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Enzymatic synthesis of banana flavour (isoamyl acetate) by *Bacillus licheniformis* S-86 esterase

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Abstract

New carboxylesterase from organic-solvent-tolerant *Bacillus licheniformis* S-86 strain was characterized. The enzyme named as type II esterase showed an optimal activity in the temperature range 60-65°C and pH 8.0. The enzyme was moderatly thermostable (half-life of 1h at 50°C), but remarkable stable at extremely alkaline pH, retaining 100% of its activity at pH 10.0-11.0. Furthermore, the esterase showed high stability in detergents (86% residual activity in 10% SDS), and also 0.1 % ionic and non-ionic detergents are inducers of enzyme activity. PMSF, a serine protease inhibitor, did not show any effect on the activity. The immobilized type II esterase was able to synthesize isoamyl acetate from isoamyl alcohol and *p*-nitrophenyl acetate (acyl donor) in *n*-hexane. The resulting ester yield (42.8%), obtained at a low temperature (28°C) and with a very low amount of enzyme ($4.6 \cdot 10^{-5}$ mg ml^{-1}), indicates a high potential for type II esterase in isoamyl acetate synthesis for production purposes.

Key words: Esterases; *B. licheniformis* esterase; characterization; transesterification; isoamyl acetate.

1. Introduction

Carboxylesterases (E.C. 3.1.1.1, carboxyl ester hydrolases) are enzymes widely distributed among all forms of life; their physiological functions have been implicated in carbon source utilization, pathogenicity, and detoxification (Ewis *et al*., 2004). Carboxylesterases have a number of unique enzyme characteristics such as substrate specificity, regio-specificity, and chiral selectivity (Jung *et al*., 2003). These enzymes preferably catalyse the hydrolysis of esters composed of short-chain fatty acids, but they also can catalyse ester synthesis, and transesterification reactions (Bornscheuer, 2002). Particularly, the potential application of these enzymes for the synthesis of short-chain esters has attracted the interest of a broad range of industrial fields like foods, pharmaceuticals and cosmetics. Among these esters, flavor acetates from primary alcohols constitute compounds with a great application due to their characteristic fragrance and flavor (Romero *et al*., 2005a). One of these esters, isoamyl acetate is one of the most important flavour compound used in the food industries (74,000 kg/year) (Krishna *et al*., 2001). However, despite this compound is a short-chain ester, all the enzymatic synthesis reactions of isoamyl acetate studied up to now, were carried out using lipases.

Other useful reactions performed by esterases that can be highlight are the resolution of racemic mixtures by transesterification, or the enantioselective hydrolysis of esters for obtaining optically pure compounds (Bornscheuer, 2002). The majority of these synthesis reactions are performed in organic media, where enzymes have the ability to conduct reactions that are suppressed in water environments (Torres & Castro, 2004). Unfortunately, wild-type esterases are seldom suited for catalysis in non-aqueous systems, particularly due to low activity and poor robustness (Maqbool *et al*., 2006). For this reason, biocatalysts able to remain stable in presence of organic solvents could be very useful industrially.

There is a great deal of interest regarding these enzymes from solvent-tolerant bacteria because of their stability in organic solvent (Hüsken et al., 2001). A screening program for bacterial esterases developed in our laboratory has lead to the isolation of a wild-type organic solvent-tolerant *Bacillus licheniformis* S-86, an organic solvent-stable esterases producer strain (Torres & Castro, 2003; Torres et al., 2005). *Bacillus* species has the advantages of extracellular enzyme production and lack of toxicity (most of *Bacillus* species are considered GRAS, Generally Regarded as Safe by FDA, USA). Additionally, species of the genus *Bacillus* have a history of safe use in the elaboration of traditional fermented food products, such as condiments and sauces produced from grains, legumes and seafood (Hwang et al., 2007; Ouoba et al., 2007). Also, safety industrial use of these bacteria and their enzymes was reported, including the production of food additives and probiotic products (Hong et al., 2005; Texeira et al., 2008). One of the esterases from *B. licheniformis* S-86, called type II esterase, was previously purified in a five-step procedure (Torres et al., 2008). This enzyme was demonstrated to be a carboxylesterase specific for short-chain acyl esters, and stable and active in the presence of hydroxylic organic solvents (Torres et al., 2008). In this paper, we report the biochemical characterization of type II esterase produced by *B. licheniformis* S-86. Furthermore, a study on the application of this enzyme in the biotransformation of isoamyl alcohol into isomayl acetate is also described.

2. Materials and methods

2.1. Bacterial strain, culture conditions and enzyme purification

The wild-type strain *B. licheniformis* S-86 has been isolated from soil in our laboratory by culture in a synthetic medium containing tributyrin as sole carbon source at 55ºC, and later by selection in a medium supplemented with isoamyl alcohol (Torres & Castro, 2003).

