Purification and characterization of a new glucoamylopullulanase from thermotolerant alkaliphilic *Bacillus subtilis* DR8806 of a hot mineral spring

Ahmad Asoodeh [a,b,*], Milad Lagzian [a,c]

[a] *Cellular and Molecular Research Group, Institute of Biotechnology, Ferdowsi University of Mashhad, Mashhad, Iran*
[b] *Departments of Chemistry, Faculty of Science, Ferdowsi University of Mashhad, Mashhad, Iran*
[c] *Biotechnology Division, Faculty of Veterinary Medicine, Ferdowsi University of Mashhad, Mashhad, Iran*

ARTICLE INFO

ABSTRACT

We have introduced a novel glucoamylopullulanase from thermostable alkaliphilic *Bacillus subtilis* DR8806 from a hot mineral spring in Iran. The enzyme was purified by ion-exchange chromatography following to ammonium sulphate precipitation. The molecular weight of the purified enzyme was estimated to be 65.5 kDa using denaturing acrylamide gel electrophoresis. The enzyme showed high activity over a wide pH range, from pH 5.0 to pH 11.0 with the optimum pH 9.5. Our results also indicated an optimum temperature of the enzyme activity at 70 °C. These features justify the characteristics of the alkaliphilic and thermostable bacterial proteins and enzymes. The enzyme did not require calcium and showed extreme stability with regard to surfactants, including SDS and Triton X-100, and oxidizing agents such as H_2O_2. These features of the enzyme suggest a promising potential for application in laundry industry. Furthermore, the enzyme was active on pullulan by 68% relative to normal activity on starch. Such characteristics have not already been reported for this type of enzyme, hence we propose that this is a new alkalophilic and thermostable enzyme.

© 2012 Elsevier Ltd. All rights reserved.

1. Introduction

Nowadays, enzymes play a crucial role in the industries, particularly food and laundry industries as well as waste management, mining and even petroleum. Enzymes that are due to be used in these industries should have some special characteristics that may not be possible to be found in their traditional counterparts, for example, a higher thermostability, enhanced activity, and extreme pH profile. In addition they should be resistant to general enzyme inhibitors and independent of the metal ions or bear retain their activity toward oxidizing agents and organic reagents [1]. Though an enzyme might show all or some of the such features.

Unicellular microorganisms, especially bacteria, are suitable sources of the broad range of the industrial enzymes [2]. Among enzymes, hydrolases composed of EC 3 have of prime importance. The 3 most important groups of hydrolases are proteases, lipases and glycosidases [3].

Glycosidases are enzymes, which act on the glycosidic bonds in glycogen, cellulose, starch as well as disaccharides. Among glycosidases, those which act on starch commonly are known as amylases. Amylases are preferentially used in the food and laundry industries [1]. Among amylases those which retain their activity in the alkaline pH are used in the formulation of laundry powders. However, such amylases should meet other criteria, especially the proper activity in the oxidizing environment and detergents such as SDS or bleaching agents. Another important requirement for an enzyme used in the industry, particularly food industry, is metal ion-independency; as it is true for most amylases, which are used in food industry, which need calcium for their optimum activity and thermostability [4]. Since removal of calcium is required for food processing, it demands a further process as well as increasing the cost of final product in the market, a calcium independency is an advantage for amylases. This specificity is important in laundry industries because calcium is one of the culprits in water hardness. Only a limited number of amylases comply with the required properties for application in the industry. Due to this necessity, identifying new amylases is an ongoing investigation by many research groups [1].

This study describes the purification and characterization of an exo-acting glucoamylase with significant pullulanase activity

* Corresponding author at: Cellular and Molecular Research Group, Institute of Biotechnology, Ferdowsi University of Mashhad, Mashhad, Iran.
Tel.: +98 511 8795457; fax: +98 511 8795457.
E-mail address: Asoodeh@um.ac.ir (A. Asoodeh).

from *Bacillus subtilis* DR8806 isolated from Dig Rostam hot mineral spring in Kerman-Iran. The enzyme is a calcium independent, thermo-tolerant and alkaliphilic glucoamylase as indicated by fine activity in the high pH ranges. In addition, the enzyme shows proper activity in the presence of oxidizing agents and some detergents. These features along with the isolation location of the microorganism indicate that this protein could be considered as a novel amylolytic enzyme.

2. Materials and methods

2.1. Materials

All culture mediums and supplements were provided by Merck & Himedia (Merck, USA & Himedia Laboratories, India). PCR and DNA extraction reagents were bought from Qiagen (Qiagen, USA), DNS (3,5-dinitrosalicylic acid), maltooligosaccharide, TLC plate, starch, amylose and amylopectin, were obtained from Sigma (Sigma–Aldrich, USA). Q-Sepharose was purchased from Pharmacia (Pfizer and Pharmacia, Sweden). All other used chemicals were of analytical grade.

2.2. Isolation of microorganism

The strain microorganisms were collected from Dig Rostam hot mineral spring in the southeast of Iran under the sterile condition. Samples were grown on the super LB medium at 60 °C and 180 rpm on a rotary shaker incubator for 48 h and subsequently subcultured on the LB agar plate to isolate single colonies. All mediums were prepared with the spring's water to simulate the native living conditions of the microorganisms. A total three different colonies were detected and screened for α-amylase activity on the 1% starch agar plate supplemented with 1% (w/v) peptone, 2% (w/v) yeast extract and 0.2% (w/v) NaCl. The positive colony, which left a white halo-zone pattern, was selected by staining with Lugol's solution. Finally, one colony confirmed as α-amylase producer strain.

