Bioresource Technology 100 (2009) 896–902

Contents lists available at ScienceDirect

Bioresource Technology

journal homepage: www.elsevier.com/locate/biortech

An organic-solvent-tolerant esterase from thermophilic *Bacillus licheniformis* S-86

Sebastián Torres [a], M. Alejandra Martínez [a], Ashok Pandey [b], Guillermo R. Castro [c,d,*]

[a] Planta Piloto de Procesos Industriales Microbiológicos (PROIMI), Av. Belgrano y Pasaje Caseros, T4001 MVB Tucumán, Argentina
[b] National Institute for Interdisciplinary Science and Technology, Trivandrum 695 019, India
[c] CINDEFI (Institute of Applied Biotechnology) and INIFTA (UNLP – CCT La Plata – CONICET), Facultad de Ciencias Exactas, Universidad Nacional de La Plata, Calle 50 y 115 (B1900AJL) La Plata, Argentina
[d] Department of Biomedical Engineering, School of Engineering, Tufts University, 4 Colby Street, Medford, MA 02155, USA

ARTICLE INFO

Article history:
Received 23 March 2008
Received in revised form 2 July 2008
Accepted 12 July 2008
Available online 23 August 2008

Keywords:
Esterases
Solvent tolerance
B. licheniformis
Stress
Non-aqueous biocatalysis

ABSTRACT

A thermophile, halotolerant and organic-solvent-tolerant esterase producer *Bacillus* sp. S-86 strain previously isolated was found to belong to *Bacillus licheniformis* species through morphological, biochemical, 16S rRNA gene sequence analyses and rDNA intergenic spacers amplification (ITS-PCR). The strain can grow at 55 °C in presence of C2–C7 alkanols ($\log P = -0.86$ to 2.39), and NaCl concentrations up to 15% (w/v). This bacterium showed optimal growth and esterase production at 50 °C. Two different molecular weight esterase activities were detected in zymographic assays. PMSF inhibited type I esterase activity, showing no inhibitory effect on type II esterase activity. *B. licheniformis* S-86 was able to grow in presence of hydroxylic organic-solvents like propan-2-ol, butan-1-ol and 3-methylbutan-1-ol. At a sub-lethal concentration of these solvents (392 mmol l^{-1} propan-2-ol; 99 mmol l^{-1} butan-1-ol, 37 mmol l^{-1} 3-methylbutan-1-ol), adequate to produce 50% cell growth inhibition at 50 °C, an increment between 1.9 and 2.3 times was observed in type I esterase production, and between 2.2 and 3.1 times in type II esterase production.

1. Introduction

Enzymes synthesized by extremophile microorganisms offer new opportunities for Green chemistry arena in where biocatalysis and biotransformations play a central role. Among them, *Bacillus* species are one of the common microbial enzyme producers at industrial level, especially hydrolases.

Esterases (E.C. 3.1.1.1, carboxyl ester hydrolases) have attracted considerable attention in Biotechnology because of current applications and the perspective of new compound synthesis to be used in food, pharmaceutical, and chemical industries (Gupta and Roy, 2004). Useful reactions performed by hydrolases include resolution of racemic mixtures by transesterification, enantio- and regioselective hydrolysis and synthesis of natural and non-natural pro-drugs, detergents, polyesters, and additives (Gupta and Roy, 2004). Generally, most of these reactions occur in non-aqueous environments. Conversion of *R,S*-naproxen esters to *S*-naproxen, the synthesis of cephalosporin-derived antibiotics, and selectively conversion of heroin into morphine are typical examples of industrial applications of carboxylesterases in non-aqueous media (Bornscheuer, 2002). Also, lipases and proteases, some of the most important industrial enzymes, were extensively studied as biocatalysts in organic-solvents, particularly for biodiesel production and peptide synthesis, respectively (Ranganathan et al., 2008; Tang et al., 2008).

The vast majority of synthetic reactions are performed in organic media. Biotransformation in organic-solvents offer unique industrially attractive advantages, such as changes in the enantioselectivity of the reaction, the reversal of the thermodynamic equilibrium of hydrolysis reactions, suppression of water-dependent side reactions, and resistance to bacterial contamination. However, the major drawbacks of biocatalysis in organic media are system heterogeneity, low reaction rates and diffusional molecular coefficients, and also enzyme deactivation under extreme environmental conditions (e.g., temperature, pH, etc.). In addition, the biocatalytic activity is reduced about 10^3 folds or more in non-aqueous media. This fact could be ascribed to the biocatalyst screening and selection processes typically made in aqueous environments. Consequently, enzyme properties can not be predicted in organic-solvents and enzymatic synthesis of desired compounds in non-conventional media are generally random and time-consuming processes that fail sometimes (Torres and Castro, 2004).

In recent years, isolation of wild-type organic-solvent-tolerant extremophile microorganisms able to produce highly stable enzymes in non-aqueous environments has been proven to be

* Corresponding author. Address: CINDEFI (Institute of Applied Biotechnology) and INIFTA (UNLP – CCT La Plata – CONICET), Facultad de Ciencias Exactas, Universidad Nacional de La Plata, Calle 50 y 115 (B1900AJL) La Plata, Argentina. Tel./fax: +54 221 483 37 94x132/103.
E-mail address: grcastro@gmail.com (G.R. Castro).

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doi:10.1016/j.biortech.2008.07.009

successful (Tang et al., 2008). However, most of organic-solvents are extremely toxic for microorganisms; even at very low and sub-saturating concentrations in aqueous media (Heipieper et al., 2007). It is currently accepted that toxicity of organic-solvents towards microorganisms can be estimated by the log*P*, defined as the logarithm of the solvent partitioning coefficient between *n*-octanol and water. In particular, pure and mixed solvents with log*P* values lower than 2.0 were reported as exceptionally toxic to microorganisms (Ogino et al., 1994).

