Journal of
Microbial & Biochemical Technology

Papagianni and Papamichael, J Microb Biochem Technol 2017, 9:3
DOI: 10.4172/1948-5948.1000353

Research Article OMICS International

Purification, Amino Acid Sequencing and Thermostability of an Extracellular Low Molecular Weight Esterase Produced by Bacillus Subtilis NRRL 41270 in Fermentation

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Abstract

Extracellular esterase activity in *Bacillus subtilis* NRRL 41270 fermentation broths was found to reside in a small protein with a molecular weight less than 10 kDa. Following purification, esterase activity on fluorescein dibutyrate was estimated at 12 U/min/mg proteins. Enzyme saturation was observed at 5 μM substrate concentration. The produced esterase hydrolysed tributyrin. Its specific activity was estimated to be 17.8 μmol acid released/min/mg proteins. The small protein was subjected to size exclusion chromatography, SDS-PAGE and amino acid sequencing. Analysis revealed a sequence of the following amino acid residues: eevaetysfyhitphdystshispapvqffspap, according to which the molecule has 34 amino acid residues and a calculated molecular mass of 3853, which was in accordance with the gel filtration and SDS-PAGE results. Sequence based analysis and use of bioinformatics tools showed no significant similarity with known proteins while revealed a strongly hydrophobic molecule, with a α-helical conformation in the N-terminal, the rest of the molecule being β-sheet-rich. The enzyme appeared to be thermostable with more than 85% of the original activity maintained after 120 h incubation at 60°C. The producer organism and the features of the micro enzyme, suggest the case of a biotechnologically interesting biocatalyst that should be further researched in terms of its stability and production characteristics.

Keywords: *Bacillus subtilis*; Esterase; Micro enzyme; Purification; Sequence

Introduction

Bulk production of industrial enzymes, e.g. proteases, lipases, amylases and others, is mainly through fermentation with Gram-positive bacteria and fungi. Certain Bacillus species are regarded as important microbial cell factories. The capacity of selected strains of *B. subtilis*, *B. licheniformis* and others, to produce and secrete large quantities (20-25 g/L) of extracellular enzymes (proteases, lipases, amylases, esterases) has placed them among the most important industrial enzyme producers [1,2].

B. subtilis has attracted early research interest on various aspects of its physiology, production and applications, and it is the best-characterized among Bacillus species. The early sequencing of *B. subtilis* genome and extensive proteome analysis generated knowledge through the years so that the particular organism provided an excellent model in Systems Biological studies [1]. Since most of *B. subtilis* applications are related to the high-level secretion of proteins, major research has been focused on the cell factory's secretion machinery [1,3,4].

As a natively soil bacterium, *B. subtilis* secretes numerous enzymes to degrade a variety of substrates, enabling the bacterium to survive in a continuously changing environment [5]. Several *B. subtilis* strains are known as excellent producers of proteases and lipases in submerged and solid-state fermentation. These enzymes are produced commercially and their production represents the major part of the industrial-enzyme market. A relatively large number of proteases, lipases and esterases produced by *B. subtilis* species have been purified, characterized biochemically and their fermentation conditions optimized for maximum yields. A common limitation of industrial application of lipases and esterases is their limited thermostability at high temperatures, as well as the limited pH stability in operating industrial conditions. Therefore, searching for novel microbial enzyme sources is of great importance in the development of new thermostable enzymes and subsequent applications. Several thermostable esterases have been reported from *Bacillus* species, including *B. stearophilus*, *B. subtilis*, *B. licheniformis* S-86 *and Bacillus* sp. RN2 and *B. cereus* [6-10].