2.3. Microorganism identification

For microorganism identification and classification, biochemical assays carried out according to "Bergey's Manual of Determinative Bacteriology" [5]. Along with these assays, molecular identification of the microorganism was also done by extraction, amplifying and sequencing 16s rDNA of the isolate. Total genomic DNA extraction was accomplished according to Sambrook et al. [6]. For amplification, a pair of universal primers was used as such the forward primer was composed of; 5′-AGTTTGATCCTGGCTCAG-3′ and the backward; 5′-GGCTTACCTTGTTACGACTT-3′. The PCR conditions as were described previously [7]. The amplified segment was sequenced, refined and evolutionarily analyzed using CLC Main Workbench ver.5.7.2 (CLCBio, Denmark) and then submitted to GenBank.

2.4. Optimization of microorganism growth and enzyme production

In order to choose the best supporting conditions for growth of the isolate and enzyme production, several media were studied [8] according to the following schemes; (all g/l): *medium #1*: Beef heart infusion, 500.0 g; soluble starch, 20.0 g; tryptose, 10.0 g; NaCl, 5.0 g; *medium #2*: white soybeans, 100.0 g; starch, 20.0 g; *medium #3*: glycerol, 20.0 g; L-glutamic acid, 4.0 g; citric acid, 2.0 g; K$_2$HPO$_4$, 0.5 g; starch, 20.0 g; ferric ammonium citrate, 0.5 g; MgSO4, 0.5 g; *medium #4*: bacteriological peptone, 60.0 g; MgSO$_4$, 5.0 g; KCl, 5.0 g; starch, 20.0 g; *medium #5*: nutrient broth, 32.0 g; fructose, 10.0 g; peptone, 10.0 g; starch, 20.0 g. In addition different conditions for temperature (37 °C, 45 °C, 55 °C, 60 °C) and pHs (4, 7, 10) were optimized and analyzed for the evaluation of each medium. In all experiments, LB broth supplemented with 2% potato starch was used as a supporting medium.

2.5. Enzyme purification

A 500 ml cultured sample medium was centrifuged at $10,000 \times g$ for 10 min. Ammonium sulfate (85% of saturation) was directly added to the obtained supernatant and the mixture was kept in the cold room at 4 °C for 24 h. Afterward, centrifugation at $15,000 \times g$ was done for 20 min and enzyme activity assay was carried out. Since the assay showed no detectable enzyme activity present in the supernatant fraction the subsequent purification steps were done on the precipitated material. The precipitated material was dissolved in 25 mM phosphate buffer (pH 6) and dialyzed at 4 °C against the same buffer for 24 h. Subsequently, the solution was applied onto a "HiTrap Q-Sepharose Fast Flow™" column (3 cm × 15 cm) pre-equilibrated with the above buffer and washed with three column volumes to remove unbound proteins. Elution of the bound proteins was done using a gradient of the same buffer containing 0.1–1 M NaCl at a flow rate of 1 ml/min. Fractions containing protein (absorbance at 280 nm)

were analyzed for the enzyme activity. Positive fractions were dialyzed against 25 mM Tris–HCl pH 8. The second chromatography separation was performed again at the second buffer to obtain a pure active enzyme. The active fractions was concentrated using an Amicon ultrafiltration apparatus equipped with 10 kDa NMWL Ultrafiltration Discs (Millipore, USA), and then again applied to the chromatography column to collect active enzyme. In the third step. The buffer pH of the column was decreased from 8 to 7.5 to obtain a single peak in the chromatogram. The active enzyme peak was pooled and lyophilized for long storage. Quatification of the protein concentration was performed in all purification steps by the method of Bradford [10] using bovine serum albumin as standard.

2.6. Amylase activity assay

Amylase activity was assessed with DNS according to Bernfeld [9] using 1% starch dissolved in a 50 mM Tris buffer pH 9.5 at 70 °C (standard assay conditions). One unit of amylase activity was defined as the amount of the enzyme that released 1 μM/min of reducing sugar at 70 °C in the standard assay conditions. The amount of produced reducing end sugar was compared with D-maltose as standard of the reaction.

2.7. Gel electrophoresis and zymography

In order to evaluate the quality of purification and the molecular weight of the purified enzyme, denaturing polyacrylamide gel electrophoresis (SDS-PAGE) was used according to Simson [11] on a 5% (w/v) stacking gel and 12% (w/v) resolving gel. The gel condition of native gel electrophoresis (PAGE) was as above without SDS and stacking gel, in a 10% gel, including 1% starch in order to detect enzyme activity. The sample was mixed with β-mercaptoethanol free 4× loading buffer without heating. Electrophoresis was done at 100 V at room temperature until. Subsequently, Lugol's solution was used for detection of amylase activity in gel. A colorless area in the dark blue background is an indicator of starch digestion and active amylase.