Growth of *B. licheniformis* S-86, esterase production and enzyme purification were carried out as previously described (Torres et al. 2008). The production of type II esterase from B. *licheniformis* S-86 were conducted in a synthetic medium 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; yeast extract, 1.50; maltose, 10.0; and olive oil (0.05%, v/v) (pH=7.5). *B. licheniformis* S-86 was grown in 400-ml airlift reactor 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.

Type II esterase was purified to homogeneity from the culture supernatant of *B.licheniformis* S-86 after five steps of purification that included ammonium sulfate fractionation and concentration; ultrafiltration of the concentrated enzyme solution in a 100-kDa cut-off centrifugal filter device (Centricon, Millipore, MA, USA); thermal treatment (65°C during 15 min) for the elimination of thermolabile proteins; hydrophobic interaction chromatography (Octyl-Sepharose 4 Fast Flow; GE, NJ, USA); and finally, ion-exchange chromatography (DEAE Sepharose CL-6B; GE) (Torres et al. 2008).

2.2. Enzyme assays

Esterase activity was assayed by measuring the enzymatic hydrolysis of 0.25 mmol l^{-1} *p*-nitrophenyl ester of acetate (*p*NP-acetate) to *p*–nitrophenol spectrophotometrically at 400 nm (Metrolab 1250, R. Corswant, Argentina). The extinction coefficient of *p*-nitrophenol, 7.17×10^3 l mol^{-1} cm^{-1} (r^2 = 0.9997), was determined at 400 nm using a standard solutions of *p*-nitrophenol in Tris-HCl buffer (30 mmol l^{-1}, pH= 7.0). Unless otherwise noted, 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 water. The enzyme

reaction was started by adding 5 µl of 20 mmol l^{-1} *p*NP-acetate (dissolved in pure acetone) into the mixture, and incubated at 37°C for 15 minutes. One enzymatic unit (EU) was defined as the amount of enzyme producing 1 µmol of *p*-nitrophenol per minute.

2.3. Determination of protein concentration

Coomassie Blue G-250 reagent was used to determine samples protein content using Bovine Serum Albumin (Fraction V) as standard (Sedmak & Grossberg, 1977).

2.4. Determination of kinetic parameters

Kinetic measurements were carried out with *p*NP-acetate under the standard assay conditions (30 mmol l^{-1} Tris-HCl buffer, pH 7.0; 37°C). The turnover number (K_{cat}) was calculated from the following equation: K_{cat} = [S.A.] M_r, where [S.A.] is the specific activity of the enzyme and M_r is the molecular mass previously estimated (Torres et al., 2008). The catalytic efficiency (K_{cat}/K_m ratio) was calculated from the K_m value previously reported (Torres et al., 2008).

2.5. Effect of temperature on type II esterase activity and stability

The optimum temperature of the type II esterase was determined at pH 7.0 (30 mmol l^{-1} Tris-HCl buffer) by the standard esterase assay with *p*NP-acetate as substrate in the range of 37 to 75°C. The thermostability of the esterase was examined by incubating the enzyme for 1 h in 30 mmol l^{-1} of Tris-HCl buffer (pH 7.0), at temperatures ranging from 37 and 75°C. Then, the residual activities were measured at 37°C by the standard assay as described above.

2.6. Effect of pH on type II esterase activity and stability

The optimum pH of the type II esterase was investigated in 30 mmol l^{-1} citrate-phosphate (pH range 4-6), phosphate (pH range 6-8), Tris-HCl (pH range 7-10) and glycine-NaOH (pH range 10-11) buffers in the presence of 0.25 mmol l^{-1} *p*NP-acetate at 37°C. Esterase pH stability was studied by incubation of the enzyme at various pHs (pH range 4.0-11.0; using 50 mmol l^{-1} buffer concentration) for 1 h at 28°C. The residual esterase activities were measured at 37°C and pH 7.0 (30 mmol l^{-1} phosphate buffer) as described above.

2.7. Effect of detergents on type II esterase activity

The effect of detergents on esterase activity was analyzed by incubation of enzyme for 1 h at 28°C in 30 mmol l^{-1} Tris-HCl buffer (pH 7.0), containing 0.1% (w/v) of the following detergents: Tween 20, Tween 40, Tween 60, Tween 80, Tritón X-100 and SDS. The ionic detergent SDS was also assayed in the same concentation used in SDS-PAGE, 10% (w/v). The residual esterase activities were measured at 37°C as described above.

