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THERMOPHILIC *BACILLUS* LICHENIFORMIS RBS 5 ISOLATED FROM HOT TUNISIAN SPRING CO-PRODUCING ALKALINE AND THERMOSTABLE α-AMYLASE AND PROTEASE ENZYMES

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ABSTRACT

Bacillus licheniformis RBS 5 was isolated from thermal spring in Tunisia. The isolate coproduce α-amylase and protease enzymes. The α-amylase activity showed an optimal activity at approximately 65°C and in wide pH interval ranging from 4 to 9. This enzyme was stable over the range of 45 to 70°C after 30 min of incubation and in the pH range of 8 to 10. Protease activity was optimal; at 80°C, pH 12. This enzyme was stable until 60°C over the pH range of 10 to 12. EDTA at concentration of 5 mM reduces slightly both activities evoking the serine alkaline protease. Cationic ions (Ca^{2+}, Cu^{2+}, Zn^{2+}, and Mg^{2+}) have an inhibition effect on α-amylase. However, protease activity was enhanced by Ca^{2+}, Cu^{2+} and Mg^{2+}); the other cations reduce slightly the proteolytic activity. SDS and H_2O_2 were found as inhibitors for both activities whereas Triton X-100 and perfume have no effect. Taken together, these traits make protease activity of *B. licheniformis* RBS 5 as efficient for use in detergent industry.

Keywords: Thermophilic *Bacillus licheniformis*, α-amylase, protease, detergent additive

INTRODUCTION

α-Amylases (EC 3.2.1.1) or α (1-4) D-glucan-4-glucanhydrolase are endohydrolases, that specifically cleave the glycosidic bonds in starch to produce glucose, maltose, or dextrins, and glucoamylase (EC 3.2.1.3). They cut α (1-4) and α (1-6)-glycosidic linkages, releasing glucose from the non-reducing ends of starch, they are widely used in the industrial conversion of starch into sugars **(Xiao *et al.*, 2006)**. Amylases have a significant commercial importance and they occupy ca. 25% of the world enzyme market **(Burhan *et al.*, 2008)**. These enzymes have numerous applications in various industrial processes including the nutritional, textiles **(Gupta *et al.*, 2003)** and starch industry **(Prakash *et al.*, 2009)**. Thermostable α-amylases have been isolated from diversified sources including plants, animals, and microbes, where they play a dominant role in carbohydrate metabolism. However, microbial sources, namely fungal and bacterial, are used for the industrial production due to advantages such as cost effectiveness, consistency, less time and space required for production, and ease of process modification and optimization. Proteases play a crucial role in physiology and pathology **(Qiao *et al.*, 1997; Hedstrom, 2002)**. Microbial proteases account for ca. 59% of the total worldwide enzyme sales **(Wei, 2007),** they play an important role in biotechnological processes **(Sellami *et al.*, 2008)**. Proteolytic enzymes can be classified as acidic, neutral and alkaline proteases with regard to their pH working range. Neutral and alkaline proteases hold great potential for application in the detergent- **(Rao *et al.*, 1998; Banerjee *et al.*, 1999; Anwar and Saleemudin, 2000; Gupta *et al.*, 2003)**, pharmaceutical- and medical fields **(Kudrya and Simonenko, 1994)**, and in the food- **(Anwar and Saleemudin, 1998),** tannery (**Wellingta and Weire, 2003)** and leather industries **(Anwar and Saleemudin, 1998)**.
Thermophilic bacilli that grow optimally over the temperature range of 45 to 70 °C have been isolated from both thermophilic and mesophilic environments. The interest for these bacteria arises from their biotechnological importance as sources of thermostable enzymes (proteases, amylases, pullanases, glucose-isomerases, lipases, xylana-ases and DNA restriction endonucleases) and products for industrial use (**Burhan, 2008**; **Adiguzel *et al.*, 2009).**
Research on thermo- and extremophiles, as promising sources for highly stable enzymes, has remained an active research subject **(Adiguzel *et al.*, 2009)**. Thermophiles represent an obvious source of thermostable enzymes, it being reasonable to assume that such character will confer on their proteins a high thermal stability **(Szilagyi and Zavodszky, 2000)**. Enzymes isolated from these microorganisms are not only thermostable and active at high temperature, but are also often resistant to and active in the presence of organic solvents and detergents **(Saeki *et al.*, 2007)**.
During the screening for thermophilic bacteria with extracellular hydrolyses from thermal Tunisian springs, a thermophilic *Bacillus lichenifomis* isolate (strain RBS 5) that displays high thermostable protease and α-amylase was isolated and identified. In addition characterization of these protease and α-amylase were performed.