2.8. Isoelectric focusing

Isoelectric focusing (IEF) was performed by focusing on PhastGel™ p*I* 3–9 (GE Healthcare Life Sciences) to calculate the isoelectric point of the purified enzyme. The gel was stained with PageSilver™ (Fermentas Life Science, USA) all in accordance with the manufacturer's recommendation.

2.9. Effect of pH on the enzyme activity and stability

To study the behavior of enzyme with respect to pH, four different buffer systems, which covered a broad range of pH from 3 to 11 were used. The buffering systems were as follows: 50 mM sodium acetate/acetic acid for pH 3–5.5; 50 mM dipotassium hydrogen phosphate/potassium dihydrogen orthophospate for pH 6–7.5; 50 mM Tris/hydrochloric acid pH 8–9.5 and 50 mM sodium bicarbonate/sodium hydroxide for pH 10–11.

In order to analyze the pH profile, the enzyme was incubated 5 min at 42 °C with 1% starch dissolved in each of the above-mentioned buffers. Subsequently, the reaction was terminated by the addition of 1 ml DNS and relative activity was determined by measuring the absorbance at 540 nm. BLA (*B. licheniformis* α-amylase) was used as the standard of the reaction.

In order to determine pH stability, the enzyme was dialyzed against each pH solution (from 3 to 11 with 0.5 unit intervals) for 3 h at 4 °C. Assuming 100% activity for the enzyme at the start of the reaction, the residual activity was determined in the standard assay conditions. Data was compared against BLA as standard of the reaction.

2.10. Temperature effects on the enzyme activity and stability

The activity of enzyme in response to temperature was studied at 7 different applied temperatures from 30 to 90 °C, at 10 degree intervals. The purified enzyme was incubated with 1% starch in the optimum pH at each temperature for 5 min, afterwards the enzyme activity was determined in standard assay condition as described above. Activity of the enzyme at the start of the reaction was considered as 100% activity.

Thermal stability was studied by incubating the enzyme at different temperatures from 30 to 90 °C for 60 min and then added 1% starch in the optimum pH to the reaction medium following by 5 min incubation at the optimum temperature for enzyme activity. Finally, the residual activity of the purified enzyme was calculated in the standard assay condition. The activity of the enzyme in initiation of the reaction was considered as 100% activity. Thermal stability was also studied in the presence of CaCl$_2$ and then compared to CaCl$_2$ free condition to determine whether the enzyme thermostability is dependent on CaCl$_2$ or not.

The half-life of the purified enzyme was calculated at different temperatures as follows: the enzyme was incubated for 180 min at each temperature; samples were

withdrawn at 30 min intervals and assayed for the residual activity as described above. In all experiments, BLA was used as the reference sample.

2.11. Enzyme–ligand interactions

The effect of Ag^{2+}, Al^{3+}, Ba^{2+}, Co^{2+}, Cu^{2+}, Fe^{2+}, Ca^{2+}, Mg^{2+}, Mn^{2+}, Li^+, Zn^{2+} and Na^+ metal ions on the enzyme activity was investigated. Metal ions were incorporated separately in the standard enzyme assay mixture at 5 and 10 mM concentrations and left for 5 min to react; subsequently, the enzyme activity was assessed. The impact of enzyme inhibitors on the enzyme activity was also studied using 5 mM dodecyltrimethylammonium bromide (DTAB), 5 mM [5,5′-dithiobis-(2-nitrobenzoic acid) (DTNB)], 5 mM p-hydroxymercuribenzoate, 10 mM phenylmethylsulfonyl fluoride (PMSF), β-mercaptoethanol and ethylenediaminetetraacetic acid (EDTA). In addition, the effects of chemicals on the enzyme activity were studied using 1 M H_2O_2 as oxidizing agent plus 1% Trition X-100 and 10% SDS as detergents. The purified enzyme was pre-incubated with these agents at 42 °C for 60 min. Afterwards, the residual activity was measured using 1% starch in the standard enzyme assay condition. The activity of the enzyme in the absence of additive was considered as 100% activity.

2.12. Thin layer chromatography

For determining the final product of enzymatic hydrolysis of starch, thin layer chromatography was performed according to Zhang et al. [12] with minor modifications. The purified enzyme was incubated with 1% starch in the standard assay condition for 24 h. Samples were taken at 1/2 h, 1 h, 2 h, 4 h, 6 h and 24 h intervals following the start of reaction and applied onto a precoated silica gel plate (Merck 60 HPTLC plate, Darmstadt, Germany) and running was done using n-butanol/acetic acid/water, 5:8:2 (v/v) as buffer. Spots were developed by immersing the plate in a developing solution containing 1 ml of 37.5% HCl, 2 ml of aniline, 10 ml of 85% H_3PO_3, and 100 ml of ethyl acetate and 2 g of diphenylamine.

2.13. Substrate specificity

To assess the activity of enzyme against different substrates, four different polymeric carbohydrates (1.0% w/v in 50 mM Tris–HCl pH 9.5), including glycogen, α/β-cyclodexterin, pulullan and maltooligosaccharide were used. Starch (1.0% w/v) in 50 mM Tris–HCl pH 9.5 was used as a reference substrate for comparing the activity of enzyme. The enzyme was mixed with each substrate at 1:9 ratios (v/v), and the assessment was performed in the standard assay conditions.