2.8. Effect of metal ions and inhibitors on type II esterase activity

The effects of metal ions on esterase activity were determined by incubating the enzyme for 1 h at 28°C in 30 mmol l^{-1} Tris-HCl buffer (pH 7.0), containing various metal salts (NaCl, KCl, $MgCl_2$, $MnCl_2$, $CaCl_2$, $FeCl_3$, $ZnSO_4$ and $CuSO_4$) at final concentrations of 1 mmol l^{-1}. The effects of inhibitors on esterase activity were examined using the following compounds: phenylmethanesulfonyl fluoride (PMSF), 2-mercaptoethanol, ethylenediaminetetraaceticacid (EDTA), $AgNO_3$ and $HgCl_2$, at final concentrations of 1 mmol l^{-1}. The enzyme was incubated with each inhibitor at 28°C for 1 h in 30 mmol l^{-1} of Tris-HCl buffer (pH 7.0). The residual esterase activities were measured at 37°C as

described above. The esterase activity of the enzyme with the addition of neither metal ions nor inhibitors was defined as 100%.

2.9. Effect of salt concentration on type II esterase activity

The effect of NaCl concentration on enzyme activity was investigated by incubation of the enzyme for 1 h at 28°C in 30 mmol l^{-1} Tris-HCl buffer (pH 7.0), containing various salt concentrations in the range 0.001 – 1.000 mol l^{-1}. The residual esterase activities in each NaCl concentration were measured at 37°C as described above and compared with the activity of the enzyme without the addition of NaCl that was set as 100%.

2.10. Isoamyl acetate synthesis using type II esterase

Isoamyl acetate synthesis was carried out in a stirred reactor with a capacity of 2 ml, in *n*-hexane solvent, using immobilized type II esterase and *p*NP-acetate as acyl donor. 60 µl of enzyme solution (0.046 µg of type II esterase, equivalent to 6.2 10^{-3} U, in 15 mmol l^{-1}Tris-HCl buffer, pH 8.0) were mixed with 50 µl of DEAE Sepharose CL-6B (suspended in ethanol 20%). Then this suspension containing the enzyme immobilized in DEAE Sepharose was dried for 1 h in a SAVANT Speed Vac Plus UVS400A at room temperature. The resulting powder was suspended in 1.0 ml of *n*-hexane and 10 µl of 3-methylbutan-1-ol. When the reaction temperature was reached (28°C), the esterification reaction was started by adding 3 µl of 160 mmol l^{-1} *p*NP-acetate dissolved in pure acetone. The reaction mixture was incubated at 28°C for 24 hours.

The isoamyl acetate concentration was measured in *n*-hexane (reaction medium) by gas chromatography, using an Agilent 6890 N gas chromatographer equipped with an automatic injector Agilent 7683 and a Agilent 19091J – 413 Column (HP-5; 30.0 m x 320

µm x 0.25 µm nominal). The following conditions were applied: injection temperature 250°C (splitless); oven temperature 60°C for 1 minute, then increase of 10°C/min until 240°C and 240°C for 2 minutes; detector temperature 275°C; and carrier gas N2 (4.49 psi). The yield was calculated based on the conversion of the *p*NP-acetate to isoamyl acetate, comparing the ester formed with the initial *p*NP-acetate concentration. Control experiments were also conducted without esterase under similar conditions.

2.11. Reagents

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

2.12. 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.

3. Results and discussion

3.1. Kinetic studies

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 (Torres et al. 2008). The kinetic parameters of type II esterase were studied using the ester *p*NP-acetate in the range 0.025 - 2.000 mmol l^{-1}. The M_r of the native enzyme was estimated to be 94.0 kDa, and its K_m value equal to 80.2 µmol l^{-1} (Torres et al., 2008). The turnover number K_{cat} of type II esterase was deduced using the M_r of the native enzyme (Table 1). The catalytic efficiency

K_{cat}/K_m of the enzyme for *p*NP-acetate showed a high efficiency of type II esterase for the hydrolysis of this ester in comparison with that observed in many esterases (Table 1) (Zimmer et al., 2006; Kim and Lee, 2004; Degrassi et al., 1999; Giver et al., 1998).

3.2. *Influence of temperature on esterase activity and stability*

Activity of the esterase was investigated over a temperature range from 37 to 75°C. The results (Fig. 1.A) indicate that the enzyme is active over a broad temperature range. The esterase activity increased very gradually from 61% at 37°C to the maximum of 100% at 60-65°C and then decreased suddenly to 55% at 70°C. The optimal reaction temperature reported here falls into the range of optimum temperatures observed in some thermophilic esterases such as those from *Ralstonia* sp. M1 or *Thermotoga maritima* (tm0053) (Quyen et al., 2007; Kakugawa et al., 2007). The Arrhenius plot of the data (Fig. 1.B) yielded an activation energy (E_a) of 38.4 kJ/mol in the range of 37-65°C. This E_a is similar to the values obtained for esterase Est 55 from *Geobacillus stearothermophilus* (35.7 kJ/mol) (Ewis et al., 2004) and the esterase from *B. licheniformis* LCB40 (32.6 kJ/mol) (Alvarez-Macarie et al., 1999). The esterase was found to be moderately thermostable, displaying at 50°C a half-life for thermal inactivation of 1 h, but it was not stable at high temperatures, retaining only 10 and 7% of its activity after 1 h at 65 and 75°C, respectively (Fig. 1.A). The stability of type II esterase from *B. licheniformis* S-86 was lower than that observed in esterases from the extremophile microorganisms *Pyrobaculum calidifontis* (Hotta et al., 2002), *B. acidocaldarius* (Manco et al., 1998), *Sulfolobus solfataricus* P2 (Kim and Lee, 2004), or *B. circulans* (Kademi et al., 2000), which are among the more thermostable esterases known. However, the stability of type II esterase was similar to the stability of the thermostable lipase produced by *Pseudomonas* sp. S5 (Rahman et al. 2005b). Additionally, it was more stable than the esterase BsubE of *B. subtilis*, almost completely