MATERIALS AND METHODS

Isolation and identification of the strain

RBS5 strain was isolated from thermal springs in Korbos, Tunisia, using specific protocol as reported previously (**Adiguzel *et al.*, 2009)**. This isolate produce clear zones on starch and/or milk nutrient agar plates. The isolate is rod-shaped and is able to grow from 55 °C to ca. 80 °C. The biochemical tests were carried out using the API 50CHB system **(Edward *et al.*, 2003)**.
The DNA from bacterial cultures was extracted using a Wizard Genomic DNA Purification Kit. The amplification of the 16S ribosomal ribonucleic acid (rRNA) gene was performed using universal primers Fd1 and Rd1 (Fd1, 5′-AGAGTTTGATCCTGGCTCAG-3′ and Rd1, 5′-AAGGAGGTGATCCAGCC-3′). PCR product of ~1.5 kb corresponding to base positions 8-1542 based on *Escherichia coli* numbering of the 16S rRNA gene **(Winker and Woese, 1991)**. The sample was placed in a hybrid thermal reactor thermocycler (BIOMetra, Leusden, The Netherlands), denatured for 1 minute at 95 °C and subjected to 30 cycles for 20 seconds at 95 °C, 30 seconds at 55 °C, and 1 minute and 30 seconds at 72 °C. This was followed by a final elongation step for 5 minutes at 72 °C. The PCR product was analyzed on 1.5% (w/v) agarose gel and sent to GATC (Germany) for sequencing. The nucleotide sequence of the 16S rRNA gene was determined using the dideoxy chain-termination method**.** The homology search was performed using the BLAST algorithm.

Culture conditions

For the screening of amylolytic activity, RBS 5 was grown on starch agar plate containing 1 g soluble starch, 0.4 g yeast extract, 0.1 g KH_2PO_4, 0.15 g Mg SO_4.7 H_2O and 15 g Agar in 1 L of distilled water at 70°C for 48 h **(Saxena *et al*., 2007)**. The bacterial isolate was inoculated on skim milk agar plates prepared with 250 ml skim milk, 3 g yeast extract, 5 g peptone, 20 g agar in 1 L of distilled water adjusted to pH 8.0 and incubated at 55 °C for 24 h (**Johnvesly and Naik, 2001)**. A clear zone of hydrolysis revealed the presence of proteolytic activities depending on the clearance and diameter zone of substrate hydrolysis.

Detection of enzyme activity by zymography

RBS 5 enzyme extract was subjected to electrophoresis (Mini Protein II Electrophoresis Cell, Bio-Rad) on 30% acrylamide gel under denaturing conditions. The SDS-PAGE procedure was completed with a 12.5% gel, following the method of **Laemmli (1970).** The gel was washed with Triton X-100 (2.5%) for one hour to remove the SDS. Electrophoresis was performed at 100 mA for 1 hour at 4 °C in 1.5 M Tris-HCl buffer at pH 8.8 **(Hmidet *et al*., 2009)**. After migration, the gel was soaked in a 1.0 M Tris-HCl buffer at pH 6.8. α-amylase and protease activities were revealed by the iodometric technique using Lugol solution to detect α-amylase and a 1% of casein solution to detect protease enzymes with the specific enzyme substrate method. Detection of protease activity was performed on polyacrylamide gel by staining for 1 H in Coomassie blue (G-250).