In order to improve biocatalyst properties in organic-solvents and high temperatures, a screening procedure was developed in our laboratory. Wild-type thermophilic microorganisms showing esterase activity were isolated from soil samples from different geographical areas of Latin America in presence of the very toxic 3-methylbutan-1-ol (isoamyl alcohol, log*P* = 1.3). Based on enzyme activity and cellular concentrations at 55 °C, strain S-86 was selected. Despite the presence of 3-methylbutan-1-ol reduced the growth rate of this strain, when this solvent was supply to the culture media an increase in esterase activity in the culture supernatant was observed (Torres et al., 2005). Crude extracts of strain S-86 displayed high esterase activity in 50% hydroxylic organic-solvents, and in some cases, enhanced regarding to aqueous buffer systems. Furthermore, esterase activity in crude extracts was optimum between 65 and 70 °C in aqueous and aqueous-cosolvent mixtures (Torres and Castro, 2003).

In a first approach, S-86 strain was identified by biochemical and morphological tests as a member of *Bacillus subtilis* cluster (Torres and Castro, 2003). In the present work, we described additional characterization of *Bacillus* sp. S-86 mainly performed by 16S rRNA gene sequence analysis, and the effect of organic-solvents on esterases production and cell growth of this strain.

2. Methods

2.1. Bacterial strain and culture conditions

Growth and enzyme production experiments of *Bacillus* sp. S-86 were carried out in a synthetic medium named EP as previously described (Torres et al., 2005). The strain was grown in Erlenmeyer flasks (125 ml) containing 15 ml of medium at 50 °C (or the desired temperature) for 48 h on a rotary shaker at approximately 200 rpm. The culture media was inoculated with an exponentially growing pre-culture (1×10^8 UFC ml^{-1}; 2% v/v) prepared in the same medium at 50 °C (or at the desired temperature). Growth was monitored by measuring optical density at 560 nm (OD_{560}) and correlated to colony forming units (CFU) in nutrient agar plates.

2.2. Growth in the presence of alkanols

EP media were initially supplemented with the following aliphatic water-miscible alkanols: propan-2-ol (392 mmol l^{-1}), butan-1-ol (99 mmol l^{-1}), and 3-methylbutan-1-ol (37 mmol l^{-1}). Alkanol concentrations were adjusted to produce 50% growth inhibition (IC_{50}). Growth inhibition caused by 3-methylbutan-1-ol was measured by comparing the differences in specific growth rate μ (h^{-1}) between intoxicated cultures with that of control cultures. The growth inhibition was defined as the percentage of the growth rates of cultures grown with 3-methylbutan-1-ol and that of control cultures. 3-Methylbutan-1-ol (37 mmol l^{-1}) was also assayed in the 9–55 mmol l^{-1} range. Enzyme induction experiments were carried out by adding the alkanol at 1.4 h of growth (OD_{560} = 0.3), in exponential growth phase. Esterase production was monitored in those cultures by adding 3-methylbutan-1-ol to get 37 mmol l^{-1}. Extracellular crude extracts and cells were recovered one hour later (still in the exponential growth phase), from the cultures supplemented and without 3-methylbutan-1-ol (control). Cells were washed and resuspended in 50 mmol l^{-1} Tris–HCl buffer (pH = 7.0) followed by French Press disruption (Thermo, WI, USA). Enzyme activities and protein concentration were measured in extracellular and intracellular extracts.

2.3. Effect of temperature on growth and enzyme production

The effect of temperature on bacterial growth and enzyme production was studied in EP media without organic-solvent and supplemented with 37 mmol l^{-1} 3-methylbutan-1-ol in the 28–60 °C range, for 48 h on a rotary shaker at approximately 200 rpm. Esterase activity and protein concentration were determined at 24 and 48 h. Growth rate μ (h^{-1}) of *Bacillus* sp. S-86 was determined in each temperature assayed.

2.4. Molecular strain characterization

Genomic DNA extraction as well as16S rRNA gene and 16S–23S intergenic transcribed spacers (ITS-PCR) PCR amplifications were performed as was previously described (Martinez et al., 2002). A 1.5 kb PCR product from 16S rRNA amplification was recovered from agarose-gels using the Prep-A-Gene DNA purification system (Bio-Rad, CA, USA), cloned into a pGEM®-T Easy Vector System II (Promega Corporation, Madison, WI, USA) and a single colony carrying a proper insert was sequenced by GATC Biotech (D-Konstanz, Germany). Strain S-86 16S rRNA gene sequence was registered in the GenBank Data Library under accession no. AY017347. To search for 16S rRNA gene sequence identities, alignments with sequences in public databases were performed by using BLAST and Seqmatch algorithms from the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov/BLAST/) and from the Ribosomal Database Project II v. 9 (http://rdp.cme.msu.edu/seqmatch/seqmatch_intro.jsp), respectively. Some of the significative hits retrieved with identities $\geqslant$98% were used for further analyses conducted using *MEGA* versus 4 (Tamura et al., 2007).

ITS-PCR fingerprints were compared with that obtained from the related reference strains *B. subtilis* 1 A1, *Bacillus licheniformis* 5 A1, and *Bacillus amyloliquefaciens* 10 A1, all kindly provided for the *Bacillus* Genetic Stock Center (http://www.bgsc.org) (Martinez et al., 2002).

2.5. Enzyme assays

Enzyme activities were determined in cell-free extracts by centrifuging the cultures at 8000*g* for 10 min (4 °C). Esterase activity was assayed by measuring the enzymatic hydrolysis of 0.92 mmol l^{-1} *p*-nitrophenyl (pNP) ester of acetate to *p*–nitrophenol in a spectrophotometer 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 from the absorbance at 400 nm of standard solutions of *p*-nitrophenol at pH 7.0 (30 mmol l^{-1} Tris–HCl buffer). The reaction mixture for standard assay contained 60 μl of 200 mmol l^{-1} Tris–HCl buffer (pH 7.0), 30 μl of the enzyme solution and 305 μl of water. The enzyme reaction was started by adding 5 μl of 74 mmol l^{-1} pNP ester (dissolved in pure acetone) into the mixture, and incubated at 37 °C for 15 min. One mmol l^{-1} phenylmethylsulfonyl fluoride (PMSF) 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 (U) was defined as the amount of enzyme producing 1 μmol of *p*-nitrophenol per minute.