3. Results and discussion

3.1. Biochemical and molecular identification

The biochemical studies (Table 1) and comparison with Bergey's manual of Determinative Bacteriology [5] indicates that the isolate is *B. subtilis.* Searches in databases against the sequenced segment of the 16s rRNA gene, resulted to categorize the isolate in *B. subtilis* that it was in accordance to biochemical tests. The sequence was submitted to GenBank which could be identified with an accession number; JF309277 and under the name of *Bacillus subtilis* DR8806.

3.2. Microorganism growth condition optimization

pH: Alkaline pH range (around 9) was found to be the preferred pH range for the microorganism growth (Fig. 1). The values for this pH were significantly higher than two other pHs (4 and 7). The optimum growth pH point for previously identified strains of *B. subtilis* was a smaller amount of 7.5 according to the records in literatures [13–17].

Temperature: The optimum temperature for growth strain was found to be 60 °C (Fig. 2). Although the DR8806 grows well in temperatures below 60 °C, the growth behavior was not verified for temperatures above 60 °C. According to our knowledge, this optimum temperature was not previously reported for any type of *B. subtilis* strains. The recorded data for temperatures of 37 and 42 °C did not show any significant differences as compared to 50 and 60 °C. Values for bacterial growth ($OD_{600\,nm}$) were recorded during 34 h.

Mediums: Among culture mediums, the medium no. 3 was found to have a large effect on the microorganism growth and enzyme production (Fig. 3c). The medium was supplemented with citrate as
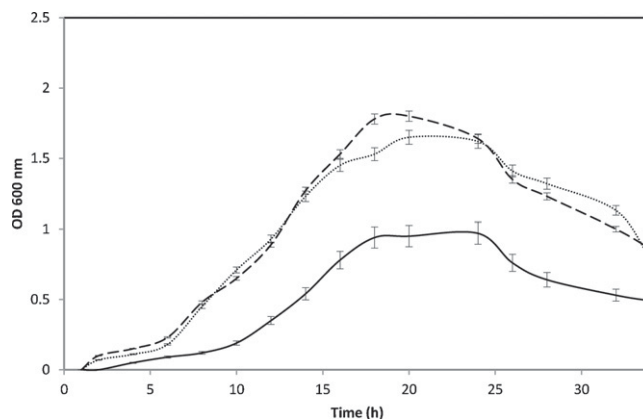

Fig. 1. Effect of pH on the growth pattern of *B. subtilis* sp. DR8806, pH 4 (—), pH 7 (•••), pH 9 (—–). The optimum pH was determined around 9. The standard errors were less than 5% of the means.

carbon source and other minerals and organic compounds, resembled to a native condition of DR8806. The medium no. 5 was found to be less suitable than the medium no. 3 for production of the enzyme (Fig. 3e). As shown in this figure, the enzyme production started with a 10-h delayed phase compared to other mediums. Our further assessments indicated that the medium no.1 and medium no. 4 (Fig. 3a and d) showed lower values for the investigated parameters. The medium no. 2 was not suitable for the growth strain and enzyme production (Fig. 3b).

3.3. Enzyme purification

The whole of culture medium (500 ml) was first precipitated using ammonium sulfate salt that enriches the enzyme to 1.1-fold. The precipitant was dialyzed and then applied onto the Q-Sepharose ion-exchange column as the first purification step. Proteins were eluted using 25 mM phosphate buffer pH 6 (buffer A) with a linear salt gradient from 0.1 to 1 M NaCl in buffer A and constant monitoring at 280 nm (Fig. 4a). Results showed that enzyme elutes in the unbound fractions along with a number of other proteins by applying the buffer A, thus the next step of ion-exchange chromatography was performed by changing pH from 6 to 8 and reapplication of the unbound fraction from the previous step for separation of the enzyme from other undesired proteins (Fig. 4b). The most active fractions were eluted at 0.35–0.4 M NaCl in buffer B (25 mM, Tris–HCl, pH 8) and then reapplied to the column to gain a single peak by decreasing the pH of buffer B from 8 to 7.5 as

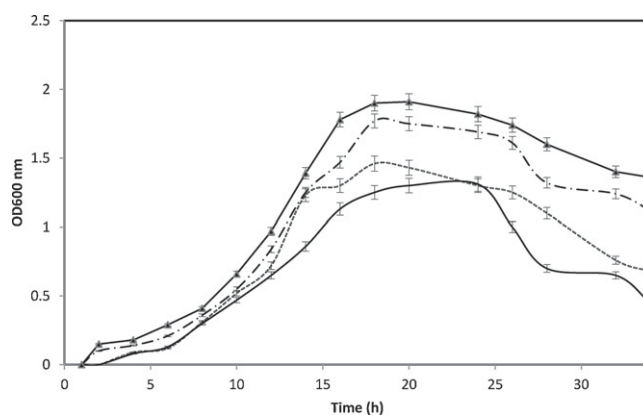

Fig. 2. Effect of different temperatures, 37 °C (—), 45 °C (—–), 55 °C (—•—), 60 °C (—▲—), on the growth rate of the microorganism. The best growth occurred at 60 °C. The standard errors were less than 5% of the means.

Table 1
Biochemical properties of *Bacillus subtilis* DR8806.

