Innovative Romanian Food Biotechnology Vol. 16, Issue of March, 2015
 Received January 25, 2015/Revised February 17, 2015/Accepted February 18, 2015

RESEARCH ARTICLE

CO-PRODUCTION OF THERMOSTABLE, CALCIUM-INDEPENDENT A-AMYLASE AND ALKALI-METALLO PROTEASE FROM NEWLY ISOLATED *BACILLUS LICHENIFORMIS* DV3

Fatma MATPAN BEKLER[1*], Ömer ACER[1], Kemal GÜVEN[1]

[1]Dicle University, Science Faculty, Department of Biology, TR 21280, Diyarbakir/Turkey, Tel.: 0090 412 248 85 50/3229; fax: 0090 412 248 83 00.

[*]Corresponding author: fatmatpan@hotmail.com

Abstract: Extracellular thermostable α-amylase and protease from one fermentation of a newly isolated thermophilic *Bacillus licheniformis* DV3 was studied. The optimum pH and temperature for the enzyme activity were 7.0 and 70°C for the α–amylase, while it was 10.0 and 50°C, respectively for the protease activity. Different carbon and nitrogen sources were investigated for the first time in terms of their effect on the co-production of extracellular thermostable α-amylase and protease. Among carbon and nitrogen sources, soluble starch and urea increased both α-amylase and protease production to a great extend. The α-amylase activity was enhanced in the presence of Mn^{2+}, inhibition was obtained in the presence of $Ca^{2+}$and Cu^{2}, and it was strongly inhibited by Hg^{2+}. The protease activity was increase in the presence of Ca^{2+} and Zn^{2+}, whereas the activity was decreased by $Mn^{2+}$and Hg^{2+}, and the activity inhibited by EDTA and PMSF.

Key words: *Bacillus licheniformis*; α-amylase; protease; co-production.

Introduction

A large number of bacteria, fungi, and yeasts produce extracellular enzymes that degrade starch in different environmental niches (Sharma and Satyanarayana, 2011). Enzymes used in industrial fields, although being of plant and animal origin, are heavily isolated from microorganisms (Eren Kiran *et al.*, 2006). With the advance in the biotechnology and purification of the enzymes, the number of enzyme applications has multiplied and a lot of new opportunities have appeared for industrial processes thanks to the obtainable thermostable enzymes.

Thermostable enzymes, have found a number of commercial applications (Demirjian *et al.*, 2001). Bacteria belonging to the *Bacillus* genus and the genera closely related to *Bacillus* secrete thermostable amylases, cellulases, lipases and proteases. Amylases, biosynthesized by the bacteria, show unique characteristics such as thermophilic, thermotolerant, alkaline, and acidophilic properties, and are of great significance in present day biotechnology. These enzymes are used for baking, brewing, fermentation applications, food, detergent, paper, and textile industries to analyses in clinical and medicinal chemistry as well as pharmaceutical industries (Hmidet *et al.*, 2008; Asoodeh *et al.*, 2010). Bacterial proteases are mostly extracellular, easily produced in larger amounts, thermostable, and active at a wider pH range (Banik and Prakash,

2004). Proteases find application in detergents, food, leather, pharmaceutical industry, waste management (Anwar and Saleemuddin, 1997; Gupta *et al*., 2002; Maurer, 2004; Haddar *et al*., 2009; Swati and Satyanarayana, 2013), and bioremediation processes (Deng *et al*., 2010), silk degumming and silver recovery (Swati and Satyanarayana, 2013), cosmetics, skin care ointments and contact lens cleaners (Jellouli *et al*., 2011; Asker *et al*., 2013).

Amylase and proteases are used together for several industries. In previous studies, there are few reports about co-production of α -amylase and protease (Hmidet *et al*., 2009; Negi and Banerjee, 2009; Corrêa *et al*., 2011; Kumar *et al*., 2013). Most industrial enzymes are secreted by microorganisms into the growth medium in order to break down the carbon and nitrogen sources. It is by far the most important to obtain a microorganism that grows very quickly on inexpensive medium, while at the same time producing large amount of the target enzymes.

Thus, the aim of the present work was to investigate the co-production of two important enzymes (α -amylases and protease) by thermophilic strain *Bacillus licheniformis* DV3 under the same conditions, as well as studying some properties of the enzymes produced.

Materials and methods

Bacterial strain

In this study, the strain *Bacillus licheniformis* DV3, which were isolated from the Davut hot water springs of Diyadin township of Ağri Province (39° 27′ 52″ N, 43° 40′ 53″ E) (temperature 78°C, pH 7.7), in northeastern Turkey, were used. The strain *Bacillus licheniformis* DV3 was isolated and identified by morphological, biochemical tests and 16S rRNA sequence analysis (Matpan, 2007).

Growth medium and optimization of the culture

The microorganism was grown at 55°C in nutrient broth (NB: beef extract, 10 g/L; peptone, 10 g/L; and NaCl, 5 g/L) and the pH was adjusted to 7.0 prior to sterilization. The media were autoclaved at 120◦C for 20 min. The cultivations were performed in a shaker for 4–64 h at 55◦C in 250 mL Erlenmeyer flasks with a working volume of 33 mL.

