K_2HPO_4, 6.0; $MgSO_4 \cdot 7H_2O$, 0.2; $CaCl_2$, 0.05; $MnSO_4 \cdot H_2O$, 0.01; $ZnSO_4 \cdot 7H_2O$, 0.001; peptone, 2.50; and yeast extract, 1.50 (pH=7.5). SM medium was supplemented or not with the following oils (0.1 and 0.5% *v*/*v*): olive oil, soybean oil, corn oil, sunflower oil, and grape oil; and

esters (0.1% *v*/*v*): polyoxyethylene sorbitan mono-laurate (Tween 20), polyoxyethylene sorbitan mono-palmitate (Tween 40), polyoxyethylene sorbitan mono-stearate (Tween 60), polyoxyethylene sorbitan mono-oleate (Tween 80), Sorbitan trioleate (Span 85), and Tributyrin. *B. licheniformis* S-86 was grown in Erlenmeyer flasks (125 ml) containing 15 ml of media at 50 °C for 48 h on a rotary shaker at approximately 3.3 Hz. The culture medium was inoculated with an exponentially growing pre-culture prepared in the same medium at 50 °C (1×10^8 UFC ml^{-1}; 2% *v*/*v*). Growth was determined by measuring optical density at 560 nm (OD_{560}) and correlated to colony forming units (CFU) in nutrient agar plates.

Esterase Production for Enzyme Purification

Esterase production was conducted in SM medium supplemented with maltose (10.0 g l^{-1}) and olive oil (0.05%) at pH 7.5 in 400-ml airlift reactors at 50 °C for 48 h. The culture medium was inoculated with an exponentially growing pre-culture prepared in the same medium at 50 °C (1×10^8 UFC ml^{-1}; 2% *v*/*v*). Culture supernatants were obtained by centrifugation at 10,000×*g* for 15 min at 4 °C.

Enzyme Assays

Enzyme activities were determined in cell-free extracts by centrifuging the cultures. Esterase activity was assayed by measuring the enzymatic hydrolysis of 0.92 mmol l^{-1} *p*-nitrophenyl (*p*NP) ester of acetate to *p*-nitrophenol in a spectrophotometer at 400 nm (Metrolab 1250, R. Corswant, Argentina) [14]. The extinction coefficient of *p*-nitrophenol, 7.17×10^3 l mol^{-1} cm^{-1} (r^2=0.9997), was determined at 400 nm of standard solutions of *p*-nitrophenol in Tris–HCl buffer (30 mmol l^{-1}, pH=7.0). The reaction mixture for standard assay contained 60 μl Tris–HCl buffer (200 mmol l^{-1} pH 7.0), 30 μl of the enzyme solution, and 305 μl of solvent–water mixtures. The enzyme reaction was started by adding 5 μl of 74 mmol l^{-1} *p*NP ester (dissolved in pure acetone) into the mixture and incubated at 37 °C for 15 min. PMSF (1.0 mmol l^{-1}) was added to reaction mixture to inhibit serine-type enzymes. Total esterase activity was quantified without PMSF, and non-PMSF-sensitive esterase was determined in presence of the inhibitor. The PMSF-sensitive esterase activity was calculated from the difference between total and non-PMSF-sensitive esterase activities. One enzymatic unit (EU) was defined as the amount of enzyme producing 1 μmol of *p*-nitrophenol in aqueous medium per minute.

Determination of Protein Concentration

Coomassie Blue G-250 reagent was used to determine sample protein content using bovine serum albumin (fraction V) as standard [15].

Purification of Type II Esterase

Type II esterase was purified to homogeneity following a five-step procedure, which was performed (unless was specified) at room temperature.

1) *Ammonium sulfate fractionation*. $(NH_4)_2SO_4$ was added to the culture supernatant to 40% saturation. This solution was centrifuged at 15,000×*g* for 15 min at 0 °C, and the pellet was discarded. The resulting supernatant was brought to 80% $(NH_4)_2SO_4$ saturation at 0 °C, and the precipitate was collected by centrifugation at 15,000×*g* for

15 min at 4 °C. The precipitate was dissolved in 20 mmol l^{-1} phosphate buffer (pH 7.0) and dialyzed against the same buffer at 4 °C.