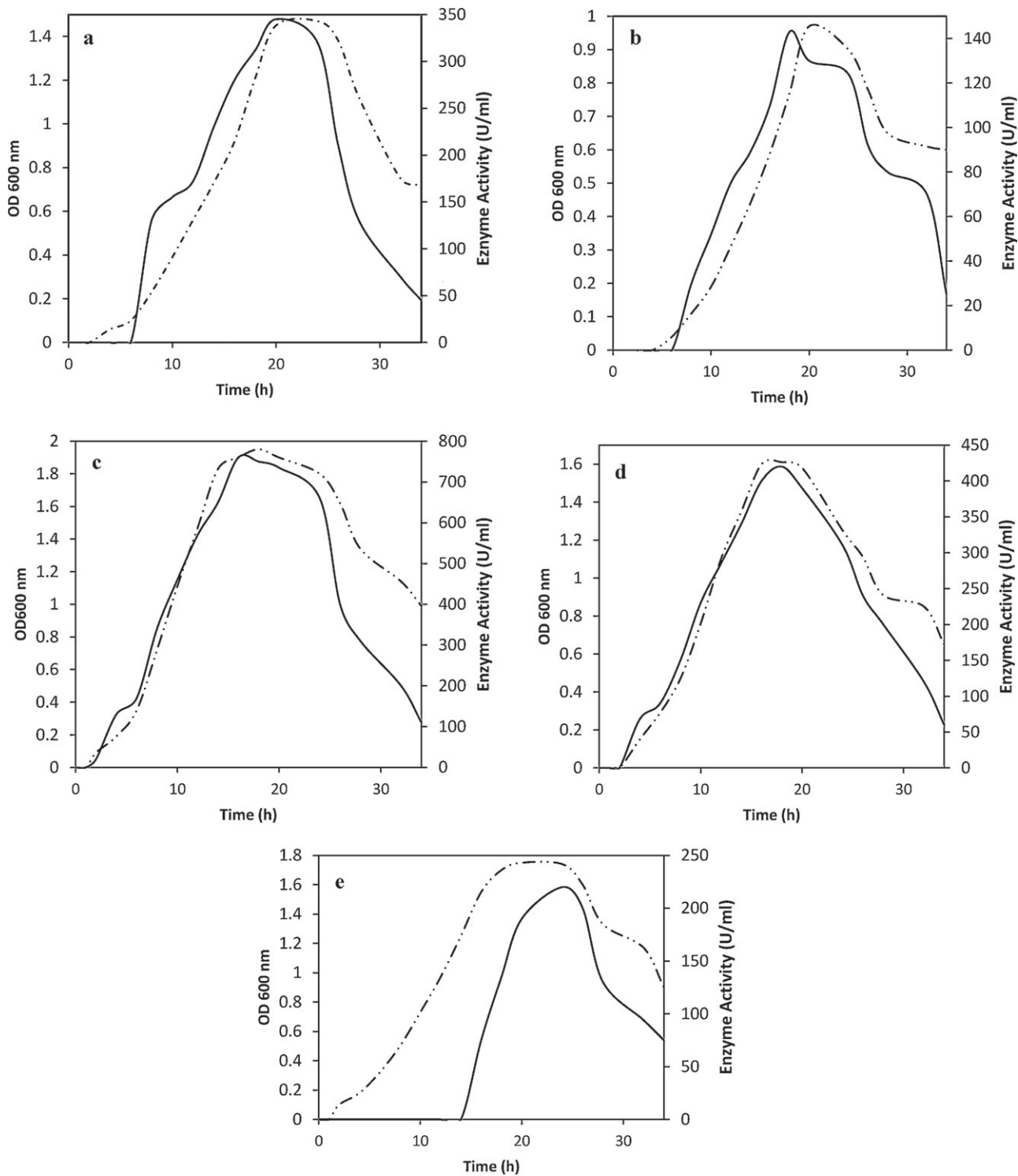| Morphology			Nutrient utilization							Polysaccharide degradation				Physical growth conditions		
Gram Staining	Shape	Spore Forming	Citrate	Catalase	MR-VP Test	Glc	Mal	Lac	Xyl	Starch	Cellulose	Gelatin	Xylan	pH	Temperature	Salt (NaCl)
+	Rod	+	+	+	–	+	+	+	+	+	+	+	+	4–11	Up to 60 °C	Up to 10%