2.6. Electrophoresis

Esterase zymograms were performed in native 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. PMSF was added at 1.0 mmol l^{-1} concentration to determine serine type enzyme, prior the electrophoresis were performed.

2.7. 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 and Grossberg, 1997).

2.8. Reagents

All reagents used in this work were of analytical or microbiological grade from Sigma (St. Louis, Mo, USA) or Merck (Darmstad, Germany).

2.9. Statistical analysis

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

3. Results

3.1. Strain characterization

Bacillus sp. S-86 strain is a thermophile, halotolerant and organic-solvent-tolerant microorganism able to produce esterases that, as was previously demonstrated, were active in homogeneous mixtures of organic-solvents and water (Torres and Castro, 2003). Some of the culture characteristics of the S-86 strain are included in Table 1. This organism grew in media with a range of 0–15% (w/v) NaCl, at temperatures up to 55 °C, and in the presence of very toxic alkanols, such as ethanol (857 mmol l^{-1}), propan-2-ol (522 mmol l^{-1}), butan-1-ol (109 mmol l^{-1}) and 3-methylbutan-1-ol (55 mmol l^{-1}).

As a whole, 16S rRNA sequence analysis, ITS-PCR fingerprinting, oxidase and catalase assays in addition to physiological, morphological and biochemical characterization applying API CBH50 system data (Torres and Castro, 2003) displayed rather faint differences with that described for *B. licheniformis* species. ITS-PCR fingerprint of S-86 strain revealed strong differences between S-86 strain and *B. subtilis*, and *B. amyloliquefaciens* reference strains profiles, both members of the *subtilis* cluster. In contrast, the band pattern, which ranged between 200 and 700 bp from S-86 and *B. licheniformis* 5 A1 reference strain, showed few differences in terms of band presence or absence at 450 and 500 bp band sizes (Fig. 1).

Alignments performed against NCBI and RDP II databases resulted in an extensive list of highly significant hits retrieved (⩾98%), results which clearly indicated that strain S-86 belongs to *B. licheniformis* species. Further phylogenetic and molecular evolutionary analyses with *MEGA* versus 4 software illustrate this in Fig. 2.

3.2. Effects of organic-solvents on esterases production

Analysis of crude supernatants from *B. licheniformis* S-86 batch cultures, using native gel electrophoresis showed two distinctive bands, both with esterase activities suggesting the presence of two isoenzymes (Fig. 3A). These two esterases were detected in media containing or not organic-solvents (Fig. 3A). One esterase activity (named type I) was inhibited by PMSF, indicating a serine type hydrolase; but the inhibitor has no effect on the other enzyme activity (named type II). These results supported the idea of two esterases with unrelated enzymatic structures and likely activities.

In order to improve enzyme production in *B. licheniformis* S-86, different batch culturing conditions were evaluated. Effect of temperature on bacterial growth in the 25–60 °C range was studied in presence or absence (control) of 3-methylbutan-1-ol (37 mmol l^{-1}). Maximum growth and enzyme production was found in both media at 50 °C (Fig. 4). The highest specific growth rates of *B. licheniformis* S-86 were 1.52 and 3.12 h^{-1} in medium supplemented or not with 3-methylbutan-1-ol, respectively (Fig. 4). Total esterase specific activity was maximal in medium supplemented with 3-methylbutan-1-ol at stationary growth phase (48 h, 5.52 U mg^{-1}), and it was enhanced 2.3-fold compared to the control without the alkanol (Fig. 4).

Table 1
Characteristics of growth of strain S-86 and other extremophile microorganisms

Strain	Growth characteristics				Reference
	Temperature (°C)		NaCl (%)	OS[a] tolerance	
	Maximum	Optimum	Maximum		
Bacillus licheniformis S-86	55	50	15	Alkanols	Present work
Thermobacillus composti	61	50	4.4	ND[b]	Watanabe et al. (2007)
Geobacillus thermoglucosidasius	80	60	ND	Ethanol	Fong et al. (2006)
Bacillus axarquiensis	45	32	12	ND[b]	Ruiz-Garcia et al. (2005)
Bacillus oshimensis	41	28–32	20	ND[b]	Yumoto et al. (2005)
Bacillus bogoriensis	40	37	12	ND[b]	Vargas et al. (2005)
Methylothermus thermalis[c]	67	57–59	3.0	Methanol	Tsubota et al. (2005)
Methylohalobius crimeensis[c]	42	30	15	Methanol	Heyer et al. (2005)
Bacillus cereus R1	35	ND	2.0	Toluene	Matsumoto et al. (2002)
Bacillus pallidus	60	ND	ND	Isopropanol	Bustard et al. (2002)
Pyrobaculum calidifontis	100	90–95	0.8	ND[b]	Amo et al. (2002)
Oceanomonas baumannii	30	ND	12	Phenol	Brown et al. (2000)
Bacillus licheniformis BAS50	55	40–45	13	ND[b]	Yakimov et al. (1995)
Pseudomonas aeuroginosa LST-3	41	ND	ND	Alkanes	Ogino et al. (1994)
Pseudomonas aeuroginosa ST-001	41	ND	ND	Heptanol	Aono et al. (1992)

[a] OS: organic-solvent.
[b] ND: not determined.
[c] Obligate methanotroph bacteria, which requires methane or methanol to survive.