2) *Ultrafiltration*. The concentrated enzyme solution was ultrafiltered in a 100-kDa cut-off centrifugal filter device (Centricon, Millipore, MA, USA). This procedure was carried out to eliminate a polymer that interfered with the following chromatographic steps.
3) *Thermal treatment*. Thermolabile proteins were eliminated by heating the enzyme solution at 65 °C during 15 min and then centrifuging at 15,000×*g* for 15 min at 4 °C. The resulting pellet containing denatured proteins was discarded.
4) *Hydrophobic interaction chromatography*. $(NH_4)_2SO_4$ was added to a final concentration of 1.7 mol l^{-1} to the enzyme solution and loaded onto a column (diameter, 1.3 cm; height, 11.0 cm) of Octyl-Sepharose 4 Fast Flow (GE, NJ, USA) equilibrated with 40 mmol l^{-1} phosphate buffer (pH 7.0) containing 1.0 mol l^{-1} $(NH_4)_2SO_4$. After the column was washed with the same buffer, proteins were eluted with a discontinuous gradient of $(NH_4)_2SO_4$ (from 1.0 to 0 mol l^{-1}) in phosphate buffer at 1.0 ml min^{-1} flow rate. Collected fractions were dialyzed against 20 mmol l^{-1} phosphate buffer (pH 7.0).
5) *Ion-exchange chromatography*. The active fractions from the Octyl-Sepharose column were applied onto a DEAE Sepharose CL-6B (GE) column (diameter, 0.9 cm; height, 9.5 cm) equilibrated with 20 mmol l^{-1} phosphate buffer (pH 7.0). Proteins were eluted with a discontinuous gradient of NaCl (from 0.125 to 1.0 mol l^{-1}) in phosphate buffer at a flow rate of 1.0 ml min^{-1}. Collected fractions were dialyzed against 20 mmol l^{-1} phosphate buffer (pH 7.0), and active fractions were concentrated by ultrafiltration using a Centricon centrifugal filter device (Amicon, MA, USA) with a membrane of 10 kDa cut-off. The samples were then stored at −20 °C until used.

Electrophoresis

Polyacrylamide gel electrophoresis (PAGE) under non-denaturing conditions and sodium dodecyl sulfate PAGE (SDS-PAGE) were carried out as described by Laemmli [16]. Gel electrophoresis was performed in a Mini-PROTEAN II electrophoresis cell (Bio-Rad, CA, USA). For both non-denaturing and SDS-PAGE, 8% (*w*/*v*) polyacrylamide gels were used. Proteins were stained using the silver staining procedure [17]. Esterase zymograms were performed in both native PAGE and in SDS-PAGE submerging the gels into 100 mmol l^{-1} Tris–HCl buffer (pH 7.0), containing 20.0 mg ml^{-1} of α-naphtyl acetate/Fast Blue RR salt at 45 °C for 20 min. Bands showing hydrolytic activity were detected by Fast Blue release.

Estimation of Molecular Mass

The molecular mass (M_r) of denatured type II esterase was estimated by gel electrophoresis (SDS-PAGE 8%). Rabbit muscle myosin (200.00 kDa), *Escherichia. coli* β-galactosidasa (116.25 kDa), rabbit muscle phosphorylase b (97.40 kDa), bovine serum albumin (66.20 kDa), hen egg white ovalbumin (45.00 kDa), bovine carbonic anhydrase (31.00 kDa), and soybean trypsin inhibitor (21.50 kDa) were used as molecular mass standards (Bio-Rad). Proteins were stained by the silver staining method.

The M_r of native type II esterase was also estimated by gel electrophoresis (SDS-PAGE 8%). This could be possible due to the stability of the enzyme in presence of SDS and 2-mercaptoethanol. The electrophoresis was done in the same conditions but without heating at 100 °C the sample containing the purified enzyme. In this way, the type II esterase kept its catalytic activity, and from the activity band, the M_r of the native enzyme could be estimated.

Determination of Kinetic Parameters

The effect of the substrate (*p*NP-acetate) concentration (range from 0.025 to 2.000 mmol l^{-1}) on the reaction rate was determined under the standard assay conditions (30 mmol l^{-1} Tris–HCl buffer, pH 7.0; 37 °C). The kinetic constants (K_m and V_{max}) with *p*NP-acetate were estimated in the range 25 to 250 μmol l^{-1} of substrate concentration by the Lineweaver and Burk plot [18].