inactivated after few minutes at 50ºC (Henke & Bornscheuer, 2002). Despite its moderate thermal stability, the optimum activity of type II esterase occurred at major temperatures, which were higher than the optimum temperatures observed in enzymes with similar or greater stability, such as the aforementioned lipase from *Pseudomonas* sp. S5 or the estarases produced by the thermophilic *B. circulans* and *B. licheniformis* LCB40, which display maximum activities at temperatures below 60°C (Rahman et al. 2005b; Kademi et al., 2000; Alvarez-Macarie et al., 1999). A similar effect of the temperature on activity and stability was observed in a carboxylesterase from *Archaeoglobus fulgidus* that displays optimal activity at 70°C, but almost no long-term thermostability even at moderately temperatures (Rusnak et al., 2005).

3.3. Influence of pH on esterase activity and stability

The activity of type II esterase was evaluated at pH values ranging from 4.0 to 10.0. As shown in Fig. 2, the enzyme displayed high levels of activity under neutral to alkalophilic conditions. The enzyme exhibited the highest activity at pH 8.0 and high acitvity (around 85% of the maximum) still retained in the pH range of 9.0-10.0. However, a significant decrease in the enzyme activity was observed bellow pH 7.0 with almost no activity at pH 4.0. The esterase from *B. Licheniformis* S-86 showed optimum activity in a slightly alkaline pH, similar to that for most esterases (Manco et al., 1998; Costa & Peralta, 1999). Nevertheless, the most interesting feature of type II esterase was the unusual spectrum of pH stability. The enzyme exhibited the highest stability at pH range of 10.0-11.0 (Fig. 2), with no loss of activity after 1 h of incubation in these conditions, while it retained almost 80% residual activity at pH 7.0, but only 10% at pH 4.0. The pH range of activity and stability of the esterase from *B. licheniformis* S-86 is clearly on the alkaline side, similar to that for some alkalo-stable esterases, such as the enzymes from *Ralstonia*

sp. M1 (Quyen et al., 2007), *B. circulans* (Kademi et al., 2000) and *Kluyveromyces marxianus* CBS 1553 (Monti et al., 2008).

3.4. Effect of detergents on esterase activity

The effect of 0.1% (w/v) of detergents including ionic and non-ionic ones was measured by incubating the esterase for 1 h at 28°C in 30 mmol l^{-1} Tris-HCl buffer (pH 7.0). At this concentration all the detergents induced the esterase activation (Table 2). However, the major activation of the enzyme was observed with non-ionic ones, particularly in presence of Triton X-100 that strongly increased the esterase activity by up 70%. The minor activation of the enzyme was measured with SDS. The esterase activity was increased by 28% when incubating with 0.1% SDS. Besides, type II esterase was stable in presence of high concentrations of this detergent, like the used in SDS-PAGE (10%). After 1 h of incubation at this concentration the esterase activity was not significantly affected and it only decreased 14%. 10% SDS largely exceeds the critical micellar concentration (cmc), above which most of the proteins are denaturalized (Mogensen et al., 2005). Also, unlike most hydrolases, type II esterase showed activation in the presence of both ionic and non-ionic detergents (Jung y col., 2003; Maqbool et al., 2006).

3.5. Effect of metal ions and inhibitors on esterase activity

The effect of 0.1 mmol l^{-1} of various metal ions was determined by incubating the esterase for 1 h in 30 mmol l^{-1} Tris-HCl buffer (pH 7.0). Except for Fe^{3+} and Cu^{2+}, all assayed metal ions exerted a positive influence on type II esterase activity by 7-35% (Table 3). The incubation of the enzyme with Fe^{3+} and Cu^{2+} resulted in a severe loss of activity. These metals together with Zn^{2+} have been reported to be highly toxic for most lipases and

esterases (Kaiser et al., 2006; Monti et al., 2008). However, Zn^{2+} exhibited the highest stimulatory effect on the enzyme activity. The enhancement of the enzyme activity by this metal, as well as Ca^{2+}, Mg^{2+} or Mn^{2+} may probably be due to the stabilization of an enzyme conformation that favors the catalysis (Kim et al., 2000), or to the neutralization of liberated fatty acids during the hydrolysis reaction (Kaiser et al., 2006).