Cultures were incubated at 160 rpm at 55°C for 24 h in a horizontal shakerThe cultures were centrifuged at 8,200 x*g* at 4°C for 10 min, and the cell-free supernatants were used for estimation of α -amylase and protease activities. Growth was determined by measuring the increase in OD_{470}.

α-Amylase activity

The enzyme activity was determined according to the Bernfeld method (Bernfeld, 1955). One unit (U) of amylase activity was defined as the amount of enzyme that released 1 µmol of maltose per minute per mL under the assay conditions.

Protease assay methods

Protease activity was determined by the method of Leighton *et al.* (1973). The protein content in the extracellular extracts was determined by the Lowry method (Lowry *et al.*, 1951) using bovine serum albumin as a standard in this procedure. One unit of protease was defined as the amount of enzyme required to release 1 µmol of tyrosine per min under assay conditions (55°C, pH 7.0).

Effect of the incubation time on the α-amylase and protease co-production

100 mL of liquid media (NB) was prepared autoclaved and the flasks were inoculated with 1 mL of an overnight cell suspension ($1{,}2x10^8$ cells/mL) and stirred in a water bath at 160 rpm at 55°C .

Next, a sample was taken at the end of every 4 hours over a 48 h and was centrifuged at 8,200 x*g*, and the bacteria were precipitated by centrifugation. A supernatant was used for the *α*-amylase and protease activity measurement.

Effect of the carbon and nitrogen sources on the α-amylase and protease co-production

In order to determine the effect of various carbon and nitrogen sources on the *α*-amylase and protease production, 250 µL overnight culture of dtrain DV3 was inoculated into soluble starch-free basal medium (BM) 0.2% yeast extract, 1% beef extract, 0.02% $CaCl_2$ and 0.01% $MgSO4.7H_2O$) containing 2% concentration of various carbon sources such as glucose, lactose, sucrose, fructose, galactose, and soluble starch, and various nitrogen sources, such as bacto liver, casaminoacid, glycine, tryptone, urea, and ammonium sulphate in 100 mL Erlenmeyer flasks.

 Received January 25, 2015/Revised February 17, 2015/Accepted February 18, 2015

RESEARCH ARTICLE

The partial purification of α-amylase and protease

The culture supernatant containing the extracellular α-amylase and protease was first subjected to ammonium sulphate precipitation 60-80% (w/v) and centrifuged at 10,000 g, suspended in a minimal volume of buffer and then dialysed. The enzyme solution was used in the following experiments.

Effect of pH on the activity and stability of the α-amylase and protease

The effect of pH on the activity of the α-amylase and protease was measured in the range of 4.0–11.0 [0.1 M citric acid buffer (4.0-6.0), 0.1 M Tris-HCl buffer (6.0-9.0) and 0.1 M glycine-NaOH (9.0-11.0)] under standard assay conditions. For the measurement of the pH stability, both enzymes were preincubated in buffers at different pH values in the range of 4.0–11.0 at 55°C for 1 h. Aliquots were withdrawn and the residual amylolytic and proteolytic activities were determined under standard assay conditions.

Effect of temperature on the activity and stability of the α-amylase and protease

The effects of temperature on the α-amylase and protease activity was studied at temperatures from 30 to 90°C for 30 min in 0.1 M Tris-HCl buffer (pH 7.0) for α–amylase and 0.1 M glycine-NaOH buffer (pH 10.0) for protease. The thermostability of the partially purified enzymes was examined by incubating the enzymes for 30 min at different temperatures (60-80°C for amylase 45, 50 and 55°C for protease). Aliquots were withdrawn to test the remaining activity under standard conditions. The unheated enzymes were considered as the control (100%).

Effects of metal ions and enzyme inhibitors on the α-amylase and protease activity

The effects of various inhibitors on the enzyme activity were investigated by preincubating the enzyme in the presence of substances with a final concentration of 1.5 mM [Ca^{2+}, Mn^{2+}, Cu^{2+}, Hg^{2+}, ethylene diamine tetra acetic acid (EDTA) and phenyl methyl sulfonyl fluoride (PMSF)] for 30 min at the optimum temperatures, followed by measuring the residual α-amylase and protease activity. The activity in the absence of any additives was taken to be 100%. The residual activity was measured using the standard assay conditions.

Statistical analysis

The results are represented as the mean ± SD of at least 3 experiments. The Kruskal Wallis test was done with the SPSS Statistical software, for Windows.

Results and discussion

Time course of bacterial growth and enzyme production

In this study, an isolate from hot spring water in Ağri, Turkey, were examined for their potential in the co-production of α-amylase and protease. This is the first report on the bacteria and their enzymes in Davut hot water springs. The strain was Gram-positive, spore-forming and rod-shape. According to some biochemical and physiological tests and partial 16S rDNA sequence analysis, strain DV3 was identified as a strain of *B. licheniformis*. The 16S rRNA gene sequence was submitted to GenBank under the accession number HQ864575. The results of the time-course studies on the growth of *B. licheniformis* DV3, and α-amylase and protease production are shown in Fig. 1. The maximum growth for *B. licheniformis* DV3 was obtained after 24 h of incubation. Similarly, the highest α-amylase activity was found after 24 h of incubation (831 U/mL), after which amylase levels began to fall down to minimal level at 48 h. Protease activity was found to increased up to 36 h of incubation (898 U/mL), after which sharply decreased (Fig. 1).