Substrate Specificity

Substrate preference of type II esterase was determined spectrophotometrically (pH 7.0; 37 °C) using the following *p*NP esters (0.25 mmol l^{-1}): acetate (C_2), propionate (C_3), butyrate (C_4), caproate (C_6), caprate (C_{10}), laurate (C_{12}), palmitate (C_{16}), and estearate (C_{18}). The activity towards *p*NP-acetate and propionate was measured in 30 mmol l^{-1} Tris–HCl buffer. Towards *p*NP-butyrate, caproate, caprate, laurate, palmitate, and estearate, the reactions were carried out in the same buffer containing 0.35% (*w*/*v*) Triton X-100 and 0.8% (*w*/*v*) arabic gum and compared with the activity towards *p*NP-acetate in the same experimental conditions. The results were expressed as a percentage of the esterase activity measured with *p*NP ester of acetate which was set at 100% [14].

Effect of Water-Miscible Organic Solvents on Type II Esterase

The effect of water-miscible organic solvents in enzyme stability and activity was investigated in 30 mmol l^{-1} Tris–HCl (pH 7.0) containing 50% (*v*/*v*) organic solvent. The activity of type II esterase in organic solvents was measured using *p*NP-acetate 0.25 mmol l^{-1} as substrate at 37 °C. The enzyme activity in each organic solvent was compared with the activity in water which was set at 100% [14]. Type II esterase stability was measured by incubating the enzyme in the reaction mixtures at 28 °C for 1 h. After that, the enzyme activity in each solvent was measured and compared with the initial activity (before the incubation) in the same solvent, which was set at 100%. The respective extinction coefficients in each organic solvent were 5.04×10^3 l mol^{-1} cm^{-1} (methanol), 4.91×10^3 l mol^{-1} cm^{-1} (ethanol), 3.45×10^3 l mol^{-1} cm^{-1} (1-propanol), 4.12×10^3 l mol^{-1} cm^{-1} (2-propanol), 6.11×10^3 l mol^{-1} cm^{-1} (ethylenglycol), 4.62×10^3 l mol^{-1} cm^{-1} (1,3-propanediol), 5.44×10^3 l mol^{-1} cm^{-1} (propylenglycol), 6.64×10^3 l mol^{-1} cm^{-1} (glycerol), 7.17×10^3 l mol^{-1} cm^{-1} (2,3-butanediol), and 4.78×10^3 l mol^{-1} cm^{-1} [dimethyl sulfoxide (DMSO)].

Statistical Analysis

Determinations were made in duplicate, and results shown are the average of two or more independent experiments. Data are represented by the mean±standard deviation. Analysis of variance was performed on data sets using a significance of p values lower than 0.05.

Results and Discussion

Effect of Carbon Source on Esterase Production

In this work, different oils and esters (surfactants) were screened for their capacity to support *B. licheniformis* S-86 growth and esterase production. As indicated in Tables 1, 2,

Table 1 Effect of oils on esterase activity, protein concentration, and growth of *B. licheniformis* S-86 at 50 °C during 48 h of growth.

Oils (0.1% *v*/*v*)	Growth (UFC ml^{-1} 10^8)	Protein (μg ml^{-1})	Esterase activity (U mg^{-1})		Yield index (fold)	
			Type I	Type II	Type I	Type II
Control	1.39±0.07	12.6±0.7	2.30±0.42	2.23±0.33	1.0	1.0
Olive	2.07±0.11	14.4±0.4	3.08±0.38	2.11±0.28	1.3	0.9
Soybean	1.79±0.09	13.6±0.6	3.31±0.35	2.46±0.18	1.4	1.1
Corn	2.82±0.15	22.1±0.4	3.09±0.31	2.38±0.18	1.3	1.1
Sunflower	1.79±0.10	14.6±0.6	3.30±0.37	2.76±0.23	1.4	1.2
Grape	2.75±0.15	16.0±0.4	3.82±0.41	3.48±0.38	1.7	1.6
Tributyrin	2.26±0.07	17.0±0.2	3.00±0.32	2.44±0.28	1.3	1.1

Data represent the average±standard deviation.