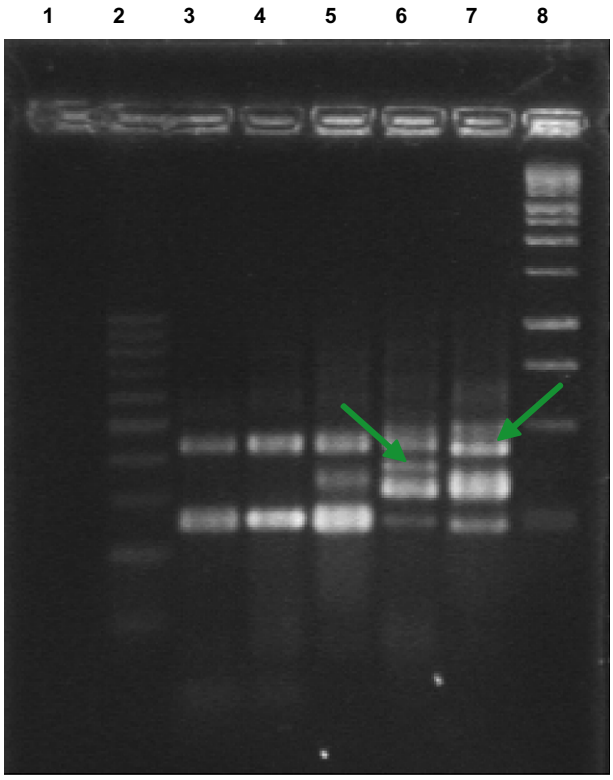

Fig. 1. Evolutionary relationships between 16S rDNA sequences retrieved from a BLAST analysis were inferred using the neighbor-joining method. Evolutionary distances were computed using the maximum likelihood method (Tamura et al., 2007). Scale bar shows the units of the number of base substitutions per site; all positions containing gaps and missing data were eliminated from the dataset and there were a total of 1483 positions in the final dataset. Phylogenetic analyses were conducted in MEGA4 (Tamura et al., 2007). GenBank accession numbers are indicated.

In order to establish the effect of 3-methylbutan-1-ol on esterases production, 9–55 mmol l^{-1} of alkanol range were tested. Maximal esterase specific activities (per mass of protein) were detected using 37 mmol l^{-1} 3-methylbutan-1-ol at 48 h (Fig. 5). Concentrations of 3-methylbutan-1-ol up to 37 mmol l^{-1} increased the production of both enzymes, but esterase type II production was slightly enhanced in all range of alkanol concentrations tested, and only statistically significant ($p < 0.05$) differences were found at 9 and 37 mmol l^{-1} 3-methylbutan-1-ol (approximately 1.3-fold higher than type I esterase specific activity) (Fig. 5). Above 37 mmol l^{-1} 3-methylbutan-1-ol in the medium, the specific activities began to decrease, and when the concentration of 3-methylbutan-1-ol in culture medium reached the highest concentration of 92 mmol l^{-1}, massive cell lysis and death was observed (data not shown).

B. licheniformis S-86 displayed 1.7 to 2.7-fold higher total esterase specific activities in cultures supplemented with propan-2-ol ($\log P = 0.28$), butan-1-ol ($\log P = 0.8$) and 3-methylbutan-1-ol ($\log P = 1.3$) compared to the control (no solvent added) between 24 to 48 h of growth at 50 °C. Representative values of alkanols effect on esterase specific activities at 24 and 48 h of cell growth are displayed in Tables 2A and 2B. The highest total esterase activity was observed at 48 h of growth in presence of butan-1-ol (2.7-fold; 6.52 U mg^{-1}) (Table 2B), while at 24 h, the greater activity was measured in the cultures supplemented with 3-methylbutan-1-ol (2.7-fold; 4.31 U mg^{-1}) (Table 2A).

Also, enzymes induction was studied by adding 3-methylbutan-1-ol during the exponential phase. Zymograms of intracellular crude extracts of *B. licheniformis* S-86 showed, after the induction with the alcohol, the presence of both type I and type II esterases activities (Fig. 3B). Total esterase specific activity in intracellular crude extracts was improved 1.9-fold in presence of 3-methylbutan-1-ol compared with the control (without alkanol). Intracellular enzyme activity of type I esterase was higher than type II esterase in medium supplemented with or without 3-methylbutan-1-ol. Nevertheless, in presence of 3-methylbutan-1-ol, type