Enzyme assay α-amylase activity

The culture was grown at 70 °C for 72 h in a shaking incubator (150 rpm). The pH of the medium is 7.0. The broth was centrifuged at 10,000 rpm at 4 °C for 20 min and the clear supernatant was used as crude enzyme **(Saxena *et al*., 2007).** α-amylase activity was detected phenotypically: starch forms a deep blue complex with iodine **(Gupta *et al*., 2003),** and with the progressive hydrolysis of the starch, the color changes to red brown. The α-amylase activity was determinate by incubating of 0.5 mL of each enzymatic aliquot with 1% of soluble starch dissolved in 0.1M phosphate buffer (pH 6.5) at 70 °C for 15 min **(Konsoula and Liakopoulou, 2007)**. The reaction was stopped by adding 1ml of 0.1 N of HCl and the final volume was adjusted to 40 mL with distilled water **(Konsoula and Liakopoulou, 2007)**. The optical density of the precipitate was determined at 620 nm. All the experiments were performed in triplicates.

Protease enzyme assay

The Protease was produced in 1% glucose (w/v), 0.5% casein (w/v) and 0.1% $MgSO_4$.7 H_2O (w/v). The culture was grown for 72 hours at the optimum temperature for bacterial growth (**Johnvesly and Naik, 2001)**. Protease activity in the cell-free supernatant was measured; alkaline protease activity was determined by using 1% casein as a substrate in 25 mM Glycine-NaOH buffer (pH 11). The assay was carried out routinely in a mixture containing 0.5 mL of a suitably diluted enzyme with 0.95 ml of Glycine-NaOH solution and 1 ml casein solution. After 20 min incubation in the optimum temperature for bacterial growth, the reaction was terminated by the addition of 2 ml of 10% TCA (trichloroacetic acid) solution. The mixture was slightly agitated and was centrifuged at 8000 rpm for 30 min. The optical density of the precipitate was determined at 280 nm (**Johnvesly and Naik, 2001)**.

Effect of pH on the activity and stability

The optimum pH and stability of the enzymatic preparation was monitored over a pH range of 4.0 to 13.0 in different buffers and the residual enzymatic activities were determined under standard assay conditions. The following buffers were used: 100 mM glycine-HCl, pH 2.0-4.0; 100 mM Sodium acetate buffer, pH 4.0-6.0; 100 mM Tris-HCl buffer, pH 7.0-8.0; 100 mM glycine-NaOH buffer, pH 9.0-10.0; 100 mM Na_2HPO_4-NaOH buffer pH 11.0; 100 mM KCl-NaOH, pH 12-13 **(Hmidet *et al*., 2009).**

Effect of temperature on the activity and stability

To investigate the effect of temperature, enzyme activity was tested at different temperatures between 40 to 100 °C at pH 12.0 for protease activity and at pH 10.0 for α-amylase activity. Thermal stability was examined by incubating the enzyme preparation at temperature of 30 min **(Hmidet *et al*., 2009)**. The remaining enzyme activities were measured under standard assay conditions. Non-heated crude enzyme was used as a control (100%).

Effects of inhibitors and metal ions on enzymes activities

The effects of enzyme inhibitors on α-amylase and protease activities were studied using Ethylenediaminetetra acetic acid (EDTA), and β-mercaptoethanol. The RBS 5 crude enzyme was pre-incubated with inhibitors for 30 min. The remaining α-amylase and protease activities were determined under standard assay conditions. The activity of the crude enzyme assayed in the absence of inhibitors was taken as 100% **(Hmidet *et al*., 2009)**. Effects of various metal ions (at 2 and 5 mM concentrations) on α-amylase and protease activities were investigated by adding divalent metal ions (Zn^{2+}, Ba^{2+}, Ca^{2+}, Hg^{2+}, Cu^{2+} et Mg^{2+}) to the reaction mixture. The activity of the crude enzyme without metallic ions was taken as 100%.

Effect of surfactants and detergents on enzyme stability

The suitability of RBS 5 crude enzyme for use as a detergent additive was tested by studying its stability in the presence of several surfactants such as SDS (sodium dodecyl sulphate), Triton X-100, and oxidizing agents such as hydrogen peroxide (H_2O_2) and perfume. The Crude enzyme was incubated with different additives for 1 hour at 40 °C **(Hmidet *et al*., 2009)**, then the residual enzyme activities were determined under standard assay conditions.