In this work, we report the purification procedure, amino acid sequence and thermostability profile of a low molecular weight enzyme (micro enzyme) with esterase activity in *B. subtillis* fermentation broths. Esterases (carboxylic ester hydrolases) constitute a large group of enzymes that catalyze the hydrolysis, synthesis or trans esterification of ester bonds. They are found in all forms of life, including the group of hyper thermophilic bacteria and archaea. The molecular mass of esterases is usually in the range of 20-160 kDa [11]. Esterases with lower molecular masses exist but reports are rather rare and the information given is limited [12]. The full sequence of an esterase of 118 amino acids and molecular mass 13.72 kDa produced by a *B. thuringiensis* strain was reported in GenBank (AHZ51904.1) [13]. Simoes et al. described a thermostable esterase of 1.57 kDa produced by *B. stearothermophilus* NCIMB 13335 [11,14]. Other reported micro enzymes include a 5.7 kDa esterase by *Candida lipolytica*, a 9.7 kDa rennin by an unclassified Actinomycete, a disulfide bond-forming enzyme of 6.2 kDa produced by the archaebacterium *Sulfoborus solfataricus*, an 8 kDa protease by the extremophilic bacterium *Kurthia spiroforme* [15-18]. Three more micro enzymes (1.53, 1.827 and 4.275 kDa) with esterase activity produced by the thermophilic filamentous fungi *Talaromyces emersonii* and *Emericella nidulans* were reported by Xiaolin and Mattey [19]. None of these peptide biocatalysts however, has been characterized in terms of their structural features, e.g. amino acid sequence and primary structure and therefore critical analysis is restricted. Also, in most cases

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Received May 26, 2017; **Accepted** June 03, 2017; **Published** June 10, 2017

Citation: Maria P, Emmanuel MP (2017) Purification, Amino Acid Sequencing and Thermostability of an Extracellular Low Molecular Weight Esterase Produced by *Bacillus subtilis* Nrrl 41270 in Fermentation. J Microb Biochem Technol 9:117-121. doi: 10.4172/1948-5948.1000353

important information on properties that determine the enzyme-like character, e.g. substrate specificity, heat denaturation or pH profile, is not provided. The present work aims to provide substantial information on the isolated biocatalyst with esterase activity.

Materials and Methods

Microorganisms and culture conditions

Storage: Glycerol stocks of two independent clones of *B. subtilis* NRRL 41270 were maintained at -80°C (1.3 ml fresh overnight culture +50% glycerol). Long-term storage for frequent use was by means of plating on nutrient sporulation medium (NSM; 0.8% nutrient broth (**Sigma-Aldrich)**, 0.05 mM $MnCl_2$, 0.7 mM $CaCl_2$, and 1.0 mM $MgCl_2$). For frequent use, *B. subtilis* was grown on Luria-Bertani agar plates at room temperature (LB agar plates: LB broth [tryptone, 10 g/l; yeast extract, 5 g/l; NaCl, 10 g/l]+technical agar, 15 g/l).

Growth conditions: A loop full of stock culture was plated out on LB agar containing 0.4% tributyrin and inbubated at 37°C. Twenty four hours later, colonies exhibiting clear zones were inoculated individually into 250 ml Erlenmeyer flasks containing 25 ml of LB broth and shaken at 100 rpm for 24 h at 37°C. For esterase production, 5 ml of LB broth culture were inoculated into 500 ml of fermentation medium and placed in a BIOFLO 110 New Brunswick Scientific stirred tank bioreactor (STR) with a working volume of 2 l, at 37°C for 24 h with aeration. The agitation system of the reactor was consisted of two 6-bladed Rushton-type impellers (52 mm), operated at the stirrer speed of 100 rpm. The temperature was maintained at 37°C. The air flow rate was 1 vvm. The oxygen sensor was calibrated by sparging the medium with air (dissolved oxygen tension, DOT 100%) and N_2 (DOT 0%). Following sterilization (120°C /15 min), the pH of the medium was adjusted with titrants (1 M NaOH and HCl solutions) at the chosen level of pH 7.0 and remained under control during fermentation by automatic addition of titrants.