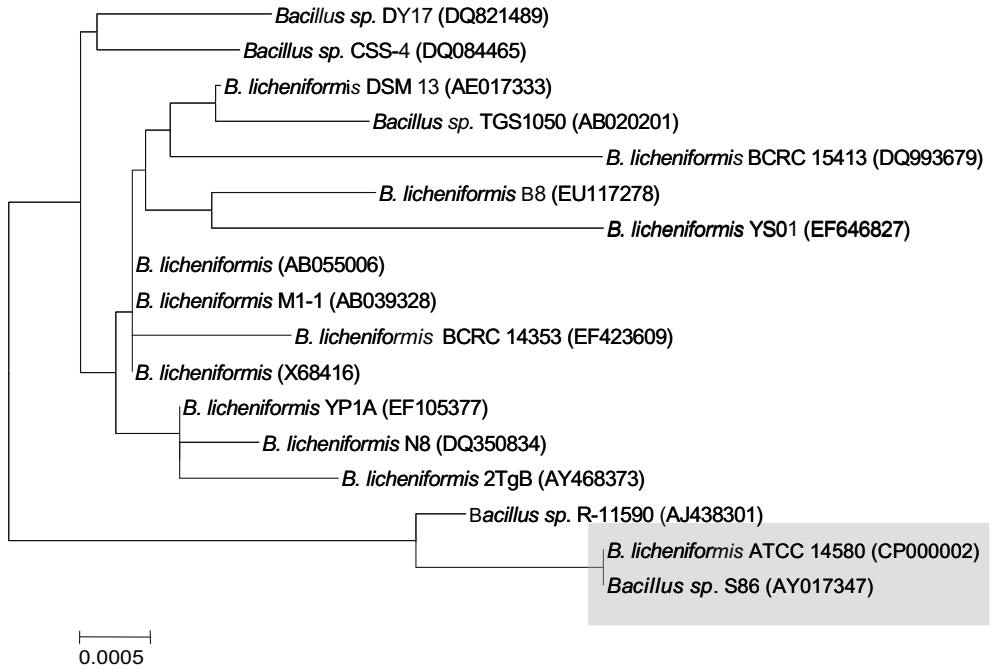

Fig. 2. ITS-PCR fingerprint in agarose 1.5% gel. Lines: (1) control lacking DNA template; (2) DNA ladder 100 bp; (3) *B. subtilis* 1 A1; (4) *B. subtilis* sp.; (5) *B. amyloliquefaciens* 10 A1; (6) *B. licheniformis* 5 A1; (7) *Bacillus* sp. S-86; (8) 1 kb ladder. The arrows indicate the band length polymorphism found between two *B. licheniformis* strains.

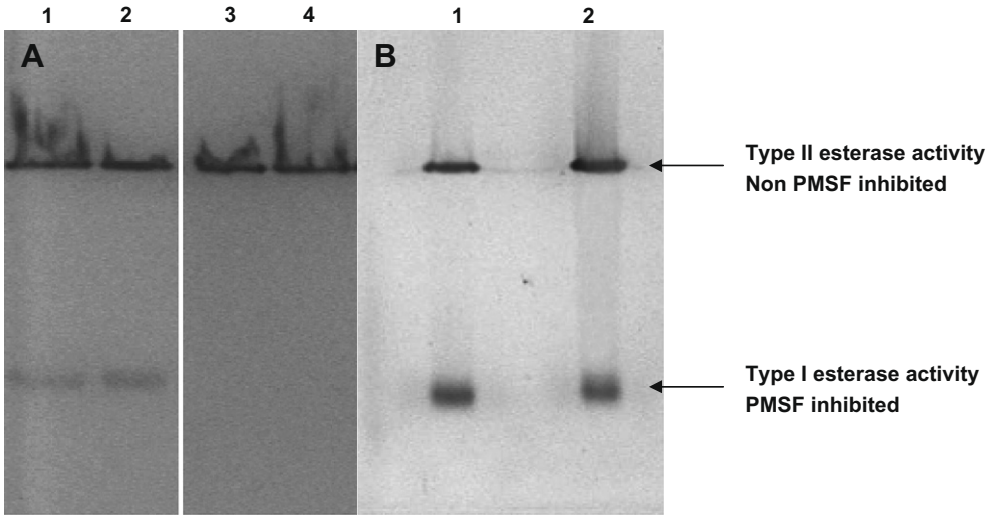

Fig. 3. Native PAGE of extracellular (A) and intracellular (B) crude extracts of *B. licheniformis* S-86. Esterase activities were determined with α-naphtyl acetate as the substrate. (A) Extracellular crude extracts were obtained in media without organic-solvent (lines 2 and 3) and supplemented with 37 mmol l^{-1} 3-methylbutan-1-ol (lines 1 and 4) at 48 h of growth. Corresponding PAGE of *B. licheniformis* S-86 crude extracts incubated with PMSF (lines 3 and 4) show that PMSF inhibits type I esterase activity, suggesting it is a serine type enzyme. (B) Intracellular crude extracts were obtained from exponentially growing cells in control media (line 1) and after induction with 37 mmol l^{-1} 3-methylbutan-1-ol (line 2).

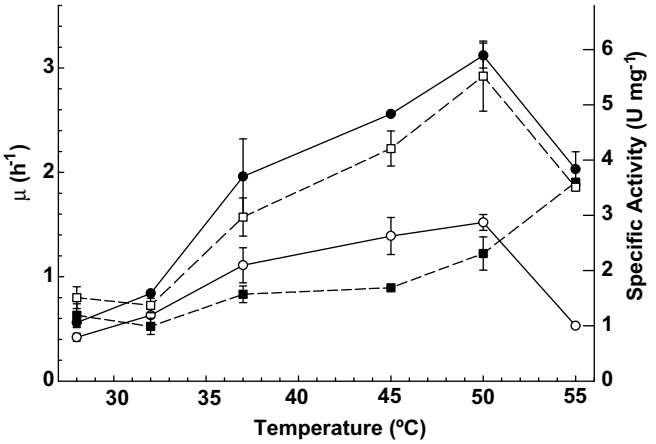

Fig. 4. Effect of temperature on growth and enzyme production of *B. licheniformis* S-86. Specific growth rate μ (h^{-1}) at different temperatures was determined in batch culture using media without 3-methylbutan-1-ol (●) and added with 37 mmol l^{-1} 3-methylbutan-1-ol (○). Total esterase specific activity (U mg^{-1}) was determined at different temperatures at 48 h, in EP media without 3-methylbutan-1-ol (■) and in media with 37 mmol l^{-1} 3-methylbutan-1-ol (□). Enzyme activities were assayed with *p*-nitrophenyl-acetate. Errors bars give the standard deviations from four independent assays.

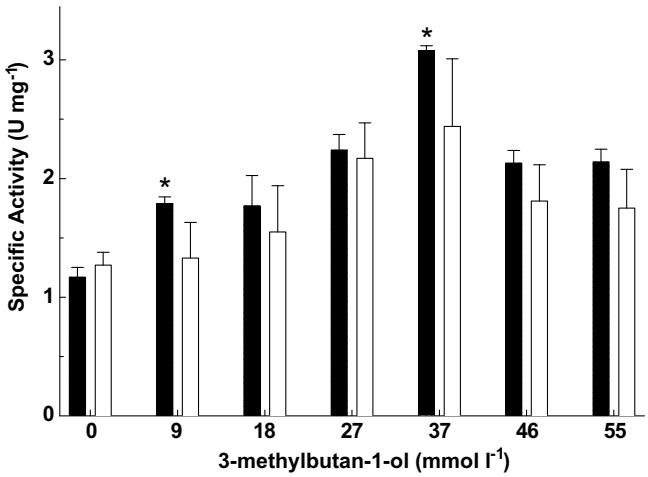

Fig. 5. *B. licheniformis* S-86 enzymes production in media supplemented with different concentrations of 3-methylbutan-1-ol after 48 h of batch culturing at 50 °C. Specific activities of type I (□) and type II (■) esterases were determined. Errors bars give the standard deviations from four independent assays. *Significantly different from type I esterase; $p < 0.05$.