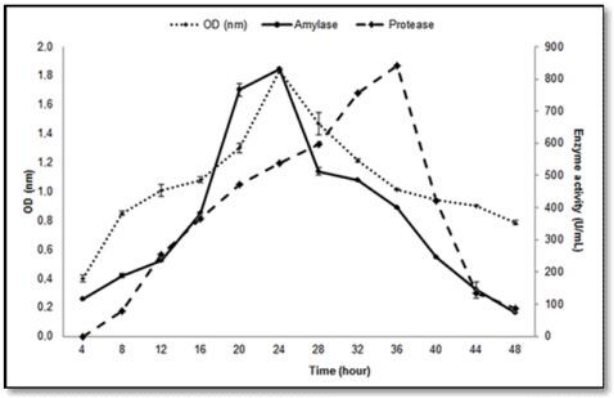

Figure 1. *Effect of time-course on bacterial growth and co-production of ~-amylase and protease*

Results represent the means of three experiments, and bars indicate ± standard deviation. Absence of bars indicates that errors were smaller than symbols

Effect of the carbon and nitrogen sources on the α-amylase and protease co-production

As shown in Fig. 2, the substrate for maximum amylase production for *B. licheniformis* DV3 was 2% soluble starch (1650 U/mL), lactose (1102 U/mL), and fructose (1018 U/mL), compared to control. It was also observed that soluble starch (1534 U/mL), lactose (1110 U/mL), and sucrose (922 U/mL) favored protease production.

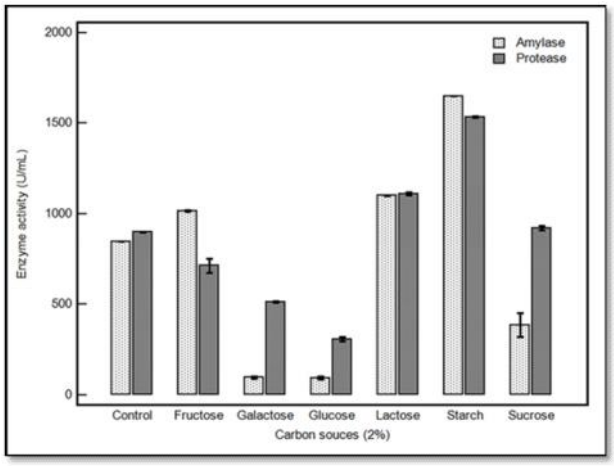

Figure 2. *Effect of carbon sources on ~-amylase and protease production*
Results represent the means of three experiments, and bars indicate ± standard deviation. Absence of bars indicates that errors were smaller than symbols

In previous studies, Ul-Haq *et al.* (2003) and Ray *et al.* (2008) determined that the production of α-amylase was increased in the presence of soluble starch by *B. licheniformis* GCBU-8 and *Bacillus brevis* MTCC 7521. Behal *et al.* (2006) reported that the maximum α-amylase production was increased by fructose. Fujiwara and Yamamamto (1987), Kumar *et al.* (2013) and Nadeem *et al.* (2008) stated that the best carbon sources was starch for protease production. Mabrouk *et al.* (1999) also found that the protease production was increased in the presence of lactose. In our study, glucose and galactose as a carbon source, reduced α-amylase and protease to minimal level (Fig. 2). It is well known that the synthesis of carbohydrate degrading enzymes in most species of the genus *Bacillus* is subjected to catabolic repression by glucose (Wind *et al.*, 1994; Asgher *et al.*, 2007) and sucrose (Qader *et al.*, 2006).

Among the various organic and inorganic nitrogen sources tested, while keeping the beef extract concentration constant, urea and tryptone at a concentration of 2% resulted in the maximum α-amylase and protease production (Fig. 3). Casamino acid and glycine none had any significant effect on the amylase and protease production, and in fact, ammonium salts had an inhibitory effect on the α-amylase and protease production, in agreement with references (Saxena *et al.*, 2007; Nadeem *et al.*, 2008).

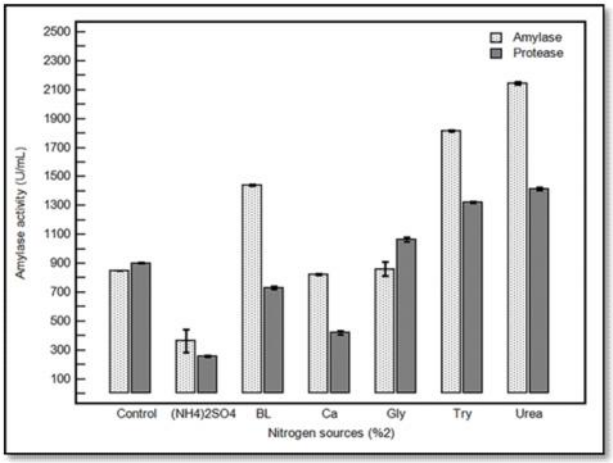

Figure 3. *Effect of nitrogen sources on ~-amylase and protease production*
Results represent the means of three experiments, and bars indicate ± standard deviation. Absence of bars indicates that errors were smaller than symbols

Biochemical properties of the partially purified α-amylase and protease

Effect of pH on the activity and stability of the B. licheniformis DV3 α-amylase and protease

The effect of pH on the α-amylase and protease activity was studied using soluble starch as a substrate at various pH values at 55°C for *B. licheniformis* DV3. The pH activity profile of this strain is shown in Fig. 4.