Fig. 3. Impact of some particular mediums on the enzyme production (—) and microorganism growth (-••). Exact composition of each medium described in Section 2.4. a, b, c, d and e show the mediums 1, 2, 3, 4 and 5 respectively. The standard errors were less than 5% of the means.
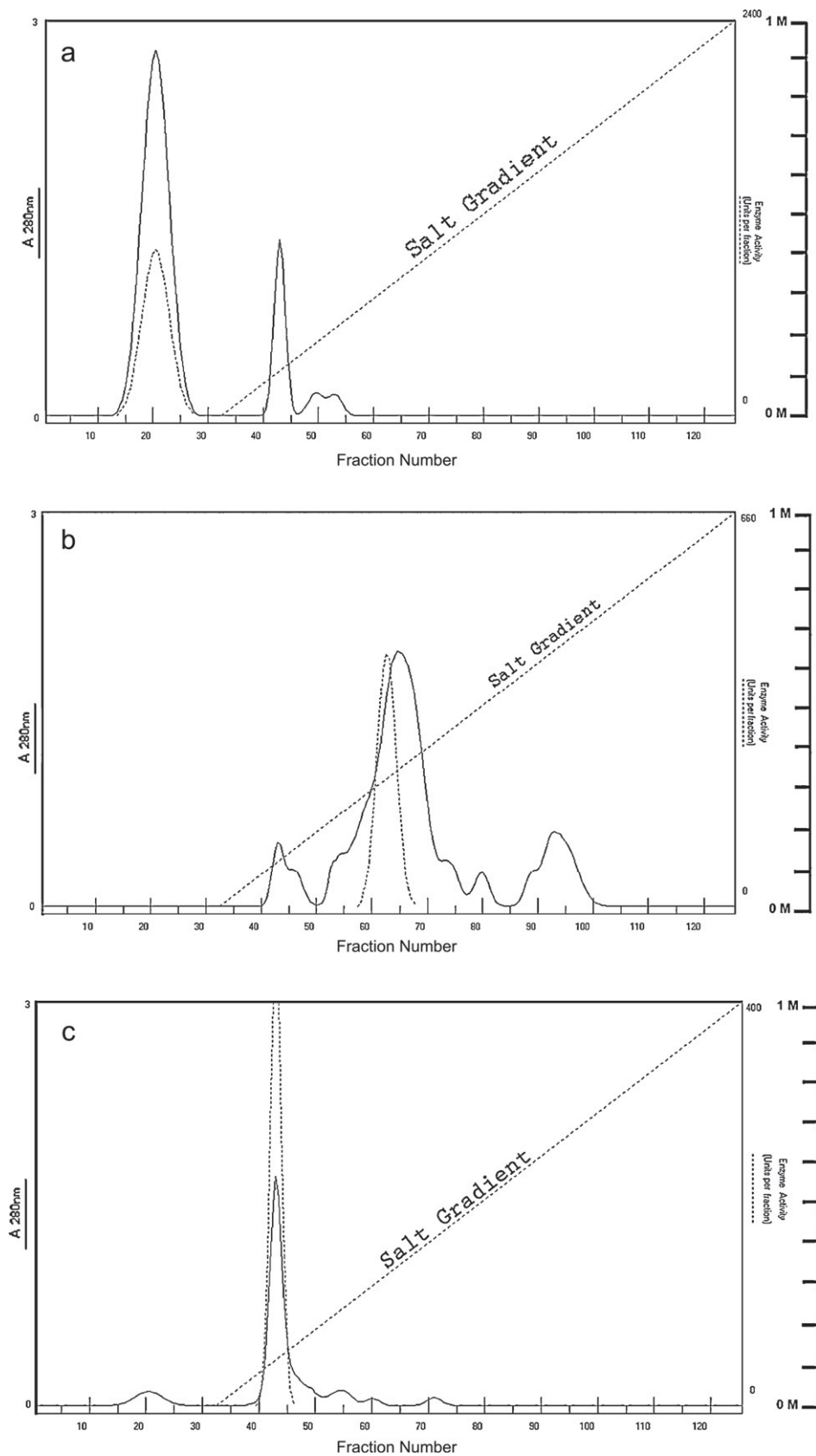
Fig. 4. Ion-exchange chromatography plots. (a) shows the chromatography when the column was equilibrated at pH 6. The protein of interest eluted in the unbound samples. In (b) the column was pre-equilibrated with buffer at pH 8. The enzyme eluted in the fractions from 57 to 67. (c) Column was pre-equilibrated with the previous buffer but at pH 7.5. The enzyme eluted as a single peak.

Table 2
Purification steps and the yeild of enzyme.

Purification step	Total protein (mg)	Total enzyme (Unit/ml)	Fold purification	Yield of enzyme
Crude extract	50.9	54.7	1	100%
Ammonium sulfate precipitation	47.4	50.5	~1	92.3%
Ion exchange chromatography[a] pH 6	7.2	48.6	6.7	88.8%
Ion exchange chromatography[a] pH 8	1.85	43.5	22	79.5%
Ion exchange chromatography pH 7.5	1.6	41	24	75%

[a] Only the highest peak was reported here.

illustrated in Fig. 4c. Fractions related to the enzyme peak were pooled and lyophilized. In the final step, the product was achieved with a 24-fold increase in the specific activity of 25.6 U/mg and 75% recovery. Purification results are summarized in Table 2. The purity of chromatography product was confirmed by detecting a single band on the SDS–PAGE and native PAGE (Fig. 5).

Our results showed that the molecular mass of purified enzyme was 65.5 kDa. The highest calculated molecular weight attributes to *B. subtilis* AX20 with 139.5 kDa [15] while the lowest molecular weight is 41 kDa, owned by *B. subtilis* purified by Orlando and colleagues [16]. Other purified amylases from *B. subtilis* are between these two extremes of molecular weights. Fig. 5B shows the zymogram of the enzyme on native PAGE.

We also determined the isoelectric pH of the enzyme by applying isoelectric focusing. Our results indicate a p*I* around 6 (data not shown) which is in accordance with ion-exchange chromatography where the enzyme was unbound to Q-Sepharose beads at pH 6. This p*I* is one unit lower than the p*I* described for *B. subtilis* AX20 by Najafi et al. [15]. No other records exist about p*I* related to *B. subtilis* amylases.