II esterase specific activity was enhanced 2.7 times, meanwhile type I esterase increase only 1.7 times (Table 3).

4. Discussion

Based on the widespread diversity and high complex taxonomy of the *Bacillus* genera, previous findings were added to molecular systematic techniques well described as powerful tools, to confirm classical identification studies (Martinez et al., 2002; Waldeck et al., 2006; Konstantinidis and Tiedje, 2007). The little differences of *Bacillus* sp. S-86 ITS-PCR fingerprint observed in comparison to that of *B. licheniformis* 5 A1 reference strain could be attributed to extensive intra-species diversity of *Bacillus* species (Martinez et al., 2002; Xu and Côté, 2003; Konstantinidis and Tiedje, 2007). Moreover, conclusive evidence for the affiliation of the isolate with *B. licheniformis* species is the fact that strain S-86 16S rRNA gene sequence showed 100% of identity with that of *B. licheniformis* ATCC14580, a type strain recently fully sequenced, Fig. 1 (Waldeck et al., 2006).

Batch cultures of *B. licheniformis* S-86 revealed the presence of two extracellular isoenzymes. Type I and type II esterase activities can be differentiated by PMSF, a typical serine type protease inhibitor. Similarly, the extremophile *B. thermoleovorans* ID-1 produced two thermostable lipases: BTID-A which was strongly inhibited by PMSF and BTID-B who was insensitive to PMSF (Lee et al., 2001).

Batch culturing at different temperatures revealed an optimal temperature of 50 °C for cell growth and enzyme production. Temperature was proven to regulate enzyme synthesis at mRNA transcription and probably translation levels (Rahman et al., 2005). It thus regulates the synthesis of several intracellular and extracellu-

Table 2A
Effect of alkanols on esterase specific activities produced by *B. licheniformis* S-86 at 50 °C and 24 h

Solvent IC_{50} (mmol l^{-1})[a]	Esterase					
	Type I		Type II		Total	
	SA (U mg^{-1})	Folds	SA (U mg^{-1})	Folds	SA (U mg^{-1})	Folds
Water (control)	0.99 ± 0.25	1.0	0.62 ± 0.09	1.0	1.61 ± 0.16	1.0
Propan-2-ol (392)	1.47 ± 0.41	1.5	1.95[b] ± 0.20	3.1	3.48[b] ± 0.21	2.2
Butan-1-ol (99)	1.22 ± 0.19	1.2	1.99[b] ± 0.09	3.2	3.21[b] ± 0.10	2.0
3-methylbutan-1-ol (37)	2.56[b] ± 0.43	2.6	1.75[b] ± 0.03	2.8	4.31[b] ± 0.40	2.7

Data represent the average ± standard deviation.
[a] IC_{50}, solvent concentration which cause 50% growth inhibition in *B. licheniformis* S-86, SA (U mg^{-1}), specific activity per mass of proteins.
[b] Significantly different from the control, $p < 0.05$.

Table 2B
Effect of alkanols on esterase specific activities produced by *B. licheniformis* S-86 at 50 °C and 48 h

Solvent IC_{50} (mmol l^{-1})[a]	Esterase					
	Type I		Type II		Total	
	SA (U mg^{-1})	Folds	SA (U mg^{-1})	Folds	SA (U mg^{-1})	Folds
Water (control)	1.27 ± 0.11	1.0	1.17 ± 0.08	1.0	2.44 ± 0.03	1.0
Propan-2-ol (392)	2.91[b] ± 0.76	2.3	2.64[b] ± 0.44	2.2	5.55[b] ± 0.32	2.3
Butan-1-ol (99)	2.88[b] ± 0.49	2.3	3.64[b] ± 0.13	3.1	6.52[b] ± 0.36	2.7
3-methylbutan-1-ol (37)	2.44[b] ± 0.67	1.9	3.08[b] ± 0.04	2.6	5.52[b] ± 0.63	2.3

Data represent the average ± standard deviation.
[a] IC_{50}, solvent concentration which cause 50% growth inhibition in *B. licheniformis* S-86, SA (U mg^{-1}), specific activity per mass of proteins.
[b] Significantly different from the control, $p < 0.05$.

Table 3
Intracellular esterase activities of *B. licheniformis* S-86 during exponential growth, after 37 mmol l^{-1} 3-methylbutan-1-ol addition

Treatment	Esterase activity (10^{-2})[a]					
	Type I		Type II		Total	
	(U ml^{-1})	(U mg^{-1})	(U ml^{-1})	(U mg^{-1})	(U ml^{-1})	(U mg^{-1})
Control	8.90[c] ± 0.56	2.90[c] ± 0.34	2.35 ± 0.35	0.70 ± 0.08	11.25 ± 0.21	3.60 ± 0.26
ISO[b]	20.88[c,d] ± 2.52	4.90[c,d] ± 0.46	8.15[d] ± 0.21	1.90[d] ± 0.06	29[d]03 ± 2.31	6.80[d] ± 0.40

Data represent the average ± standard deviation.
[a] Esterase activity and standard deviation values multiplied by 10^{-2}.
[b] Culture medium supplemented with 3-methylbutan-1-ol during mid-exponential growth phase, up to a final concentration of 37 mmol l^{-1}.
[c] Significantly different from type II esterase, $p < 0.05$.
[d] Significantly different from the control, $p < 0.05$.

lar enzymes. Besides, for extracellular enzymes, temperature influences their secretion by changing physical properties of cell membrane (Rahman et al., 2005).