The fermentation medium contained the following: D-glucose, 5 g/l; glycerol tributyrate, 4 g/l; $MgSO_4.7\ H_2O$, 0.5 g/l; KCl, 0.5 g/l; $FeSO_4.7\ H_2O$, 0.001 g/l; ammonium acetate, 5 g/l; phosphate buffer, 0.04 M, pH 7.0. An amino acids supplement was prepared and added to the above medium at the final concentration of 150 µM. The supplement contained the following (final concentration): Casamino Acids (Difco 0230-15-5), 4 g/l; L-glutamine (Sigma G8540), 0.1 g/l; L-tryptophan (Sigma T0254); 0.1 g/l; L-asparagine (Sigma 0884), 0.02 g/l; L-methionine (Sigma M9625), 0.02 g/l.

Fermentation was interrupted at 24 h from inoculation, when the level of enzyme activity was maximal. Samples were withdrawn at regular time intervals from the fermentation broth for analyses. The course of growth was monitored by measuring the absorbance at 580 nm using a BioMate 3S UV-Visible spectrophotometer (ThermoFisher Scientific). Esterase activity was determined by a fluorimetric assay after the cells and tributyrin were removed.

Purification of esterase: Cells were removed from the fermentation broth by centrifugation at 15,000x g for 15 min at 4°C. To remove excess tributyrin, the supernatant was filtered through Whatman No. 1 paper. The filtrate was passed through a tangential flow filtration (TFF) device (Mini-Ultracette, Pall Filtron) with a cut-off of 10 kDa. The filtrate was loaded on to a 1 × 100 Bio-Gel P-6 column (Bio-Rad) for size exclusion chromatography. Pre-equilibration of the column was done with 100 mM ammonium bicarbonate at pH 7.5 and elution with the same buffer at the flow rate of 0.08 ml/min. Fractions of 1 ml were examined for esterase activity and protein content. Protein concentration was measured with the ninhydrin reaction in which 0.1 ml of each fraction was mixed with 0.9 ml of 0.2% ninhydrin solution in ethanol and the absorbance was monitored at 540 nm. Positive samples were dehydrated using a centrifuge concentrator (5305 Eppendorf) and analyzed by HPLC using a Hamilton PRP-3 reverse-phase column (2.5 × 150 mm, 10 µl, 300 Å, Hamilton Company).

Enzyme assays: Esterase activity in the filtrates was measured by the fluorimetric assay described by Kramer and Guilbault [20] and Guilbault and Kramer [21]. Fluorescein dibutyrate was used as substrate at the concentration of 5×10^{-6} M. The substrate was prepared by emulsifying 10 g of tributyrin in 90 ml of 10% acacia gum solution. 0.5 ml substrate and 0.5 ml of 0.1 M phosphate buffer (pH 7.5) were added to 1 ml of sample and the mixture was incubated at 40°C for 2 h in a shaking water bath. The reaction was terminated by adding 10 ml of an acetone:ethanol 1:1 mixture. The liberated from the reaction butyric acid was determined by titration with 1 M NaOH.

Estimation of protein content: Protein concentration in the samples was determined by following the protocol of Lowry et al, using Folin phenol as coloring reagent and bovine serum albumin (BSA) as the standard. Absorbance was monitored at 660 nm [22].

Molecular mass determination: The samples derived from size exclusion chromatography were desalted by dialysis using a mini dialysis kit with 1 kDa cut off (GE Healthcare Life Sciences) and then were dehydrated at a centrifuge concentrator to concentrate the enzyme. The concentrates were re-suspended in distilled water and loaded on two 17% polyacrylamide gels in a discontinuous buffer system lacking SDS (non-denaturing) which was run in parallel. One of the gels was stained with a silver stain (Invitrogen) while the other was stained for esterase activity. The gels, processed for esterase localization were incubated in a reaction mixture containing naphthyl acetate (5.58×10^{-3} mM, pH 7.5) and 0.04 g Fast Blue RR at 25°C for 1 h. Upon development of the dark brown bands, indicating esterase activity, the reaction was terminated by fixing the gel in 7% (v/v) glacial acetic acid for 20 min, followed by preservation of the gel in 5% (v/v) acetic acid prepared in 10% methanol.