The optimum pH was found as 7.0 for α-amylase. Hmidet et al. (2008) reported similar optimal pH values of 6.0–6.5 and 5.0–7.0 for α-amylases from *B. licheniformis* 44MB82-A and *B. licheniformis* NRRL B14368, respectively. The optimum α-amylase activity has been also determined at pH 7.0 for *B. subtilis* (Asgher *et al.*, 2007) and *Bacillus* sp. WN11 (Mamo and Gessesse, 1999) and 7.5 for *B. licheniformis* (Rodríguez *et al.*, 2006).

 Received January 25, 2015/Revised February 17, 2015/Accepted February 18, 2015

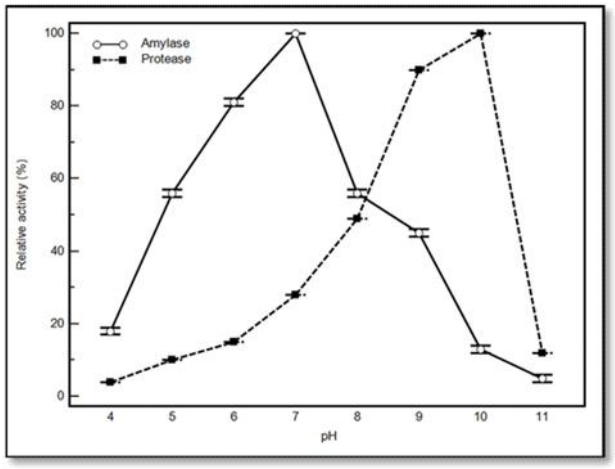

Figure 4. *Effect of pH on activity of~-amylase and protease*

Results represent the means of three experiments, and bars indicate ± standard deviation. Absence of bars indicates that errors were smaller than symbols. The values are shown as percentages of the maximum activity of enzyme

As can be seen in Figure 5, the α -amylase was highly stable at pH 7.0 and retained the activity about 100% and 83% at pH 8.0 after 30 min. Asodeh *et al.* (2010) and Behal *et al.* (2006) also reported that α-amylase was considerable stable at pH 7.0 for *Bacillus* sp. This pH-stability profile makes them interesting for their application in industrial processes to be carried out at a pH range close to neutrality.

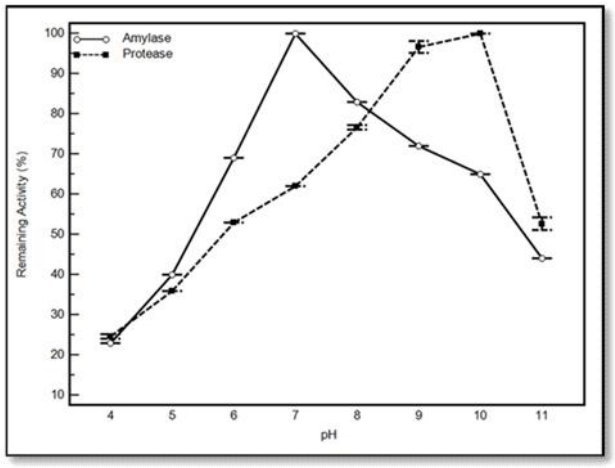

Figure 5. *Effect of pH on stability of ~-amylase and protease*

The optimum pH was found as 10.0 for protease. Optimum pH value has been found also 10.0 for the protease of *Bacillus licheniformis* MP1 and *Bacillus* sp. B001 (Deng *et al.*, 2010; Jellouli *et al.*, 2011).

Banik and Prakash (2004) and Beg and Gupta (2003) reported an optimal pH activity of 10.5-11.0 for the protease of *Bacillus cereus* and *Bacillus mojavensis*, respectively. The protease from DV3 strain was stable at pH range of 9.0-10.0 and enzyme retained about 100% and 98% activity in the pH range of 9.0–10.0, respectively (Fig. 5). Jellouli *et al.* (2011) found stability at higher pH for alkaline protease from *Bacillus licheniformis* MP1.

Therefore, enzyme stability at high pH compromises their likely use as additives in washing detergents, leather processing or other applications at higher pH.

Effect of temperature on the activity and stability of α-amylase and protease

The amylolytic and proteolytic activities were assayed at different temperatures using optimum pHs. The α-amylase activity was tested within the range of 30–90°C.

The optimum temperature for *B. licheniformis* DV3 α-amylases was found as 70°C (Fig. 6), which is comparable to that described for other *Bacillus* α-amylases (Bolton *et al.*, 1997; Mamo and Gessesse, 1999; Malhotra *et al.*, 2000; Cordeiro *et al.*, 2002; Najafi *et al.*, 2005; Asgher *et al.*, 2007; Arikan, 2008).