and 3, higher biomasses regarding to the control medium (SM medium without carbon source) were obtained at 48 h of growth with all the oil and ester substrates assayed. Among the carbon sources tested for esterase production, grape oil (0.1%, *v*/*v*) and Tween 20 to 60 enhance the enzyme production between 1.6- and 2.2-fold above the control medium (Tables 1 and 3). Tween 20 resulted the best inducer of type I esterase (2.0-fold), whereas Tween 60 resulted the best for type II esterase (2.2-fold; Table 3). However, enzyme production in microorganisms not only depends on the carbon source but also its concentration [19]. When oils were tested in higher concentrations (0.5%, *v*/*v*), no increased in the enzyme activity of type II esterase was observed, and only 1.2- to 1.3-fold increase in esterase activity type I was observed in presence of grape, soybean, and olive oil (Table 2).

The mechanisms regulating biosynthesis of enzymes vary widely in different microorganisms. Despite this, similar results claiming that oils and surfactants enhance the production of some hydrolases were previously reported [6, 20, 21]. In the case of surfactants, they could improve esterase production acting as a carbon source, and/or they could improve esterase secretion, increasing cell permeability and thereby facilitating the export of proteins across the cell membrane [19]. The effects of surfactants in growth and enzyme production have been studied in several microorganisms with different results [22]. Contrary to what is observed in *B. licheniformis* S-86, a low biomass of *Candida rugosa* was observed when Tween 80 was the sole carbon source, suggesting a possible surfactant toxicity [23].

Table 2 Effect of oils on esterase activity, protein concentration, and growth of *B. licheniformis* S-86 at 50 °C during 48 h of growth.

Oils (0.5% *v*/*v*)	Growth (UFC ml^{-1} 10^8)	Protein (μg ml^{-1})	Esterase activity (U mg^{-1})		Yield index (fold)	
			Type I	Type II	Type I	Type II
Control	1.39±0.07	12.6±0.7	2.30±0.42	2.23±0.33	1.0	1.0
Olive	2.24±0.12	19.4±0.2	2.95±0.35	2.27±0.27	1.3	1.0
Soybean	2.14±0.13	16.4±1.0	2.75±0.25	2.29±0.07	1.2	1.0
Corn	3.17±0.17	22.3±1.2	2.37±0.22	2.32±0.09	1.0	1.0
Sunflower	2.48±0.12	20.1±1.1	2.19±0.19	2.40±0.18	0.9	1.1
Grape	3.13±0.18	21.9±1.4	2.73±0.13	2.35±0.12	1.2	1.0

Data represent the average±standard deviation.

Table 3 Effect of surfactants on esterase activity, protein concentration, and growth of *B. licheniformis* S-86 during 48 h of growth at 50 °C.

Surfactant (0.1% *v*/*v*)	Growth (UFC ml^{-1} 10^8)	Protein (μg ml^{-1})	Esterase Activity (U mg^{-1})		Yield index (fold)	
			Type I	Type II	Type I	Type II
Control	1.39±0.07	12.6±0.7	2.30±0.42	2.23±0.33	1.0	1.0
Tween 20	1.73±0.10	12.9±0.4	4.68±0.39	4.72±0.05	2.0	2.1
Tween 40	3.65±0.17	25.2±0.4	4.46±0.16	4.38±0.05	1.9	2.0
Tween 60	3.71±0.20	16.0±0.4	4.48±0.45	4.89±0.53	1.9	2.2
Tween 80	1.80±0.09	19.7±0.4	2.33±0.27	2.39±0.10	1.0	1.1
Span 85	3.58±0.19	23.1±0.4	3.32±0.43	2.56±0.09	1.4	1.1

Data represent the average±standard deviation.

In a previous report, *B. licheniformis* S-86 was cultivated in SM medium supplemented with 10 g l^{-1} maltose [13]. The total esterase production in this medium at 48 h of growth was approximately 54% lower than what is observed in the control medium (SM medium without carbon source). This result suggested a repressive effect (like a catabolite repression) on the esterase production. The inhibitory effects of various carbohydrates in the production of cholesterol esterase by a *Streptomyces* sp. strain was investigated showing similar results [24]. Catabolite repression was also reported in the synthesis of some microbial lipases [6, 23].

Purification and Partial Characterization of Type II Esterase

Grape oil (0.1%, *v*/*v*) and Tween 20 to 60 gave the higher esterase yields. However, in absence of maltose in the culture media, the stability of both esterases decreased in about 80% in presence of high concentrations of ammonium sulfate. Due to ammonium sulfate fractionation methodology being selected as a purification step of type II esterase, production of the enzymes was conducted in SM medium supplemented with 10 g l^{-1} maltose.