The cell growth of *B. licheniformis* S-86 in presence of 3-methylbutan-1-ol showed the typical solvent toxic effect on cell proliferation and viability. However, total esterase specific activity was enhanced in presence of the solvent reaching a maximum in the stationary phase. These results evidenced that production of esterases occurred for a longer period of time than cell growth, and a markedly enlarge of the production or release of the enzymes by 3-methylbutan-1-ol and other alkanols. In fact, the specific activities of both esterases were augmented in presence of other alkanols such as propan-2-ol, and butan-1-ol. In addition, esterase induction levels were different for both enzymes and depended on alkanol source. These results are suggesting a specific trigger of esterases by C_3–C_5 alkanols, which allow manipulating the culture conditions in order to change the ratio between the esterases.

Gram-positive *B. licheniformis* S-86 was able to tolerate very toxic alkanols at high concentrations. In general, absence of the outer membrane in Gram-positive bacteria makes them more susceptible to organic-solvent stress. But recently, strains of Gram-positive bacteria like *Rhodococcus*, *Arthrobacter* and *Bacillus,* showed excellent tolerance to highly toxic organic-solvents (Sardessai and Bhosle, 2003). Indeed, the thermophile *B. pallidus* ST3 was able to growth in presence of propan-2-ol ($\log P = 0.28$) and acetone ($\log P = -0.23$) as sole carbon source (Bustard et al., 2002). Additionally, *Bacillus* sp. SB1 and *Bacillus* sp. BC1 exhibited excellent solvent tolerance to extremely toxic organic-solvents like butan-1-ol ($\log P = 0.8$) and chloroform ($\log P = 2.0$), respectively (Sardessai and Bhosle, 2003).

Increase of specific esterase activities could be explained by many mechanisms working alone or simultaneously. As a result of the increase in cell membrane permeability due to the organic-solvent (Heipieper et al., 2007); or by higher stability or enhanced enzyme activity conferred by the organic-solvents molecules (Sikkema et al., 1994); or as a result of cellular detoxification procedures and/or general stress response mechanism (Sardessai and Bhosle, 2002).

In conclusion, *B. licheniformis* S-86 can grow at 55 °C in presence of C_2–C_5 alkanols ($\log P = -0.86$ to 2.39), and salinity concentrations up to 15% (w/v), all characteristics of extremophiles. As for our knowledge refer, is not very common to found microorganisms with the ability of tolerate such variety of extreme conditions, which allows using this strain as very useful tool to study the mechanisms of tolerance to organic-solvents and other stresses in Gram-positive bacteria. Also, the possibilities of manipulating

the production of its esterases using organic-solvents making them very attractive to use in non-aqueous biocatalysis for non-natural hydrolysis of substrates as well as in the synthesis and or modification of molecules. Current studies in our laboratory are pursuing study the mechanisms of solvent tolerance associated with enzyme production and enzyme properties in more detail.

Acknowledgements

The authors want to thank DST (India) and MinCyT (Argentina) for support under Indo-Argentina Bilateral Collaborative Scientific Program. Also, the authors would like to thank Dan Zeigler (*Bacillus* Genetic Stock Center) for kindly providing the reference *Bacillus* strains, Mario Baigorí for helpful discussions, and Emilio Rodriguez for excellent technical assistance.

Financial support from CONICET (PIP 6203/06) and ANPCyT (PICT 14-32491, Argentina) to GRC is gratefully acknowledged.

Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.biortech.2008.07.009.

References

Amo, T., Paje, M., Inagaki, A., Ezaki, S., Atomi, H., Imanaka, T., 2002. *Pyrobaculum calidifontis* sp. nov., a novel hyperthermophilic archaeon that grows under atmospheric air. Archaea 1, 113–121.

Aono, R., Ito, M., Inoue, A., Horikoshi, K., 1992. Isolation of novel toluene-tolerant strain of *Pseudomonas aeruginosa*. Biosci. Biotechnol. Biochem. 56, 145–146.

Bornscheuer, U., 2002. Microbial carboxyl esterases: classification, properties and application in biocatalysis. FEMS Microbiol. Rev. 26, 73–81.

Brown, G., Sutclije, I., Bendell, D., Cummings, S., 2000. The modification of the membrane of *Oceanomonas baumannii* T when subjected to both osmotic and organic solvent stress. FEMS Microbiol. Lett. 189, 149–154.

Bustard, M.T., Whiting, S., Cowan, D.A., Wright, P.C., 2002. Biodegradation of high-concentration isopropanol by a solvent-tolerant thermophile, *Bacillus pallidus*. Extremophiles 6, 319–323.

Fong, J., Svenson, C., Nakasugi, K., Leong, C., Bowman, J., Chen, B., Glenn, D., Neilan, B., Rogers, P., 2006. Isolation and characterization of two novel ethanol-tolerant facultative-anaerobic thermophilic bacteria strains from waste compost. Extremophiles 10, 363–372.

Gupta, M.N., Roy, I., 2004. Enzymes in organic media-forms, functions and applications. Eur. J. Biochem. 271, 2575–2583.

Heipieper, H.J., Neumann, G., Cornelissen, S., Meinhardt, F., 2007. Solvent-tolerant bacteria for biotransformations in two-phase fermentation systems. Appl. Microbiol. Biotechnol. 74, 961–973.

Heyer, J., Berger, U., Hardt, M., Dunfield, P., 2005. *Methylohalobius crimeensis* gen. nov., sp. nov., a moderately halophilic, methanotrophic bacterium isolated from hypersaline lakes of Crimea. Int. J. Syst. Evol. Microbiol. 55, 1817–1826.

Konstantinidis, K.T., Tiedje, J.M., 2007. Prokaryotic taxonomy and phylogeny in the genomic era: advancements and challenges ahead. Curr. Opin. Microbiol. 10, 1–6.