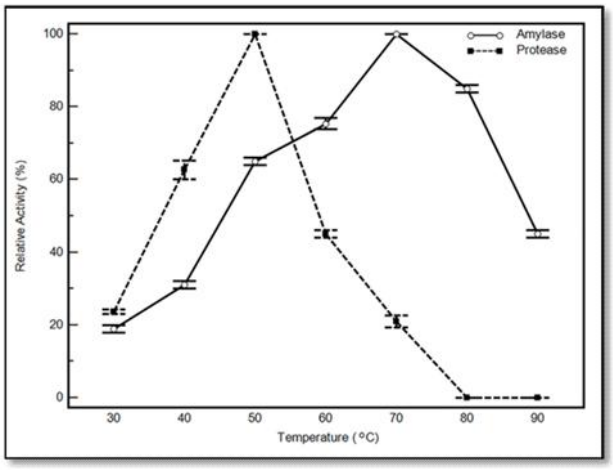

Figure 6. *Effect of temperature on activity of ~-amylase and protease*

Moreover, it was determined that α-amylase from *B. licheniformis* DV3 was stable at 60-75°C and retained original activity at 98% within 30 min (Fig. 7).

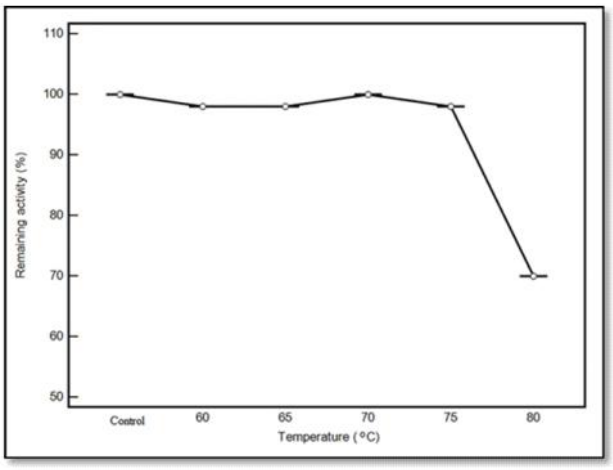

Figure 7. *Effect of temperature on stability of ~-amylase*

Asodeh *et al.* (2010) stated that amylase was comparatively stable at 75°C. The present study showed that the enzyme seemed to have considerable thermostability, which can be favorable in industrial operations for traditional brewing and food processing, particularly in industrial starch liquefaction (Stamford *et al.*, 2001; Liu and Xu, 2008).

The optimum temperature for *B. licheniformis* DV3 protease was 50°C (Fig. 6) and was highly stable and about 98% active even after 30 min of incubation at 50°C (Fig. 8).

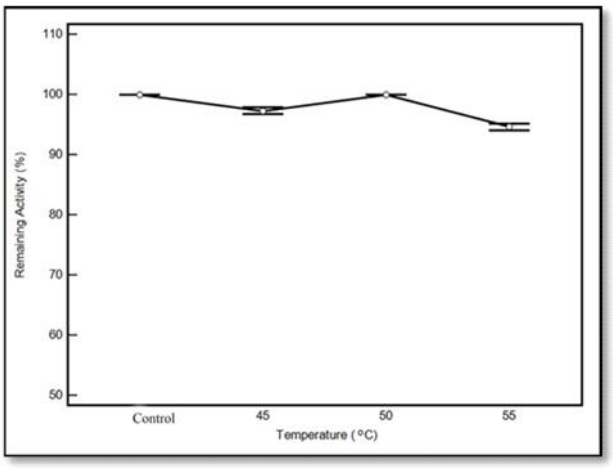

Figure 8. *Effect of temperature on stability of protease*

The stability of this protease might be advantage for using it in industrial applications such as laundry detergents formulations. In previous studies, optimum temperatures were found to be 50°C for the other *Bacillus* proteases (Mabrouk *et al.*, 1999; Banik and Prakash, 2004; Asker *et al.*, 2013; Swati and Satyanarayana, 2013; Annamalai *et al.*, 2014). Jellouli *et al.* (2011) found that alkaline-protease from *Bacillus licheniformis* MP1 was also fully active even after 2 h of incubation at 50°C.

Effects of the metal ions and enzyme inhibitors

The effect of different metal ions on the activity of the α-amylase and protease are shown in Table 1.