Stability of proteolytic crude extract of RBS 5 with commercial detergents powder

The suitability of RBS 5 crude extract to use in commercial laundry detergents was also studied. The solid detergents tested were Dixan (Henkel, Spain), Nadhif (Henkel, Tunisia) and Ariel (Switzerland). Solid detergents were diluted in tap water to give a final concentration of 7 mg/ml. The endogenous enzymes contained in these detergents were inactivated by heating the diluted detergents for 30 min at 80 °C. The crude enzyme were added to solid detergents diluted in tap water and incubated in various detergent solutions for 1 hour at different temperatures; the residual enzyme activities were determined under standard assay conditions. The stability towards commercial detergents was described with two commercial detergent enzymes under the same conditions as with the RBS 5 enzymes. The enzyme activity of the control sample (without any detergent), incubated under the same conditions, was taken as 100% **(Hmidet *et al*., 2009)**.

RESULTS

Identification of RBS 5 Strain

RBS 5 strain was isolated from thermal springs in Korbos, Tunisia. The identification of the isolate was based on both catabolic and molecular methods. Morphological and physiological characteristics showed that RBS 5 belong to the *Bacillus licheniformis*. It was a spore-forming and Gram-positive bacterium, it was rod-shaped, and it was able to grow from 50 °C to 80 °C.
The amplification of the 16S ribosomal ribonucleic acid (rRNA) gene using universal primers Fd1 and Rd1 (Fd1, 5′-AGAGTTTGATCCTGGCTCAG-3′ and Rd1, 5′-AAGGAGGTGATCCAGCC-3′) designed from conserved regions within the rRNA operon of E. coli was performed. The sequence showed a similarity of 99% with the 16S rRNA gene of *Bacillus licheniformis* strain NBSL59 (accession number JN624930). Based on the obtained data, RBS5 seemed to be closely related to the *B. licheniformis* species. Therefore, we proposed the alignment of this organism as the *B. licheniformis* RBS5 strain.

Amylolytic and proteolytic activities produced by RBS 5

Enzymatic activities produced by RBS5 were detected on polyacrylamide gels. α-amylase activity appeared on SDS-PAGE gels as unique clear band, indicating that the isolate harbored only one α-amylase enzyme (Figure 1). Similarly, protease activity was detected also as a single band (Figure 1).

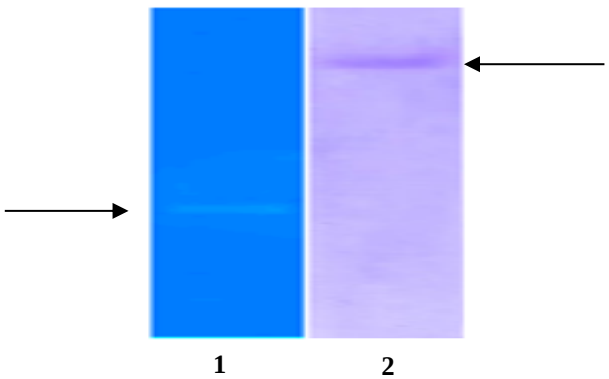


Figure 1 Zymogram showing proteolytic (1) and amylolytic (2) activities of *B. licheniformis* RBS5

Effect of pH on α-amylase and protease activities and stability

The effect of pH on proteolytic and amylolytic activities of RBS 5 crude enzyme was determined over a pH range from 4.0 to 13.0. The general activity of α-amylase was at the pH range from 4 to 9 (Figure 2). Concerning α-amylase stability, the activity was highly stable in the pH range 8.0 to 10.0 retaining more than 95% of its initial activity (data not shown) and decreased till 30% at pH 11. The RBS 5 crude extract protease was active over a broad pH range of 3.0 to 13.0 with optimal activity at pH 11.0 (Figure 2). The crude enzyme retained 100% of its proteolytic activity between pH 10.0 and 12.0, and ca. 50% of its activity at pH 13.0 (data not shown).