Type II esterase was purified to homogeneity from the culture supernatant of *B. licheniformis* S-86 after five steps of purification that included hydrophobic interaction and ion-exchange chromatographies. The enzyme was purified 76.7-fold with a specific activity of 135 EU mg^{-1}, which represented a 1.3% yield (Table 4). The purity was confirmed by the presence of a single band on SDS-PAGE (Fig. 1). The M_r of type II esterase was

Table 4 Purification of the type II esterase from *B. licheniformis S-86.*[a]

Purification step	Total activity (U)[b]	Total protein (mg)	Specific activity (U mg^{-1})[b]	Purification (fold)	Yield (%)
Culture supernatant	84.93	48.165	1.76	1.0	100.0
Ammonium sulfate fractionation	20.74	9.765	2.12	1.2	24.4
Thermal treatment (65 °C)	15.18	8.760	1.73	1.0	17.9
Octyl-sepharose	1.69	0.084	20.09	11.4	2.0
DEAE-sepharose	1.08	0.008	135.00	76.7	1.3

[a] From 570 ml of culture supernatant

[b] Esterase activity against *p*-nitrophenyl acetate

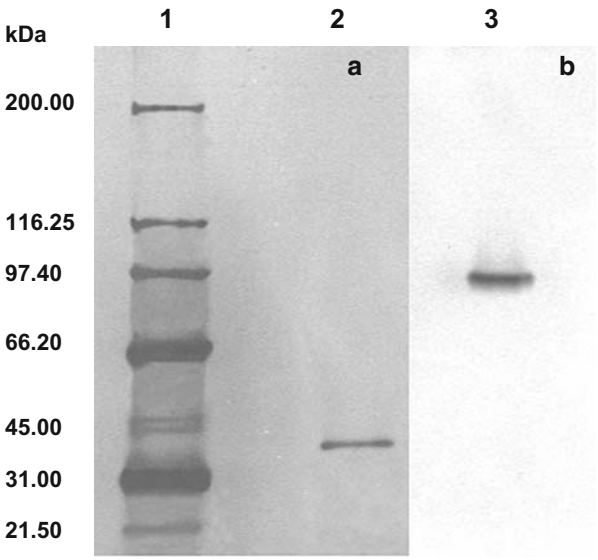

Fig. 1 SDS-PAGE (8%) of the purified type II esterase from *B. licheniformis* S-86. **a** Protein staining. *Lane 1*, Molecular mass standards; *lane 2*, purified type II esterase (1 μg of protein). Proteins were stained using the silver staining procedure. **b** Activity staining. *Lane 3*, purified type II esterase (0.03 μg of protein; nonheated sample). Esterase activity was determined with α-naphtyl acetate as substrate

estimated to be 38.4 from the logarithmic plot of the molecular mass vs. the mobility of the standard proteins on SDS-PAGE.

Type II esterase was stable in the presence of SDS and 2-mercaptoetanol in the concentrations used in SDS-PAGE according to Laemmli. When the activity of type II esterase was determined under standard conditions for SDS-PAGE, which included the heating of the sample, the activity towards α-naphtyl acetate could not be detected due to the protein denaturalization. However, if the enzyme solution, containing SDS and 2-mercaptoetanol, was not heated at 100 °C before the SDS-PAGE electrophoresis, the hydrolysis of α-naphtyl acetate could be observed (Fig. 2b). This property of the type II esterase allowed estimating the M_r of the native enzyme in 94.0 kDa. This result suggested that the protein could be a dimer or trimer with subunits of 38.4 kDa. The difference between the M_r calculated for the native enzyme (94.0 kDa.) and that calculated using two or three subunits (76.8 or 115.2 kDa, respectively) could be attributed to the fact that the native protein does not migrate as a single polypeptide of equivalent M_r [25]. Similar results were reported for the esterase produced by *Pyrobaculum calidifontis*, which consisted of a trimer with subunits of 34 kDa [26], and for the esterase of *Bacillus circulans*, also formed by three monomers of 30 kDa [25]. In comparison with other well-known esterases, the M_r of type II esterase of *B. licheniformis* S-86 was similar to the M_r of the thermostable esterase of *Bacillus acidocaldarius* (37.5 kDa) [27] and to that of the BsubE and YbfK esterases of *Bacillus subtilis* (32 kDa, each one) [28, 29] and BsteE esterase of *Bacillus stearothermophilus* (31 kDa) [29]. However, the M_r of type II esterase was also different from that of other well-known esterases [30, 31].