Table 1. *Effects of metal ions and inhibitors on the amylolytic and proteolytic activity*

Metal ions and inhibiors (1,5 mM)	Residual Activity (%) of Amylase	Residual Activity (%) of Protease
Ca^{2+}	9±0.3	123±0.05
Cu^{2+}	33±0.01	95±0.07
Hg^{2+}	0	25±0.09
Mn^{2+}	72±0.02	75±0.02
Zn^{2+}	66±0.01	122±0.02
EDTA	119±0.57	0
PMSF	0	0

*Residual activity was determined as percentage of control with no additions

The α-amylase from *B. licheniformis* DV3 was decreased by all of the chemicals and ions, except a little increase with EDTA. The inhibition effect in the presence of Ca^{2+} show that the enzyme was a member of calcium-independent amylases, in agreement with previous studies in *B. thermooleovorans* NP54 (Malhotra *et al.*, 2000), *B. licheniformis* NH1 (Hmidet *et al.*, 2008), *Bacillus* sp. WN11 (Mamo and Gessesse, 1999), and *Bacillus* sp. KR-8104 (Asoodeh *et al.*, 2010). Generally, Ca^{2+} is added to stabilize α -amylases in liquefaction step of starch process, after which Ca^{2+} ions should be removed by using some chromatographic methods prior to the isomerization of glucose to fructose due to the inhibitory effect of Ca^{2+} ions on the glucose isomerase (Hashida, 2000). This step is not required for the Ca-independent α -amylase, and this is a great advantage. In this way, the demand for Ca^{2+} independent α -amylases are increasing for industrial process.

In our study, the α -amylase activity was strongly inhibited in the presence of Hg^{+2}, Cu^{+2} and PMSF for DV3 strain. The inhibition by Hg^{2+} may indicate the importance of indole amino acid residues in enzyme function, as demonstrated for other microbial α-amylases (Gupta *et al.*, 2003). Furthermore, Hg^{2+} is known to oxidize indole rings and to interact with the aromatic rings present in

 Received January 25, 2015/Revised February 17, 2015/Accepted February 18, 2015

tryptophan residues (Sharma and Satyanarayana, 2011).

The *B. licheniformis* DV3 protease was activated by Ca^{2+} and Zn^{2+}, whereas Mn^{+2} and Cu^{2+} decreased the activity. Stronger inhibitory effect was observed in presence of Hg^{2+}, EDTA and PMSF. In previous studies, it was found that Ca^{2+} (Jellouli *et al.*, 2011; Asker *et al.*, 2013; Benkiar *et al.*, 2013; Annamalai *et al.*, 2014) and Zn^{2+} (Jellouli *et al.*, 2011; Benkiaret *et al.*, 2013) stimulated the protease activity. EDTA (Sellami-Kamoun *et al.*, 2008; Deng *et al.*, 2010) fully inhibited the protease activity. Metalloproteases need divalent cations for their activities. The metalloproteases generally contain Zn^{2+} in their structure and chelators such as EDTA inhibit enzyme activity which clearly indicates that the alkaline protease belongs to the family metalloprotease. Calcium cations known to be stabilizers of many enzymes protect them from conformational changes. In addition, Maruthiah *et al.* (2013) and Benkiar *et al.* (2013) stated that PMSF had strong inhibition effect for protease. PMSF well-known inhibitors of serine proteases. This suggested that a serine residue at least was involved in the catalytic activity of the protease of strain DV3. Due to serine proteases showing activity and stability in alkaline conditions, they are of importance in various industrial applications (Subba Rao *et al.*, 2008). This metallo-protease is also commercially produced because of its economic importance in some applications.

Conclusions

The strain DV3 was isolated from hot spring water in Davut (Ağri), Turkey and identified as *B. licheniformis*. The strain DV3 has been found to be able to secrete extra-cellular α-amylase and protease. Optimum conditions of both two enzymes were investigated. Both enzymes were found to be produced by the strain to a great extent due to use of cheap substrates such as starch, lactose, urea. Moreover, this is a first report for co-production of two enzymes which were stable at high temperature, as well as α -amylase being calcium independent and alkaline-metallo protease. These are known to be important properties in industrial applications. Neutral-thermostable amylase is important for starch liquefaction and food industries. In addition, alkaline-metallo protease shows the potential use of this enzyme in food and detergent industry. The production process can be commercialized.

References

Annamalai N., Rajeswari M.V., Balasubramanian T., (2014). Extraction, purification and application of thermostable and halostable alkaline protease from *Bacillus alveayuensis* CAS 5 using marine wastes, *Food and Bioproducts Processing*, 92, 335-342.

Anwar A., Saleemuddin M., (1997). Alkaline Proteases: A Review, *Biosource Technology*, 64, 175-183.

Arikan, B., (2008). Highly thermostable, thermophilic, alkaline, SDS and chelator resistant amylase from a thermophilic *Bacillus* sp. isolate A3-15, *Biosource Technology*, 99, 3071-3076.

Asgher M., Asad M.J., Rahman S.U., Legge R.L., (2007). A thermostable α-amylase from a moderately thermophilic *Bacillus subtilis* strain for starch processing, *Journal of Food Engineering*, 79, 950-955.

Asker M.M., Mahmoud M.G., El Shebwy K., Abd el Aziz M.S., (2013). Purification and characterization of two thermostable protease fractions from *Bacillus megaterium*, *Journal of Genetic Engineering and Biotechnology*, 11, 103-109.

Asoodeh A., Chamani J., Lagzian M., (2010). A novel thermostable, acidophilic α-amylase from a new thermophilic "*Bacillus* sp. Ferdowsicous" isolated from Ferdows hot mineral spring in Iran: Purification and biochemical characterization, *International Journal of Biological Macromolecules*, 46, 289-297.