Furthermore, several inhibitors were assayed for their effect on esterase activity at the concentration of 1 mmol l^{-1} in 30 mmol l^{-1} Tris-HCl buffer (pH 7.0) and for 1 h at 28°C. The remaining esterase activity is shown in Table 3. As it was previously demonstrated in crude extracts from *B. licheniformis* S-86 (Torres et al., 2009), the inhibitor of serine hydrolases PMSF did not affect the activity of the purified type II esterase. By a nucleophilic reaction the hydroxyl group of serine attacks the sulfonyl group of PMSF, which leads to the irreversible sulfonylation of the active serine (Karpushova et al., 2005). However, the same as type II esterase other hydrolases were not inhibited by PMSF, like the lipases from *Acinetobacter calcoaceticus* (Dharmsthiti et al. 1998) and *B. thermoleovorans* ID-1 (Lee et al., 2001), a palmitoyl thioesterase (Das et al., 2000), a cholesterol esterase (Xiang et al., 2006) or the carboxylesterases from *Archaeoglobus fulgidus* (Rusnak et al., 2005) and *B. licheniformis* LCB40 (Alvarez-Macarie et al., 1999). In these enzymes the insensibility to PMSF was a result of the spatial limitation in the catalytic cavity or the presence of an electrophilic sulfonyl-substituent that prevents its inhibitory effect (Rusnak et al., 2005). Besides, the chelating agent EDTA inhibit enzyme activity only 11%, indicating that type II esterase is not dependent on divalent metal ions.

Despite type II esterase would be formed for more than one monomer, the enzyme activity was not inhibited in the presence of disulphide bond reducing agent 2-mercaptoethanol. This observation suggests that disulphide bonds would not be accessible to the reducing reagent. A similar behavior was described in the carboxylesterase Est 30

from *Geobacillus stearothermophilus*, which in spite of being a dimer, was not affected neither for 2-mercaptoetanol nor DTT (Ewis et al., 2004). Furthermore, this reducing reagent is regarded as acting by protecting essential thiol groups in their reduced state (Podoler & Applebaum, 1971). Thus 2-mercaptoethanol seems to increase type II esterase activity by protecting the thiol groups that could be involved in the enzyme activity. A similar response to 2-mercaptoethanol was also observed by Ewis et al. (2004) for the carboxylesterase Est 30, as well in other enzymes (Aygan et al., 2008). That thiol groups are in fact essential for enzyme activity is evident from the inhibitory effect of the thiol-blocking reagent $HgCl_2$ on type II esterase activity. Indeed, this cysteine-specific inhibitor $HgCl_2$ and also the histidine-specific inhibitor $AgNO_3$, decreased the enzymatic activity by 50% and 90%, respectively. These results suggested that His and Cys residues are located at or near the active site and are related to catalytic activity of the esterase.

3.6. Effect of salt concentration on esterase activity

Due to the halotolerance of *B. licheniformis* S-86, the influence of different NaCl concentrations on the enzyme activity was determined. The esterase was quite stable in high NaCl concentrations. Nearly 70% and 50% of esterase activity remained in the presence of 0.5 mol l^{-1} and 1 mol l^{-1} NaCl, respectively. A similar effect of NaCl concentrations was observed in EstB2 esterase from marine *Bacillu*s sp. Besides, concentrations of this salt between 5 and 50 mmol l^{-1} increased the activity of type II esterase by up 13% (Karpushova et al., 2005). A larger stimulating effect on enzyme activity was observed in other enzymes, such as the lipase from a *Pseudomonas* strain and the arylesterase from *Lactobacillus casei* (Dong et al., 1999; Fenster et al., 2003). Some authors argue that the stimulant effect on catalytic activity of low concentrations of salt is due to the interaction of ions Na^{1+} with the surface of the enzyme, affecting the ionization

of some amino acid residues. This somehow led to a change in the conformation of the enzyme and thus alters its activity (Dong et al., 1999).

3.7. Isoamyl acetate synthesis

B. licheniformis S-86 was able to growth in presence of the very toxic isoamyl alcohol. In an effort to explain the mechanism of tolerance towards this organic solvent, we previously demonstrated that part of this alcohol present in the culture medium, was transformed in a more hydrophobic and less toxic ester, which suggests the involvement of esterases in this process (unpublished data). In this work we demonstrated the ability of type II esterase to catalyze the esterification of isoamyl alcohol to isoamyl acetate.