The effect of the substrate concentration on the reaction rate of type II esterase was studied using the ester *p*NP-acetate (Fig. 2). The kinetic was Michaelian with linear Lineweaver–Burk plot. The enzyme had a V_{max} corresponding to 256.4 μmol of substrate, hydrolyzed per minute for each milligram of enzyme, and a K_m value of 80.2 μmol l^{-1}. This lower value of K_m indicates a great affinity of the enzyme for *p*NP-acetate. Higher values of K_m for *p*NP-acetate were obtained in other esterases from strains belonging to the *Bacillus*

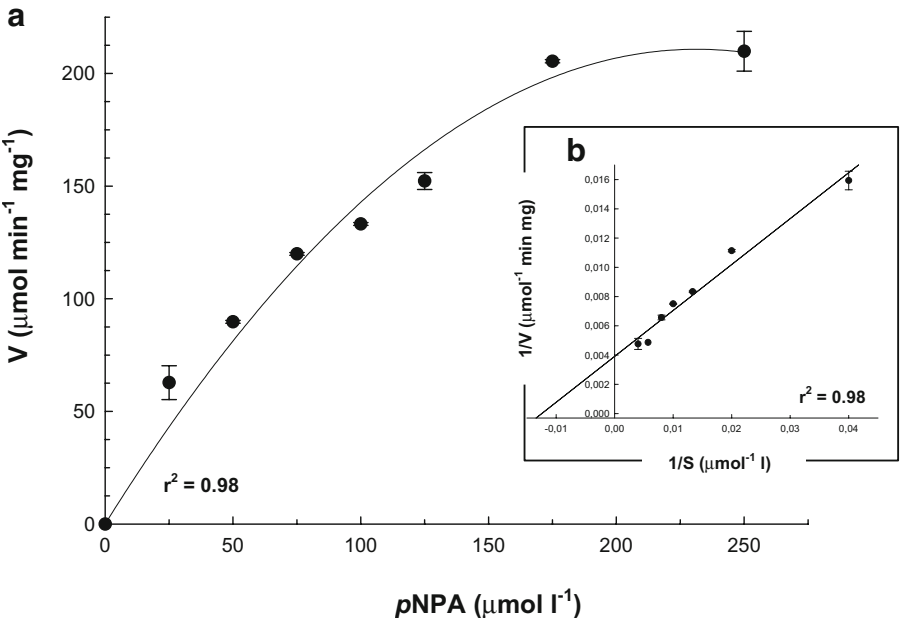

Fig. 2 Kinetic of type II esterase. **a** Rate of hydrolysis of *p*-nitrophenyl acetate (*p*NPA) in the range 25 to 250 μmol l^{-1} performed at pH 7.0 and 37 °C. **b** In the *inset*, Lineweaver and Burk plot of type II esterase activity towards *p*NPA

genus [14, 32–36], such as the esterase from *B. acidocaldarius* (124 μmol l^{-1}) [27], or the esterases Est25 of *E. coli* (1000 μmol l^{-1}) and Est3 of *Sulfolobus solfataricus* P2 [37, 38].

Table 5 shows the activity of type II esterase against various *p*NP esters having different carbon chain lengths, from C_2 (acetate) to C_{18} (estearate). The activity of the enzyme against other esters is shown relative to that against *p*NP-acetate. The highest hydrolytic activity was found towards to *p*NP-acetate. However, a pronounced decrease in the activity was observed from *p*NP-butyrate. This pattern of hydrolysis against *p*NP esters found in type II esterase was similar to that observed in acetyl esterases [25] and in a cephalosporin esterase of *Rhodosporidium toruloides* [39] and in other esterases produced by *Bacillus*

Table 5 Substrate specificity of the type II esterase.

p-Nitrophenyl ester	Esterase activity (%)[a]
Acetate[b]	100.0±2.5
Propionate	88.3±6.3
Butirate	1.4±0.1
Caproate	0
Caprate	0
Laurate	0
Palmitate	2.6±1.0
Estearate	0

[a] Data represent the average of three independent assays ± standard deviation.