Banik R.M., Prakash M., (2004). Laundry detergent compatibility of the alkaline protease from *Bacillus cereus*, *Microbiological Research*, 159, 135-140.

Beg Q.K., Gupta R., (2003). Purification and characterization of an oxidation-stable, thiol-dependent serine alkaline protease from *Bacillus mojavensis*, *Enzyme and Microbial Technology*, 32, 294-304.

Behal A., Singh J., Sharma M.K., Puri P., Batra N., (2006). Characterization of alkaline α-amylase from

Bacillus sp. AB 04, *International Journal of Agricultural and Biological Engineering*, 8, 80-83.

Benkiar A., Nadia Z.J., Badis A., Rebzani F., Soraya B.T., Rekik H., Naili B., Ferradji F.Z., Bejar S., Jaouadi B., (2013). Biochemical and molecular characterization of a thermo- and detergent-stable alkaline serine keratinolytic protease from *Bacillus circulans* strain DZ100 for detergent formulations and feather-biodegradation process, *International Biodeterioration and Biodegradation*, 83, 129-138.

Bernfeld P., (1955). Amylases, α and β, *Methods Enzymology*, 1, 149-158.

Bolton D.J., Kelly C.T., Fogarty W.M., (1997). Purification and characterization of the a-amylase of *Bacillus flavothermus, Enzyme Microbial Technology*, 20, 340-343.

Cordeiro C.A.M., Martins M.L.L., Luciano A.B., (2002). Production and properties of α-amylase from thermophilic *Bacillus* sp., *Brazilian Journal of Microbiology*, 33, 57-61.

Corrêa T.L.R., Moutinho S.K.S., Martins M.L.L., Martins M.A., (2011). Simultaneous α-amylase and protease production by the soil bacterium *Bacillus* sp. SMIA-2 under submerged culture using whey protein concentrate and corn steep liquor: compatibility of enzymes with commercial detergents, *Ciência e Tecnologia de Alimentos*, 31, 843-848.

Demirjian D.C., Morís-Varas F., Cassidy C.S., (2001). Enzymes from extremophiles, *Current Opinion in Chemical Biology*, 5, 144-151.

Deng A., Wu J., Zhang Y., Zhang G., Wen T., (2010). Purification and characterization of a surfactant-stable high-alkaline protease from *Bacillus* sp. B001, *Biosource Technology*, 101, 7100-7106.

Eren Kiran Ö., Çomlekcioglu U., Dostbil N., (2006). Some Microbial Enzymes and Usage Fields in Industry, *KSU Journal of Science and Engineering*, 9, 12-19.

Fujiwara N., Yamamoto K., (1987). Production of alkaline protease in a low-cost medium by alkalophilic *Bacillus* sp. and properties of the enzyme, *Journal of Fermentation and Technology*, 65, 345-348.

Gupta R., Beg Q., Lorenz P., (2002). Bacterial alkaline proteases: molecular approaches and industrial applications, *Applied Microbiology and Biotechnology*, 59, 15-32.

Gupta R., Gigras P., Mohapatra, H., Goswami V.K., Chauhan B., (2003). Microbial α-amylases: a biotechnological perspective, *Process Biochemistry*, 38, 1599-1616.

Haddar A., Agrebi R., Bougatef A., Hmidet N., Sellami-Kamoun A., Nasri M., (2009). Two detergent stable alkaline serine-proteases from *Bacillus mojavensis* A21: Purification, characterization and potential application as a laundry detergent additive, *Biosource Technology*, 100, 3366-3373.

Hashida M., Bisgaard-Frantzen H., (2000). Protein engineering of new industrial amylases, *Trends Glycoscience Glycotechnology*, 68, 389-401.

Hmidet N., Bayoudh A., Berrin J.G., Kanoun S., Juge N., Nasri M., (2008). Purification and biochemical characterization of a novel α-amylase from *Bacillus licheniformis* NH1, *Process Biochemistry*, 43, 499-510.

Hmidet N., El-Hadj Ali N., Haddar A., Kanoun S., Alya S.-K., Nasri, M., (2009). Alkaline proteases and thermostable α-amylase co-produced by *Bacillus licheniformis* NH1: Characterization and potential application as detergent additive, *Biochemical Engineering Journal*, 47, 71-79.

Jellouli K., Ghorbel-Bellaaj O., Ayed H.B., Manni L., Agrebi R., Nasri M., (2011). Alkaline-protease from *Bacillus licheniformis* MP1: Purification, characterization and potential application as a detergent additive and for shrimp waste deproteinization, *Process Biochemistry*, 46, 1248-1256.

Kumar M.S., Rao M., Anbuselvi S., Kumar S.S., (2013). Dual Production of Amylase and Protease Using *Bacillus Subtilis.* Research Journal of Pharmaceutical, *Biological and Chemical Sciences*, 4, 1022-1028.

Leighton T.J., Doi R.H., Warren R.A.J., Kelln R.A., (1973). The relationship of serine protease activity to RNA polymerase modification and sporulation in *Bacillus subtilis*, *Journal of Molecular Biology*, 76, 103-122.