3.4. pH, enzyme activity and stability

The optimum pHs were determined for four buffer systems as described previously. The enzyme showed appropriate activity in a broad range of pH from 4 to 11 with an optimum activity at 9.5.

To the best of our knowledge closest formerly determined optimal pH with regard to our purified enzyme was for DM-03 of *B. subtilis* as determined to be 9 [18], while for the other strains of *B. subtilis* determined optimum pHs were lower than 6.5 [13,19,20]. The best measured activity of the enzyme was approximately 92% for the pH range of 8.5–10 and exclusively 94% of the activity at pH 9.5 (Fig. 6a). The DR8806 amylase was highly stable in the pH range of 4.0–9.0 and retained more than 90% of the initial activity after 1 h incubation at 70 °C. However, the residual activity rapidly decreased at pH above 10.0 with 64 and 50% of the initial activity and was recoverable from pH 10.5 and 11 (Fig. 6b) respectively. This range of pH stability was more than those reported for *B. subtilis* strains up to the time of present study [13–15,20].

3.5. Effect of temperature on enzyme activity and stability

The optimum temperature of enzyme activity was determined by assessing its activity at different temperatures. As shown in Fig. 7a, the enzyme was active over a wide range of temperatures between 30 °C and 80 °C. The enzyme showed an optimum activity at 70 °C which has not been reported for strains of *B. subtilis* so far [13,15,18–23]. However, it has been reported that two amylases from *B. subtilis* strains had an optimum activity of 65 °C [14,23]. The relative activity of the purified enzyme at 80 °C was to be 45%; but beyond this temperature, no detectable activity could be measured.
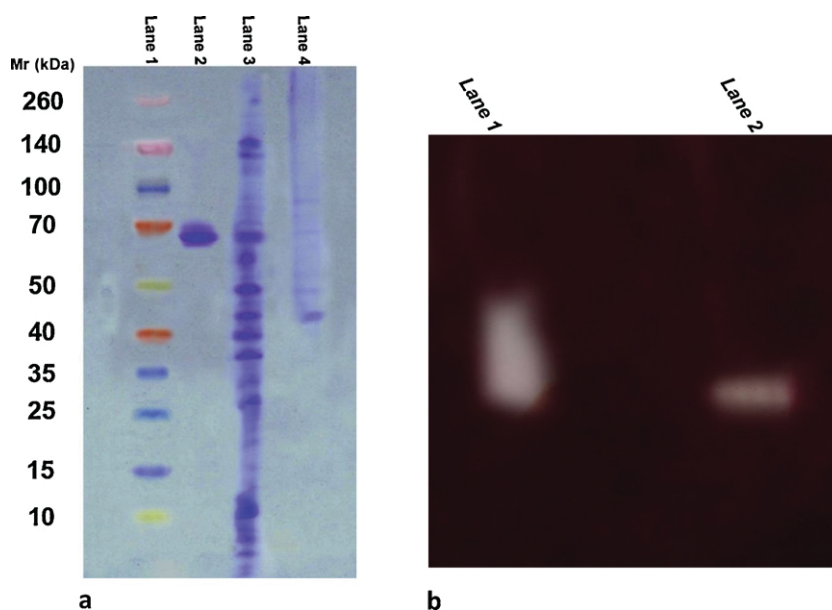

Fig. 5. SDS–PAGE (A) and native-PAGE (B) analysis of the enzyme. In (A): Lane 1, Spectra™ multicolor broad range protein ladder (Fermentas Life Science, USA); lane 2, the purified sample; lane 3, the crude enzyme extract; lane 4, the supernatant of 85% ammonium sulphate precipitate; in (B): lane 1, the crude enzyme extract; lane 2, the purified sample.
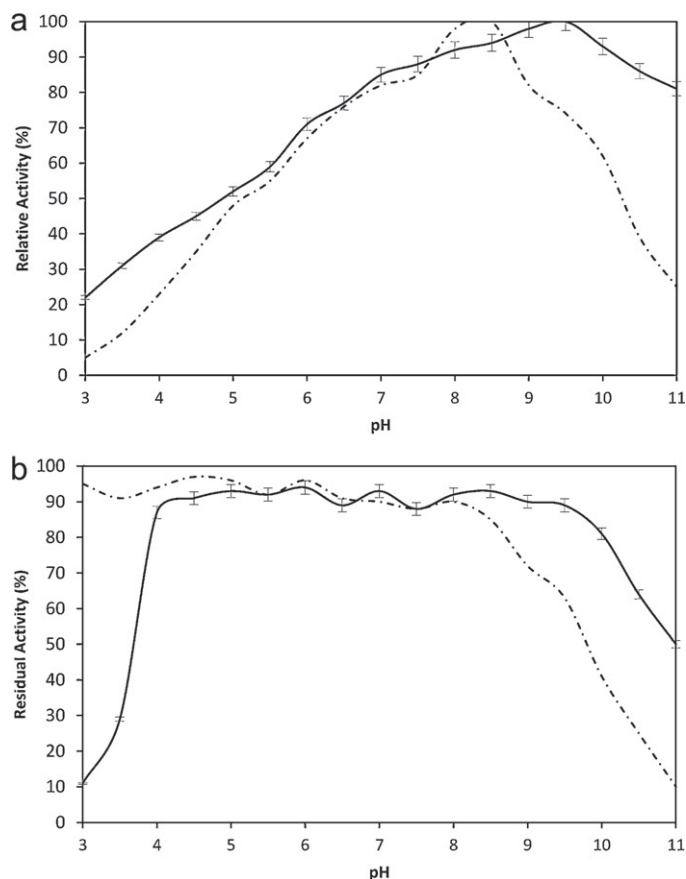
Fig. 6. pH profile for enzyme activity and stability. In (a) the effect of pH on the enzyme activity was shown. The optimum pH for the best activity demonstrated at 9.5. (b) Effect of long incubation at different pH on the enzyme stability was elucidated. Enzyme was well stable at pH from 4 to 10. The behavior of BLA was also tested as control of the experiment. The purified enzyme is shown by (—) and BLA by (—•—). Each data point represents the mean of three independent assays (the standard errors were less than 5% of the means).