The recovered from native-PAGE gels protein was subjected to discontinuous SDS-PAGE, using the Laemmli buffer system with 16.5% acrylamide and 6% bisacrylamide slab gels. Silver staining was applied. The molecular mass of the protein was roughly estimated using a protein ladder of 1.7 to 40 kDa as molecular weight markers (Thermo Scientific).

Amino acid analysis: For determination of the amino acid sequence, the protein sample was transferred from SDS-PAGE gel onto a polyvinylidene (PVDF) membrane by western blotting and the membrane carrying the protein was subjected to sequencing [23]. Amino acid sequence was determined by Edman degradation sequencing of the protein using an Applied Biosystems Procise Sequenser (ABI 494 protein sequencer).

Determination of thermostability: The stability of pure enzyme in solution was examined at 40, 50, 60, 70, 80 and 90°C. Enzyme solutions were prepared at the concentration of 0.1 mg/ml in 20 mM Tris-HCl buffer, pH 7.5. Twice a day, samples were removed from the incubators, left to cool at ambient temperature and freezed. Incubation lasted 120 h. Samples were assayed for esterase activity. Activities were expressed as percentages of the activity at the starting point (time 0).

Sequence-based analysis and bioinformatics tools: The software Clone Manager 7 (version 7.11, Sci Ed Central) was used for sequence-

based computation analyses. The following analyses were performed: Molecular weight, isoelectric point, amino acid composition profile, three-line graphs plots for the predicted values for α-helix, β-sheet and β-turn configurations, hydrophilicity analysis and plots according to Kyte and Doolittle [25], Hopp and Woods [26] and surface exposure analysis according to Janin et al. [27]. Homology searches were performed with BLAST (Basic Local Alignment Search Tool, NCBI-algorithm blastP).

Results and Discussion

Bacillus subtilis NRRL 41270 was cultivated for 24 h at 30°C and 100 rpm under full aeration, in a stirred tank bioreactor (batch culture). The fermentation medium contained tributyrin and a low concentration of glucose (Figure 1). Figure 1 shows the results of absorbance monitoring of growth and esterase activity. Maximum absorbance readings were obtained between 14-16 h of fermentation while they started decreasing from 18 h onward. Maximum esterase activity was obtained with 21 h samples of fermentation broth. Cells and excess tributyrin were removed from these samples and the filtrate was separated into fractions of less and more than 10 kDa. Esterase activity was detected only in the <10 kDa filtrates following filtration through the tangential flow filter as described in the previous section. The Bio-Gel P-6 resolved peptides in the range of 1.1 to 5.7 kDa. Size exclusion chromatography of the filtrates gave one peak of esterase activity in the range of fractions 70 to 75 (Figure 2). Low molecular weight esterase activity increased during fermentation from nil to 35 U/min/ml at 13 h of cultivation, to 52 U/min/ml at 21 h.

Following purification, esterase activity on the fluorescein dibutyrate substrate was estimated to be 12 U/min/mg proteins. Enzyme saturation was observed at the substrate concentration of 5 µM. The produced esterase hydrolysed tributyrin. The specific activity of the enzyme using the titration assay with 1 M NaOH was 17.8 µmol acid released/min/mg proteins. The results therefore show the presence