Liu X.D., Xu Y., (2008). A novel raw starch digesting α-amylase from a newly isolated Bacillus sp. YX-1:

 Received January 25, 2015/Revised February 17, 2015/Accepted February 18, 2015

Purification and characterization, *Biosource Technology*, 99, 4315-4320.

Lowry O.H., Rosebrough N.J., Farr A.L., (1951). Protein measurement with the folin phenol reagent, *The Journal of Biological Chemistry*, 265-275.

Mabrouk S., Hashem A., El-Shayeb N., Ismail A.-M., Abdel-Fattah, A., (1999). Optimization of alkaline protease productivity by *Bacillus licheniformis* ATCC 21415, *Biosource Technology*, 69, 155-159.

Malhotra R., Noorwez S., Satyanarayana T., (2000). Production and partial characterization of thermostable and calcium-independent α-amylase of an extreme thermophile *Bacillus thermooleovorans* NP54, *Letters in Applied Microbiology*, 31, 378-384.

Mamo G., Gessesse A., (1999). Purification and characterization of two raw-starch-digesting thermostable α-amylases from a thermophilic *Bacillus, Enzyme and Microbial Technology*, 25, 433-438.

Maruthiah T., Esakkiraj P., Prabakaran G., Palavesam A., Immanuel G., (2013). Purification and characterization of moderately halophilic alkaline serine protease from marine *Bacillus subtilis* AP-MSU 6, *Biocatalysis and Agricultural Biotechnology*, 2, 116-119.

Matpan F., (2007). Isolation and Characterisation of Bacteria from Hot Water Springs in Diyadin (Ağri/Turkey) and Study on Their Enzymes. Dissertation. Dicle University.

Maurer K., (2004). Detergent proteases, *Current Opinion in Biotechnology*, 15, 330-334.

Nadeem M., Qazi JI ., Baig S., Syed QA., (2008). Effect of Medium Composition on Commercially Important Alkaline Protease Production by *Bacillus licheniformis* N-2, *Food Technology and Biotechnology*, 46.

Najafi M.F., Deobagkar D., Deobagkar D., (2005). Purification and characterization of an extracellular α-amylase from *Bacillus subtilis* AX20, *Protein Expression and Purification*, 41, 349-354.

Negi S., Banerjee R., (2009). Characterization of amylase and protease produced by *Aspergillus awamori* in a single bioreactor, *Food Research International*, 42, 443-448.

Qader S., Bano S., Aman A., Syed N., Azhar A., (2006). Enhanced production and extracellular activity of commercially important amylolytic enzyme by a newly isolated strain of *Bacillus* sp. AS-1, *Turkish Journal of Biochemistry*,31 135-140.

Ray R.C., Kar S., Nayak S., Swain M.R., (2008). Extracellular α-Amylase Production byBacillus brevisMTCC 7521, *Food Biotechnology*, 22, 234-246.

Rodríguez V.B., Alameda E.J., Gallegos J.F.M., Requena A.R., López A.I.G., (2006). Thermal deactivation of a commercial α-amylase from Bacillus licheniformis used in detergents, *Biochemical Engineering Journal*, 27, 299-304.

Saxena R.K., Dutt K., Agarwal L., Nayyar P., (2007). A highly thermostable and alkaline amylase from a Bacillus sp. PN5, *Biosource Technology*, 98, 260-265.

Sellami-Kamoun A., Haddar A., Ali N.E.-H., Ghorbel-Frikha B., Kanoun S., Nasri M., (2008). Stability of thermostable alkaline protease from Bacillus licheniformis RP1 in commercial solid laundry detergent formulations, *Microbiological Research*, 163, 299-306.

Sharma A., Satyanarayana T., (2011). Optimization of medium components and cultural variables for enhanced production of acidic high maltose-forming and Ca2+-independent α-amylase by *Bacillus acidicola*, *Journal of Bioscience and Bioengineering*, 111, 550-553.

Stamford T., Stamford N., Coelho L., Araujo J., (2001). Production and characterization of a thermostable α-amylase from *Nocardiopsis* sp. endophyte of yam bean, *Biosource Technology*, 76, 137-141.

Subba Rao C., Madhavendra S., Sreenivas Rao R., Hobbs P.J., Prakasham R., (2008). Studies on Improving the Immobilized Bead Reusability and Alkaline Protease Production by Isolated Immobilized *Bacillus circulans* (MTCC 6811) Using Overall Evaluation Criteria, *Applied Biochemistry and Biotechnology*, 150, 65-83.

Swati J., Satyanarayana T., (2013). Characteristics and applications of a recombinant alkaline serine protease from a novel bacterium *Bacillus lehensis*, *Biosource Technology*, 131, 76-85.

Ul-Haq I., Ashraf H., Iqbal J., Qadeer M.A., (2003). Production of alpha amylase by *Bacillus licheniformis*

using an economical medium, *Biosource Technology*, 87, 57-61.

Wind, R., Buitelaar, R., Eggink, G., Huizing, H., Dijkhuizen, L., (1994). Characterization of a new *Bacillus stearothermophilus* isolate: a highly thermostable α-amylase-producing strain, *Applied Microbiology and Biotechnology*, 41, 155-162.