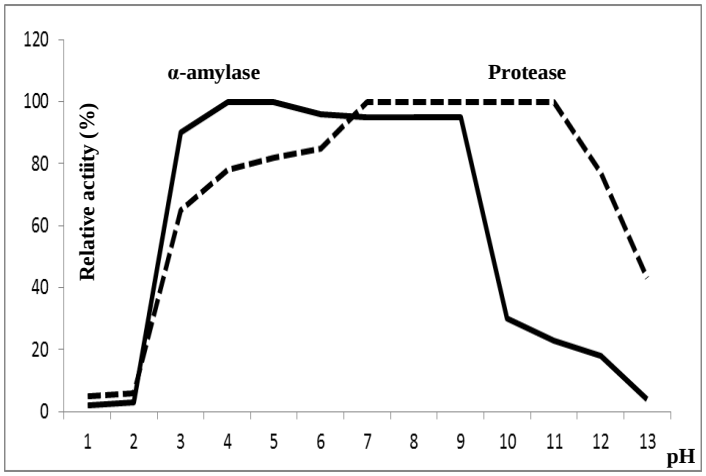


Figure 2 Effect of pH on enzyme activities of *B. licheniformis* RBS 5.

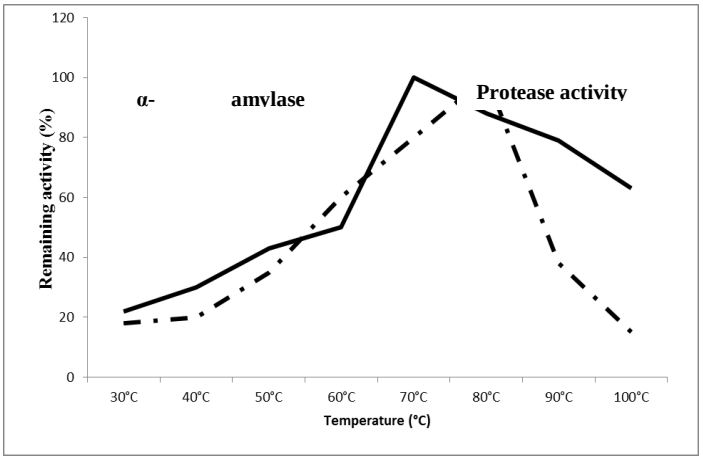
Effect of Temperature on the activity and stability of α-amylase and protease

α-amylase activity was essayed at different temperature and was active between 45 °C to 80 °C with an optimum at ca. 65 °C. For the proteases enzyme, activity increased with temperature within the range of 60-95 °C, and had optimum activity at 80 °C (Figure 4A and 4B).
The Effect of temperature on enzyme stability was studied. α- amylase retained its full activity at temperatures between 45 and 70 °C after incubation for 30 min. However, for higher temperatures, the α-amylase activity showed clear decrement and retained only 40 % of its initial activity at 80 °C. For the protease activity, the initial full activity was conserved until 60 °C during 30 min of incubation, moreover, this activity shutdown to 10% at 95 °C.

Effects of various enzyme inhibitors and metal ions on α-amylase and protease activities

The effects of various enzyme, inhibitors, were studied and are reported in Table 1**.** The amylolytic activity was slightly inhibited with 2 mM of EDTA, however, with 5 mM EDTA the retained activity was 10%. The effect of EDTA on protease activity was concentration dependent, being without effect by using 2 mM and caused 40 % - activity loss by 5 mM of EDTA. The β-mercaptoethanol showed a slightly inhibition of activity for both protease and amylase activities being 87% and 92, respectively. The effects of various cations at a concentration of 5mM on protease and α-amylase activities were studied at optimal activity conditions (Tab 2). The α-amylase activity was clearly inhibited by Ca^{2+}, Zn^{2+}, Mg^{2+}, Hg^{2+}, and Ba^{2+}, and the retained activity varried between 11% and 27%. The Cu^{2+} also exhibited inhibition effect, less than the before mentioned cations. Protease activity increased in the presence of Ca^{2+}, Cu^{2+}, Mg^{2+} by 120%, 103% and 106%, respectively. A slight inhibition was observed by using Hg^{2+}, Zn^{2+} and Ba^{2+} cations (Tab 2).