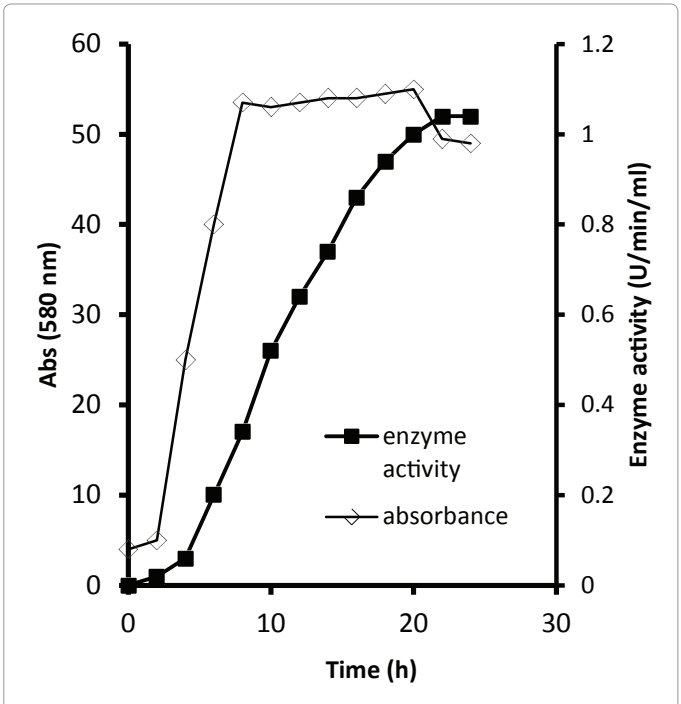

Figure 1: Time-courses of growth (absorbance monitoring) and esterase production in *B. subtilis* NCIMB 8054 fermentation.

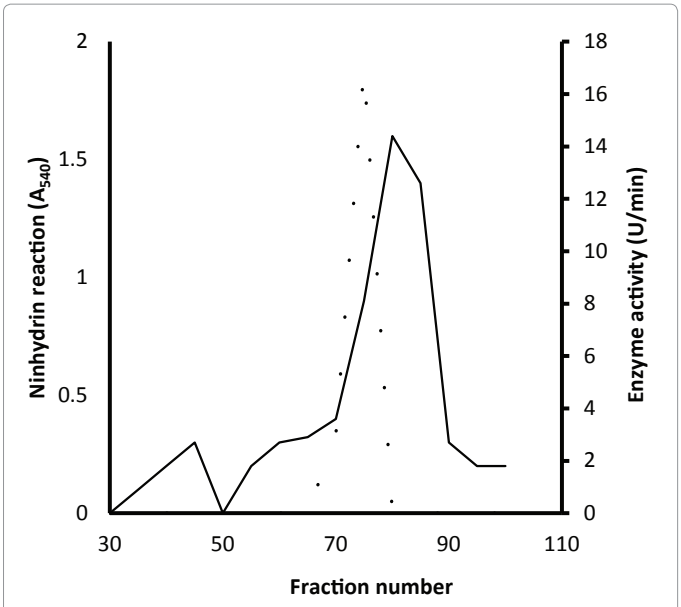

Figure 2: Elution profile of the <10 kDa filtrate from a Bio-gel P-6 column affinity chromatography. Solid line: ninhydrin reaction; Dashed line: esterase activity.

of an enzyme that catalyzes the hydrolysis of esters. Native PAGE was carried out using both silver and esterase activity stain as described in the previous section, and confirmed that esterase activity was due to the detected protein.

The approximate molecular weight of the enzyme was determined by SDS-PAGE. A band was obtained, similar to those of the native gel, with a molecular weight of about 3.8 kDa (Figure 3). Amino acid sequence analysis gave the following results: eevaetysfyhitphdystshispapvqffspap. The small protein molecule has 34 amino acid residues and according to the derived composition the calculated molecular mass is 3853.1 (Table 1). Table 1 shows the number and percentage composition of the identified 34 amino acids. The calculated isolectric point for the protein is 4.58. There is a high content in serine and proline, 5 residues each or 14.7%. A very low content of 2.9% (representing 1 amino acid) has been found for glycine and aspartate. A general BLAST (program BLASTP 2.2.24+) search carried out for the particular sequence of the esterase revealed no significant similarity with other deposited proteins or peptides.