Type II esterase was used to catalyze the esterification of 3-methylbutan-1-ol to isoamyl acetate in the presence of *n*-hexane, using *p*NP-acetate as acyl donor. After 24 h of reaction a yield of isoamyl acetate of 42.8% with regard to the initial concentration of *p*NP-acetate was obtained (Table 4). Isoamyl acetate is one of the most important flavour compound used in the food industries because of its characteristic banana flavour (Krishna et al., 2001). This ester is used as a flavoring compound in various foods and drinks, such as honey, butterscotch, artificial coffee, alcoholic beverages and perfumes. The current production of isoamyl acetate is usually available only *via* chemical synthesis by Fischer esterification mechanism (Welsh et al. 1989). The alternative enzymatic synthesis of isoamyl acetate was proposed but limited only to a laboratory scale (Krishna et al. 2001). And as for our knowledge refer, all this enzymatic synthesis reactions of isoamyl acetate studied up to now, were carried out using lipases. Despite the reaction conditions were not optimized, the yield of isoamyl acetate obtained with type II esterase was higher than that achieved with many of these lipases (Table 4) (Krishna et al., 2000; Liaquat & Owusu, 2000; Romero et al., 2005a, 2005b). In contrast with the lipases catalyzed reactions, the

concentration of the type II esterase used (0.046 μg ml^{-1}) was very low (at least 43,000 times minor), as well the reaction temperature (28°C). These two parameters, enzyme concentration and reaction temperature result very important from the economic viewpoint, making this enzyme attractive for a possible practical application (Krishna et al., 2000; Krishna et al. 2001).

4. Conclusions

B. licheniformis S-86 is an extremophile microorganism able to growth in presence of isoamyl alcohol that produces an organic solvent-tolerant carboxylesterase (Torres et al., 2008), which also was moderately thermostable, stable under extreme pH-values, high salt concentrations, and ionic and non-ionic detergents. These properties make this enzyme suitable as biocatalyst in process that required increased stability and also for the study of protein stability under such extreme conditions. Besides, one of the most important features about this enzyme is its proven utility for the synthesis in non-aqueous media of a valuable flavor compound with a great application in food industries. The use of type II esterase from *B. licheniformis* S-86 could be an alternative to be explored in order obtain isoamyl acetate by "green chemistry technology".

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Table 1. Kinetic parameters of the type II esterase for hydrolysis of *p*NP-acetate[a]. Comparison with values reported in the literature for other esterases.

Microorganism	Kinetic Parameters		References
	K_{cat}[b] (s^{-1})	K_{cat}/K_m[c] [mmol l^{-1}) s^{-1}]	
***B. licheniformis* S-86 (Type II esterase)**	**401.7**	**5008.7**	**Present work**
Thermotoga maritime (EST53 esterase)	50	43.5	Kakugawa et al., 2007
Rhodotorula mucilaginosa	3.0	7.1	Zimmer et al., 2006
Pseudomonas aeruginosa PAO1	6400	14884	Pesaresi et al., 2005
Sulfolobus solfataricus P2 (Est3 esterase)	45.3	16.2	Kim & Lee, 2004
Saccharomyces cerevisiae	8.05	23	Degrassi et al., 1999
Bacillus acidocaldarius (EST2 esterase)	660	5300	Manco et al., 1998
B. subtilis (*p*-nitrobenzil esterase)	720	390	Giver et al., 1998

a, Esterase activity was measured in 30 mM Tris-HCl (pH 7.0) at 37ºC. Data represent the average of 3 independent assays; **b,** the turnover number (K_{cat}) was calculated considering a molecular mass of 94 kDa previously estimated (Torres et al., 2008); **c,** the catalytic efficiency (K_{cat}/K_m ratio) was calculated from the K_m value (80.2 µM) previously reported (Torres et al., 2008).

Table 2. Effect of detergents on type II esterase activity.

Detergent	Concentration (%, w/v)	Relative activity (%)
Control [a]	- -	100
Tween 20	0.1	144 ± 5
Tween 40	0.1	136 ± 6
Tween 60	0.1	160 ± 5
Tween 80	0.1	155 ± 2
SDS	0.1	128 ± 5
	10.0	86 ± 1
Tritón X-100	0.1	171 ± 1

a, The esterase activity measured in the control without any detergent was set as 100%.

Table 3. Changes of esterase type II activity by effectors.

Effector (1 mmol l^{-1})	**Relative activity (%)**	**Effector (1 mmol l^{-1})**	**Relative activity (%)**
Control [a]	**100**	$CuSO_4$	31 ± 0
NaCl	107 ± 1	PMSF	97 ± 1
KCl	112 ± 1	2-Mercaptoethanol	126 ± 5
$MgCl_2$	112 ± 1	EDTA	89 ± 1
$MnCl_2$	115 ± 1	$AgNO_3$	11 ± 0
$CaCl_2$	116 ± 1	$HgCl_2$	51 ± 1
$ZnSO_4$	135 ± 5	$FeCl_3$	4 ± 1

a, The esterase activity measured in the control without any metal ion was set as 100%.