A



B

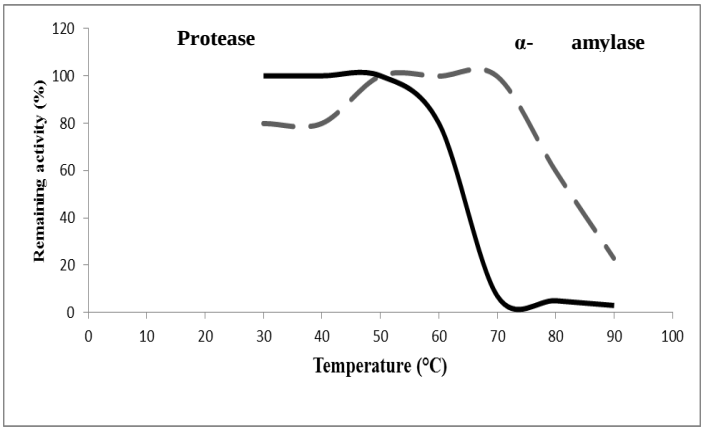


Figure 3 Effect of temperature on enzyme activities (A) and stability (B) of RBS 5.

Table 1 Effect of enzyme inhibitors on α-amylase and protease activities of RBS 5

Inhibitors	Concentrations (mM)	Relative activity	
		α-amylase	Protease
None	0	100	100
EDTA	2	80	100
	5	10	60
β-mercaptoethanol	2	92	87

Table 2 Effect of some metal ions (5mM) on α-amylase and protease activities of RBS 5

Metal ions (5 mM)	Residual activity (%)	
	α-amylase	Protease
None	100	100
Ca^{2+}	21	120
Cu^{2+}	61	103
Zn^{2+}	13	67
Mg^{2+}	11	106
Hg^{2+}	19	92
Ba^{2+}	27	90

Effect of surfactants and oxidizing agents on α-amylase and protease stability

The effect of surfactants and oxidizing agents were assayed under standard conditions (Tab 3). Both activities were highly stable in the presence of non-ionic surfactants, retaining 100% of the initial activities in the presence of 1mM Triton X-100 after 30 min of incubation at 60 °C. The assay of SDS at 5 mM caused a slight inhibition for the α-amylase and strong inhibition of protease. However, 1 mM of SDS has low inhibition effect in α-amylase and protease activities retaining ca. 94% and 93% of the initial amylolytic and proteolytic activities, respectively. In addition, we investigated the effect of the oxidizing agent hydrogen peroxide (H_2O_2) on the stability of RBS 5 enzymes after pre-incubation for 30 min at 60 °C. As shown in Table 3**,** both amylolytic and proteolytic

activities were significatively inhibited by H_2O_2. The α-amylase and protease enzymes retained 63% and 61% of its initial activities, respectively. Furthermore, a local commercialized perfume used at 1 (v/v) concentration does not affect proteolytic and amylolytic activities of RBS 5.

Table 3 Stability of α-amylase and protease of *B. licheniformis* RBS 5 in the presence of various components.

components		Concentration	Residual activities of RBS 5 (%)	
			α-amylase	Protease
	None	0	100	100
	SDS	1 (w/v)	94	93
Surfactants	SDS	5 (w/v)	82	14
	Triton X-100	1 (v/v)	100	100
Oxidizing agents	H_2O_2	0.5 (v/v)	63	61
Anti-redeposition agents	Local commercialized Perfume	1 (v/v)	100	100

Stability of proteolytic crude extract with commercial detergents powder

In order to check the suitability of the enzyme preparation with detergents powder, the crude enzyme was incubated for 1 h at 40 and 50 °C with various commercial laundry detergents. The data presented in Figure 4 showed that, RBS 5 crude enzyme was extremely stable towards detergents Ariel powder tested at 40 °C and 50 °C that retained 100% of its initial activity, After 1 hour of incubation at 40 °C more than 83% of activity with Dixan was conserved and 70% with Nadhif. Interestingly, at 50 °C, crude enzyme mixed with Ariel retained 99% and only 20% with Dixan and 14% of its activity in the presence of Nadhif. Enzyme activities of control samples without any detergent, incubated under the similar conditions, were not affected.