Primary structure analysis was carried out using the Kyte and Doolittle [25], Hope and Woods [26] and Janin et al. [27] routines to determine the hydrophilic/hydrophobic profile of the protein, while according to Garnier et al. [24] for prediction of α-helix, β-sheet and β-turn configurations [28-30] (Figures 4 and 5). According to the generated data, the *B. subtilis* esterase appears to be strongly hydrophobic, with more than 80% of its amino acid residues being hydrophobic, while it appears to have a α-helical conformation in the N-terminal, the rest of the molecule being β-sheet-rich.

The purified enzyme retained its original activity during a 120 h incubation period at 40 and 50°C (Figure 6). More than 85% of the original activity was retained after 120 hours at 60°C, while residual activity was decreased to 73% after 120 h at 70°C. At the higher temperatures tested, 80°C and 90°C, the decrease of residual enzyme activity was sharp; it should be noted however that the enzyme

maintained almost 30% of its original activity after 12 h incubation at 80°C.

Whether there is a relationship between lower mass of an enzyme and higher thermostability is not clear. It has been observed in a number of cases that the two are related and it was suggested by several authors that a general tendency exists for enzymes with lower molecular masses to have higher thermostabilities [11,14,18,28]. For others however, certain structural features such as increased intramolecular packing or reduced surface loops that have prominent stabilizing effects are not usually related to protein size and therefore the hypothesis of a general tendency cannot be supported [12].

Primary structure analysis of the purified esterase as shown in Figure 4, revealed a high degree of hydrophobic interactions in its molecule - a characteristic that is certainly related to higher thermostability [29-32]. A feature that appears in Figure 4 is the reduced surface area, since positive values represent amino acids that are likely to be exposed on the surface of the protein [27]. This feature also is in agreement with increased thermal stability of the esterase [29]. Another characteristic of the molecule of the isolated esterase that is in agreement with known

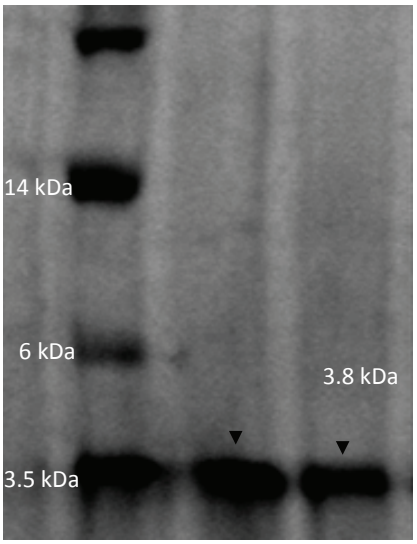

Figure 3: SDS-PAGE (16.5%) of the purified esterase from *B. subtilis* NCIMB 8054.
Line 1: MW markers; Lines 2 and 3: esterase

Amino acid	Number of residues	%
Alanine	3	8.8
Aspartic acid	1	2.9
Glutamic acid	3	8.8
Phenylalanine	3	8.8
Histidine	3	8.8
Isoleucine	2	5.9
Proline	5	14.7
Glycine	1	2.9
Serine	5	14.7
Threonine	3	8.8
Valine	2	5.9
Tyrosine	3	8.8

Table 1: Amino acid composition of the *B. subtilis* esterase.

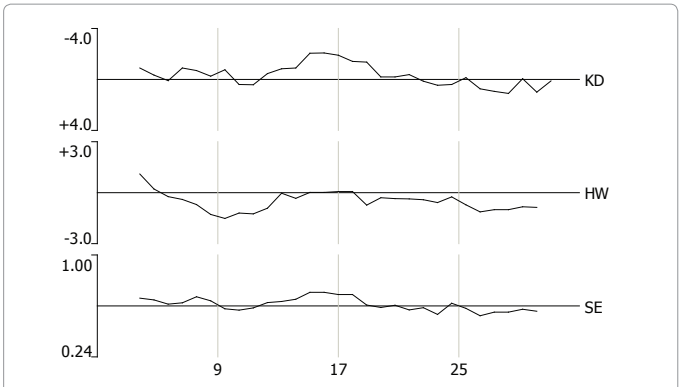

Figure 4: Plots of values calculated according to Kyte and Doolittle (KD) [25], Hopp and Woods (HW)[26] and Janin et al. [27] routines for the *B. subtilis* esterase. KD values fall within a range of +4 to -4, with hydrophilic residues having a negative score. HW values fall within a range of -3 to +3, with hydrophilic residues having a positive score. On the surface exposure (SE) graph peak values which fall above the axis line are predicted to be exposed on the surface of the protein.