Table 4. Isoamyl acetate synthesis by type II esterase from *B. licheniformis* S-86, compared with the synthesis achieved with some microbial lipases.

Enzime[a]	Reaction conditions[b]	Enzyme conc. (mg ml^{-1})[c]	Yield (%)	References
Type II esterase *B. licheniformis* S-86	***n*-hexane, *p*NP-acetate, 28°C, 24 h, enzime immobilized en DEAE sepharose CL-6B**	**4.6 x 10^{-5}**	**42.8** [d]	**Present work**
Novozym 435 *Candida antarctica,*	*n*-hexane, acetic anhidride, 40°C, 2 h, enzyme immobilized in acrylic resin	11.04	> 100.0 [d]	Romero et al., (2007)
Novozym 435 *Candida antarctica*	*n*-hexane, acetic anhidride, 40°C, 2 h, enzyme immobilized in acrylic resin	5.04	> 100.0 [d]	
Novozym 435 *Candida antarctica*	*n*-hexane, ammonium acetate, 40°C, 24 h, enzyme immobilized in acrylic resin	5.04	< 10.0 [d]	Romero et al., (2005a)
Novozym 435 *Candida antarctica*	*n*-hexane, acetic acid, 40°C, 24 h, enzyme immobilized in acrylic resin	5.04	< 25.0 [d]	
Novozym 435 *Candida antarctica*	*n*-hexane, ethyl acetate, 40°C, 24 h, enzyme immobilized in acrylic resin	5.04	~ 50.0 [d]	
Novozym 435 *Candida antarctica*	CO_2, acetic acid, 40°C, 3 h, enzyme immobilized in acrylic resin	5.00	10.0 [e]	
Novozym 435 *Candida antarctica*	CO_2, acetic anhidride, 40°C, 3 h, enzyme immobilized in acrylic resin	5.00	95.0 [e]	Romero et al., (2005b)
Lipozyme RM-IM *Rhizomucor miehei*	CO_2, acetic anhidride, 40°C, 4 h, enzyme immobilized in acrylic resin	5.00	30.0 [e]	
Lipase *Geotrichum sp.*	Water, acetic acid, 60°C, 24 h, free enzyme	10% (w/w)	24.0 [e]	Macedo et al., (2003)

a, Enzyme used for the ester synthesis, except type II esterase all the others enzymes were lipases; **b,** Reaction conditions: solvent, acyl donor, temperature, reaction time, free or immobilized enzyme; **c,** Enzyme concentration used, expressed in mg/ml, except for c* ; **d,** Yield from *p*NP-acetate; **e,** Yield from 3-methylbutan-1-ol.

Legends to figures.

Figure 1.A. Effect of temperature on type II esterase activity. Enzymatic activities were determined in 30 mmol l^{-1} Tris-HCl buffer (pH 7.0) at specified temperatures (37 to 75°C). The highest activity observed at 60-65°C was set as 100%. To study the thermal stability of type II esterase, the enzyme solution was incubated for 1 h in 30 mmol l^{-1} of Tris-HCl buffer (pH 7.0), at temperatures ranging from 37 and 75°C. The initial activity (before incubation) was set as 100%.

Figure 1.B. Arrhenius plot of temperature dependence. The logarithm (Ln) of the specific activity (U mg^{-1}) was plotted against the reciprocal of absolute temperature (T). The activation energy (E_a; kJ/mol) was calculated from the value of the slope (S) and the general gas constant (R; 8.3 J $mol^{-1}K$) with the Arrhenius equation: $E_a = -S x 2.306 x R$.

Figure 2. Effect of pH on type II esterase activity. Enzymatic activities were determined in 30 mmol l-1 Tris-HCl buffer (pH 7.0) at specified temperatures (37 to 75°C). The activity of the enzyme at different pH values (pH range 4.0 – 10.0; 30 mmol l^{-1} concentration) was measured at 37°C (Dash line). The highest activity observed at pH 8.0 was set as 100%. To study the effect of pH on the stability of type II esterase, the enzyme was incubated for 1 h in solutions with different pH values (pH range 4.0 – 11.0; 50 mmol l^{-1} concentration), at 28°C (Solid line). The activity measured before incubation (pH 7.0) was set as 100%.

Figure 1.A.