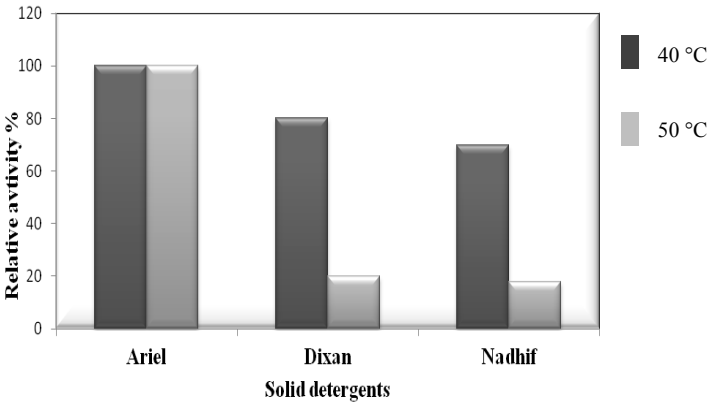


Figure 4 Stability of the protease activity in the presence of various commercial powder detergents (Ariel, Dixan and Nadhif).

DISCUSSION

In this work, we report the characterization of thermostable α-amylase and alkaline protease produced by *Bacillus licheniformis* RBS 5 strain isolated from hot Tunisian springs (korbos). The *B. licheniformis* RBS 5 was shown to co-produce thermostable α-amylase and protease. On SDS-PAGE, both activities were subsequently detected as a single band for each one suggesting the presence of two separate extracellular enzymes α-amylase and protease. Many authors have reported the occurrence of one α-amylase and more than one protease that were found in *B. licheniformis* isolate **(Akel *et al*., 2009; Hmidet *et al*., 2009).** Similarly to our finding, other reported production of unique α-amylase in thermophilic *Bacillus* sp. strain TS-23 **(Chi *et al*., 2010**) and unique protease that was produced by *Bacillus.* sp PS719 **(Nongporn *et al*., 1999)**. Both pH and temperature had the great influence on the enzymatic activities and their stability. RBS 5 coproduces two enzymatic activities: i) an α-amylase activity with an optimum production at 65 °C, and the activity remaining until 80 °C to produce 40% and ii) a protease activity with an optimum at 80° C that is still active at 95 °C conserving 10% of proteolytic activity. Similar results were reported for *Bacillus* species: *B. ferdowsicous* **(Konsoula and Liakopoulou, 2007)**, *Bacillus* sp. JB-99 (**Johnvesly and Naik, 2001)** and *B. licheniformis* NH 1 **(Hmidet *et al*., 2009**). High activity of RBS 5 α-amylase was reached at pH 4 and maintained in large pH interval, being 4 to 9. Similarly, *B. subtilis* JS-2004 has been reported to have an optimum activity at pH 8 **(Asgher *et al*., 2000)** and for *B. licheniformis* MIR 29 it was up to 9 **(Ferrero *et al*., 1996)**. Amylase production reported for *B. licheniformis* Shahed-07 **(Rasooli *et al*., 2008)** and *B. thermooleovorans* NP54 **(Malhotra *et al*., 2000)** was strictly at neutral pH. Protease produced by *B. licheniformis* RBS 5 produce higher activity at pH 11. The crude enzyme retained 100% of its proteolytic activity between pH 10.0 and 12.0, and ca. 50% of its activity at pH 13.0. Alkaline protease was reported from B. *subtilis* BP-36 with a pH range of stability 9.0 to 11.0 and an optimum pH at 9.0 **(Mashayekhi *et al*., 2012)** and at pH 10 for *B. sphaericus* **(Mourad *et al*., 2011)**. Several investigators have reported the production of alkaline proteases over this pH range **(Singh *et al*., 1999**; **Johnvesly and Naik, 2001)**. It is important to obtain enzymes with high stabilities and activities at alkaline pHs and at high temperatures **(Wellingta and Meire, 2003)**, which are established in industrial scale **(Pernilla *et al*., 2007)**. Today a large number of microbial α-amylases and protease are marketed with applications in different industrial sectors and have a biotechnological perspective **(Gupta *et al*., 2003)**.