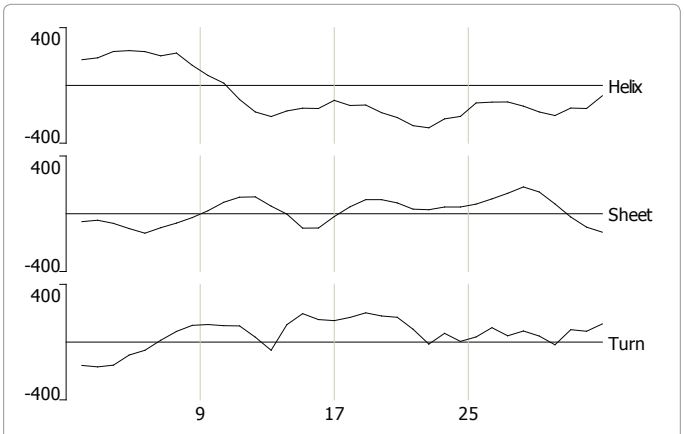

Figure 5: Garnier-Osguthorpe-Robson plots of the predicted values for α-helix, β-sheet and β-turn configurations for the *B. subtilis* esterase. Values above the axis line represent regions which are likely to form helix sheet or turn structures.

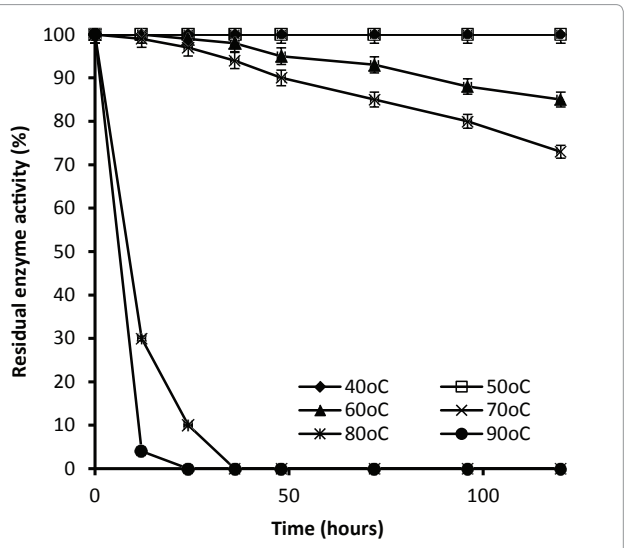

Figure 6: Residual esterase activity following incubation at various temperatures.

characteristics of thermostable proteins is the high proline content and the absence of proline residues in the α–helix area [31].

Conclusion

An overview of the literature data on small natural enzymes with less than 10 kDa molecular weight gives only a few cases of isolated micro enzymes for which critical information, e.g. structure-related data, is not provided. Here we report results on the purification, sequencing and partial structural characterization of a thermostable low molecular weight esterase produced in fermentation by a *B. subtilis* strain. The results revealed a 3.85 kDa and 34 amino acids protein, rich in proline and highly hydrophobic which also showed significant thermostability. Small biocatalysts could be versatile tools in biotechnology studies and may have various industrial applications. The present study shows that the micro esterase is secreted by *B. subtilis* and its molecule has interesting and rather rare characteristics. Further studies therefore, on the molecule itself and the producer microorganism in fermentations, are needed to highlight the production potential of the newly isolated esterase.

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