Lee, D.W., Kim, H.W., Lee, K.W., Kim, B.C., Choe, E.A., Lee, H.S., Kim, D.S., Pyun, Y.R., 2001. Purification and characterization of two distinct thermostable lipases from the gram-positive thermophilic bacterium *Bacillus thermoleovorans* ID-1. Enzyme Microbiol. Technol. 29, 363–371.

Martinez, M.A., Delgado, O.D., Breccia, J.D., Baigori, M.D., Sineriz, F., 2002. Revision of the taxonomic position of the xylanolytic *Bacillus* sp. MIR32 re-identified as *Bacillus halodurans* and plasmid-mediated transformation of *B. halodurans*. Extremophiles 6, 391–395.

Matsumoto, M., de Bont, J.A.M., Isken, S., 2002. Isolation and characterization of the solvent-tolerant *Bacillus cereus* strain R1. J. Biosci. Bioeng. 94, 45–51.

Ogino, H., Miyamoto, K., Ishikawa, H., 1994. Organic-solvent-tolerant bacterium which secrets organic-solvent stable lipolytic enzyme. Appl. Environ. Microbiol. 160, 3884–3886.

Rahman, R.N.Z.A., Geok, L.P., Basri, M., Salleh, A.B., 2005. Physical factors affecting the production of organic solvent-tolerant protease by *Pseudomonas aeruginosa* strain K. Bioresour. Technol. 96, 429–436.

Ranganathan, S., Narasimhan, S., Muthukumar, K., 2008. An overview of enzymatic production of biodiesel. Bioresour. Technol. 99, 3975–3981.

Ruiz-Garcia, C., Quesada, E., Martinez-Checa, F., Llamas, I., Urdaci, M.C., Bejar, V., 2005. *Bacillus axarquiensis* sp. nov. and *Bacillus malacitensis* sp. nov., isolated from river-mouth sediments in southern Spain. Int. J. Syst. Evol. Microbiol. 55, 1279–1285.

Sardessai, Y., Bhosle, S., 2002. Tolerance of bacteria to organic solvents. Res. Microbiol. 153, 263–268.

Sardessai, Y., Bhosle, S., 2003. Isolation of an organic-solvent-tolerant cholesterol-transforming *Bacillus* species, BC1, from coastal sediment. Mar. Biotechnol., NY 5, 116–118.

Sedmak, J.J., Grossberg, S.E., 1997. A rapid, sensitive, and versatile assay for protein using Coomassie brilliant blue G250. Anal. Biochem. 79, 544–552.

Sikkema, J., de Bont, J.A.M., Poolman, B., 1994. Interactions of cyclic hydrocarbons with biological membranes. J. Biol. Chem. 269, 8022–8028.

Tamura, K., Dudley, J., Nei, M., Kumar, S., 2007. MEGA4: molecular evolutionary genetics analysis. Mol. Biol. Evol. 24, 1596–1599.

Tang, X., Pan, Y., Li, S., He, B., 2008. Screening and isolation of an organic solvent-tolerant bacterium for high-yield production of organic solvent-stable protease. Bioresour. Technol. 99, 7388–7392.

Torres, S., Baigorí, M.D., Castro, G.R., 2005. Effect of hydroxylic solvents on cell growth, sporulation, and esterase production of *Bacillus licheniformis* S-86. Process Biochem. 40, 2333–2338.

Torres, S., Castro, G.R., 2003. Organic solvent resistant lipase produced by thermoresistant bacteria. In: Roussos, S., Soccol, C.R., Pandey, A., Augur, C. (Eds.), New Horizons in Biotechnology. Kluwer Academic Publishers, Dordrecht, Netherlands, pp. 113–122.

Torres, S., Castro, G.R., 2004. Non-aqueous homogeneous biocatalysis. Food Technol. Biotechnol. 42, 271–277.

Tsubota, J., Eshinimaev, B., Khmelenina, V., Trotsenko, Y., 2005. *Methylothermus thermalis* gen. nov., sp. nov., a novel moderately thermophilic obligate methanotroph from a hot spring in Japan. Int. J. Syst. Evol. Microbiol. 55, 1877–1884.

Vargas, V., Delgado, O., Hatti-Kaul, R., Mattiasson, B., 2005. *Bacillus bogoriensis* sp. nov., a novel alkaliphilic, halotolerant bacterium isolated from a Kenyan soda lake. Int. J. Syst. Evol. Microbiol. 55, 899–902.

Waldeck, J., Daum, G., Bisping, B., Meinhardt, F., 2006. Isolation and molecular characterization of chitinase-deficient *Bacillus licheniformis* strains capable of deproteinization of shrimp shell waste to obtain highly viscous chitin. Appl. Environ. Microbiol. 72, 7879–7885.

Watanabe, K., Nagao, N., Yamamoto, S., Toda, T., Kurosawa, N., 2007. *Thermobacillus composti* sp. nov., a moderately thermophilic bacterium isolated from a composting reactor. Int. J. Syst. Evol. Microbiol. 57, 1473–1477.

Xu, D., Côté, J., 2003. Phylogenetic relationships between Bacillus species and related genera inferred from comparison of 3′ end 16S rDNA and 5′ end 16S–23S ITS nucleotide sequences. Int. J. Syst. Evol. Microbiol. 53, 695–704.

Yakimov, M., Timmis, K., Wray, V., Fredrickson, H., 1995. Characterization of a new lipopeptide surfactant produced by thermotolerant and halotolerant subsurface *Bacillus licheniformis* BAS50. Appl. Environ. Microbiol. 61, 1706–1713.

Yumoto, I., Hirota, K., Goto, T., Nodasaka, Y., Nakajima, K., 2005. *Bacillus oshimensis* sp. nov., a moderately halophilic, non-motile alkaliphile. Int. J. Syst. Evol. Microbiol. 55, 907–911.