[b] Esterase activity with *p*-nitrophenyl acetate was set at 100%.

strains [14, 33]. The substrate specificity observed in type II esterase corresponds with its kinetics parameters against *p*NP-acetate.

Since esterases produced by *B. licheniformis* S-86 displayed high activity in 50% hydroxylic-water solvent mixtures and were active at high temperatures (60–65 °C) [5, 11], the isolated type II esterase resulted as a very attractive enzyme to study, which could be an useful tool to use in organic synthesis and industrial biocatalysis.

The enzyme activity of type II esterase was measured in reaction mixtures containing 50% of water-miscible organic solvents (Table 6). These reaction mixtures were incubated during 1 h at room temperature, and then the residual activity of the esterase was compared with the initial activity in the same solvent. Activity was observed in all cases, and a particularly high level of activity was found in 50% glycerol, ethylenglycol, and propylenglycol. Also, in these solvents, the stability of the enzyme was high, similar to that observed in water. We found that type II esterase retained more than 70% of its activity after 1 h of incubation in the presence of all the solvents assayed with the exception of 2,3-butanediol and 1,3-propanediol. Inclusive in solvents such as ethylenglycol, glycerol, and methanol, the enzyme retained entirely its initial activity. The activity of type II esterase in glycerol was higher than the activity measured for *Bacillus atrophaeus* SB-2 and *B. licheniformis* SB-3 lipases in the same concentration of the solvent (10% and 30% residual activity, respectively) [40]. And its stability was similar to that of the extremely stable esterase Est of *P. calidifontis*. However, type II esterase was much more active in 50% methanol, ethanol, and 2-propanol than the last one [26]. Furthermore, the ability of type II esterase to perform transesterification reactions was tested in an anhydrous organic solvent such as *n*-hexane, using *p*-nitrophenyl acetate as acyl donor and the alcohols ethanol or isoamyl alcohol. In these systems, the transesterification was shown by the *p*-nitrophenol formation in the absence of water, which suggested the transfer of the acyl group from the *p*-nitrophenyl ester to the alcohol (data not shown).

Table 6 Effect of water-miscible organic solvents on the activity and stability of type II esterase.

Organic solvent (50%)	Relative esterase Activity (%)[a]	
	Activity[b]	Stability[c]
Water	100	95.05±3.00
2,3-Butenodiol	26.70±2.33	63.97±5.20
DMSO	36.95±1.71	81.25±1.96
Ethanol	13.47±1.67	73.08±1.50
Ethylenglycol	79.46±5.03	98.21±6.13
Glycerol	90.19±4.00	98.67±9.71
Methanol	24.98±0.25	108.82±11.01
1-Propanol	6.85±0.90	130.76±13.02
2-Propanol	14.56±1.21	92.65±8.95
1,3-Propenodiol	4.06±0.30	45.00±7.07
Propylenglicol	60.20±2.70	87.75±2.30

[a] Data represent the average of three independent assays ±standard deviation. Esterase activity was assayed with *p*-nitrophenyl acetate at pH 7.0 and 37 °C.

[b] Esterase activity in water was set at 100%.

[c] Residual activity after 1 h incubation in 50% water-miscible organic solvents at room temperature. Remaining esterase activity in each solvent was compared with the initial activity (before the incubation) in the same solvent, which was set at 100%.

Stability and activity in organic solvents are important when using enzymes for organic synthesis. These features make type II esterase a very attractive enzyme for future application in industrial biocatalysis.

As concluding remarks, esterase type II from *B. licheniformis* S-86 can be synthesized in a simple semi-synthetic medium containing oils (e.g., from agricultural wastes) and detergents. The esterase type II has the advantage of being not sensitive to the irreversible PMSF inhibitor and constituted by subunits which allow manipulating the biocatalysis process by inducing the reversible dissociation of the enzyme. Also, the enzyme is showing very low K_m and high V_m for low molecular weight substrates, high thermoresistance, and high biocatalytic activity in 50% organic–aqueous solvent mixtures indicating a great potential for bioconversions. For this reason, further biochemical characterizations of this enzyme and the study of synthesis reactions in non-aqueous media are being carried out in our laboratory.

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