Effects of various enzyme inhibitors and metal ions on protease and α-amylase activities were investigated in this work. The addition of EDTA and β-mercaptoethanol at a concentration of 2 mM kept the protease activity and only 80% of activity was retained for α-amylase. However at 5 mM, only 10% and 60% of α-amylase and protease activities were retained, respectively, indicating the no requirement for metal cofactor. The stability of the enzyme in presence of EDTA is advantageous for use of enzyme as detergent additive **(Akel *et al*., 2009)**. It might be due to detergents that contain high amount of chelating agents, which function as water softeners and also assist in stain removal. These agents specifically bind to, and chelate metal ions making them unavailable in the detergent solution **(Beg and Gupta, 2003)**. β-mercaptoethanol is a reducing agent that affected slightly α-amylase activity; similar results was obtained by **Azad *et al*., 2009**; Stability with β-mercaptoethanol brings change in the conformation of the enzyme in the active site by breaking disulfide bonds, results in loss of enzyme activity. This indicates that the disulfide bonds play an important role in maintaining the structure of α-amylase **(Pernilla *et al*., 2007)**. β- mercaptoethanol decrease protease activity, that suggests the serine alkaline nature of our protease enzyme **(Anupama and Jayaraman, 2011).**

The effect of metal ions on α-amylase activity showed that *B. licheniformis* RBS 5 enzyme extract was strongly inhibited by Zn^{2+}, Mg^{2+}, Hg^{2+} and Ca^{2+} with residual activities values of 13%, 11%, 19% and 27% respectively. In the presence of Cu^{2+}, the activity was not very influenced, it was 61%. These findings are quite similar in part to what had been observed with *Bacillus ferdowsicous* **(Asooodeh *et al*., 2010)**. Protease activity was weakly affected by the presence of metal ions, with the exception of Zn^{2+} which inhibited the activity to 67% relative to total activity. Similar results have been reported for *Bacillus stearothermophilus* **(Jae *et al*., 2002)**. Most alkaline proteases are significantly stabilized by the addition of Ca^{2+} **(Gupta *et al*., 2003)**. The improvement of activity in the presence of $CaCl_2$ solution can be explained by Ca^{2+} binding at the catalytic site **(Lee and Jang, 2001)**. It has been reported that Ca^{2+} binding is a metal ion protects the enzyme against thermal denaturation and that it plays a vital role in stabilizing the conformation of the enzyme at high temperatures **(Wellingta and Meire, 2003)**.

We have studied the influence of surfactants, oxidizing agents and local commercialized perfume on the α-amylase and protease activities. Triton X-100, SDS and 1% of perfume kept nearly the total original activity. The protease was stable and remained active in presence of 1% of SDS.The same result has been reported for *B. mojavensis*, *Bacillus*. sp. RGR-14 and *Bacillus*. sp. KSM-KP 43 **(Gupta *et al*., 2003; Saeki *et al*., 2007)**. A 0.5% of H_2O_2, reduced the α-amylase and protease activities to 63 and 61% of the initial activities, respectively.

To assess the stability of alkaline protease and the suitability for use in commercial detergents, RBS 5 proteolytic crude extract was incubated for 1 hour at 40 °C and 50 °C, in the presence of various detergents marketed in Tunisia (Ariel, Dixan and Nadhif). The results indicated that the enzyme extract showed high stability when it was mixed with various commercial detergents powder at a concentration of 7 mg/mL at 40 °C. RBS 5 alkaline protease showed a higher stability with Ariel (100% residual activity) comparing to Dixan and Nadhif (80% and 60% of residual activity) respectively, when similar results have been reported for *B. licheniformis* RP1 and *B. mojavensis* A21 with excellent stabilities obtained with a range of commercial detergents powder at a concentration of 0.7 g/L **(Sellami Kammoun *et al*., 2008; Haddar *et al*., 2009; Jellouli *et al*., 2011)**. At 50 °C, proteolytic crude extract was not stable and activity was lost to reach 20% and 18% respectively, for Dixan and Nadhif but was stable for Ariel.

CONCLUSION

The α-amylase and protease activities produced by *B .licheniformis* RBS 5 isolate, were stable over a wide range of pH and temperature. In addition, the crude extract showed compatibility with various commercial detergents tested, it enhanced washing performance of the detergent.

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