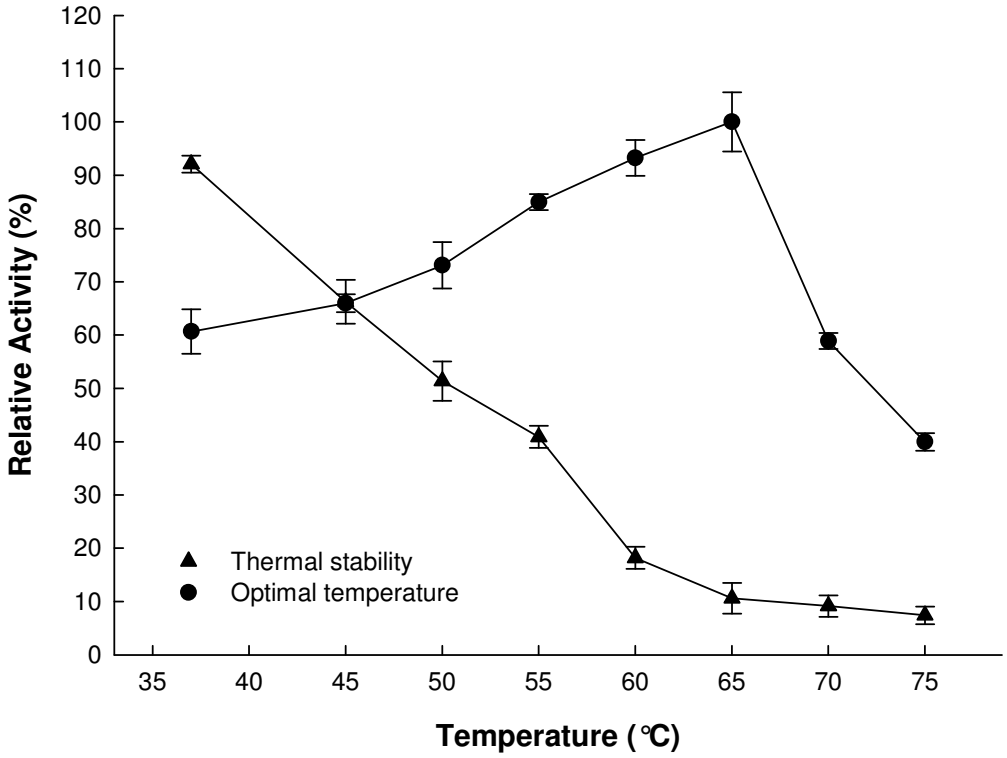

Figure 1.B.

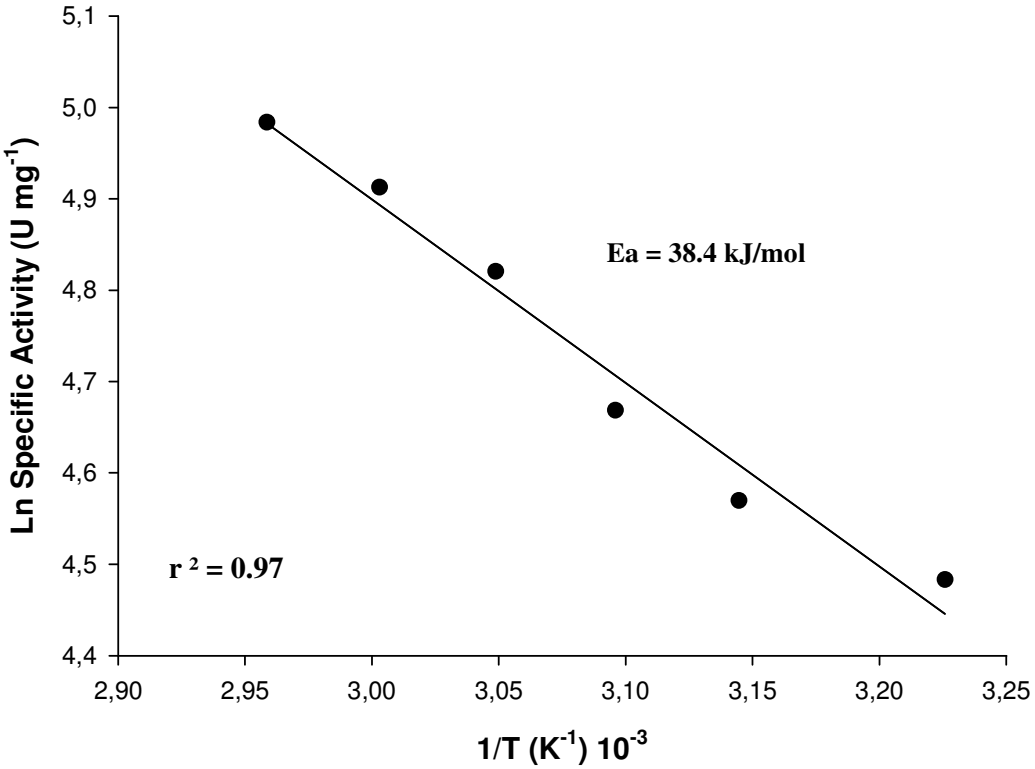

Figure 2.