Analysis of the thermal stability from 30 to 70 °C indicated that the enzyme activity was decreased less than 20% than the beginning of test although the enzyme could retain only 24% of its original activity at 80 °C. In addition, the activity of the enzyme was lost at 85 and 90 °C since no activity was detected after 5 min incubation.

To determine whether Ca^{2+} enhances thermal stability of the enzyme or not, the enzyme was incubated at 70 °C in the presence of 10 mM Ca^{2+}. As illustrated in Fig. 7b there was no significant enhancement of the thermal stability in the presence of Ca^{2+}. Calcium dependency was previously demonstrated for all amylases purified from *B. subtilis* strains [7,13–17,20,21,24]. Therefore this report is the first that suggests Ca^{2+} independency for an amylase from a strain of *B. subtilis*.

As shown in Fig. 7c, between 30 °C and 70 °C the enzyme remains active after 180 min of incubation with an activity loss of 7.5% for each 10 °C increases in the temperature. Above 70 °C, thermal inactivation curve's slope declines rapidly up to 50% of the activity. The purified enzyme has a half-life of 250 min at 70 °C. This measurement was done based on the slope of enzyme inactivation curve. At this temperature, the enzyme maintained 90% of its initial activity after 1 h of incubation. The measured half-life of the enzyme is more than other amylases from *Bacillus mojavensis* [25], *Bacillus* sp. *KR-8104* [26] and *Bacillus subtilis* AX20 [15]; however, it is lower than those for *Bacillus* sp. strain WN11 [27] and *Bacillus licheniformis* amylases [28].

Table 3
Effect of metal ions, inhibitors, surfactant and oxidizing agents on the enzyme activity. The presented figures are measurements that were done in presence of either of above agents and compared to the enzyme activity in absence of which.

Type	Agent	Concentration	Relative activity (%)
Surfactant and oxidizing agents	H_2O_2	1 M	50
	Trition X-100	1%	80
	SDS (10%)	10%	72
Metal ions	Hg^{2+}	5 mM	0
		10 mM	0
	Al^{3+}	5 mM	113
		10 mM	125
	Ba^{2+}	5 mM	100
		10 mM	91
	Co^{2+}	5 mM	108
		10 mM	100
	Cu^{2+}	5 mM	120
		10 mM	120
	Fe^{2+}	5 mM	100
		10 mM	100
	Ca^{2+}	5 mM	100
		10 mM	100
	Mg^{2+}	5 mM	91
		10 mM	75
	Mn^{2+}	5 mM	140
		10 mM	130
	Li^+	5 mM	112
		10 mM	104
	Zn^{2+}	5 mM	0
		10 mM	0
	Na^+	5 mM	100
		10 mM	105
Inhibitors	DTAB	5 mM	0
	DTNB	5 mM	0
	p-Hydroxymercuri-benzoate	5 mM	0
	PMSF	10 mM	100
	β-Mercaptoethanol	10 mM	100
	EDTA	10 mM	100

Abbreviations: DTAB, dodecyl trimethyl ammonium bromide; DTNB, 5,5′-dithiobis-2-nitrobenzoic acid; PMSF, phenylmethanesulfonylfluoride; EDTA, ethylenediaminetetraacetic acid.

3.6. Effects of metal ions, enzyme inhibitors and oxidizing agents on the enzyme activity

The effect of various metal ions on the enzyme activity has been shown in Table 3. Although Ca^{2+}, Na^+ and Fe^{2+} did not affect the enzyme activity, Mg^{2+} and Ba^{2+} showed a little decrease in the enzyme activity especially at 10 mM concentration. Hg^{2+} and Zn^{2+} completely inhibited the enzyme activity even in the minimum concentration. Other investigated metallic ions increased the enzyme activity according to the rates listed in Table 3. Among tested inhibitors; the chelating agent EDTA and β-mercaptoethanol, have no effect on the enzyme activity indicating that amylase is neither a metalloenzyme nor a disulfide stabled protein (Table 3). The effect of EDTA on the amylase from alkaliphilic *Bacillus* species varies considerably such that some are unaffected in the presence of EDTA even at a concentration of 100 mM [29], while others are completely inhibited by lowing EDTA concentration, e.g. the amylase activity of *Bacillus* sp. IMD 370 is completely inhibited by 1 mM EDTA. Other inhibitors except PMSF completely inhibit the enzyme.
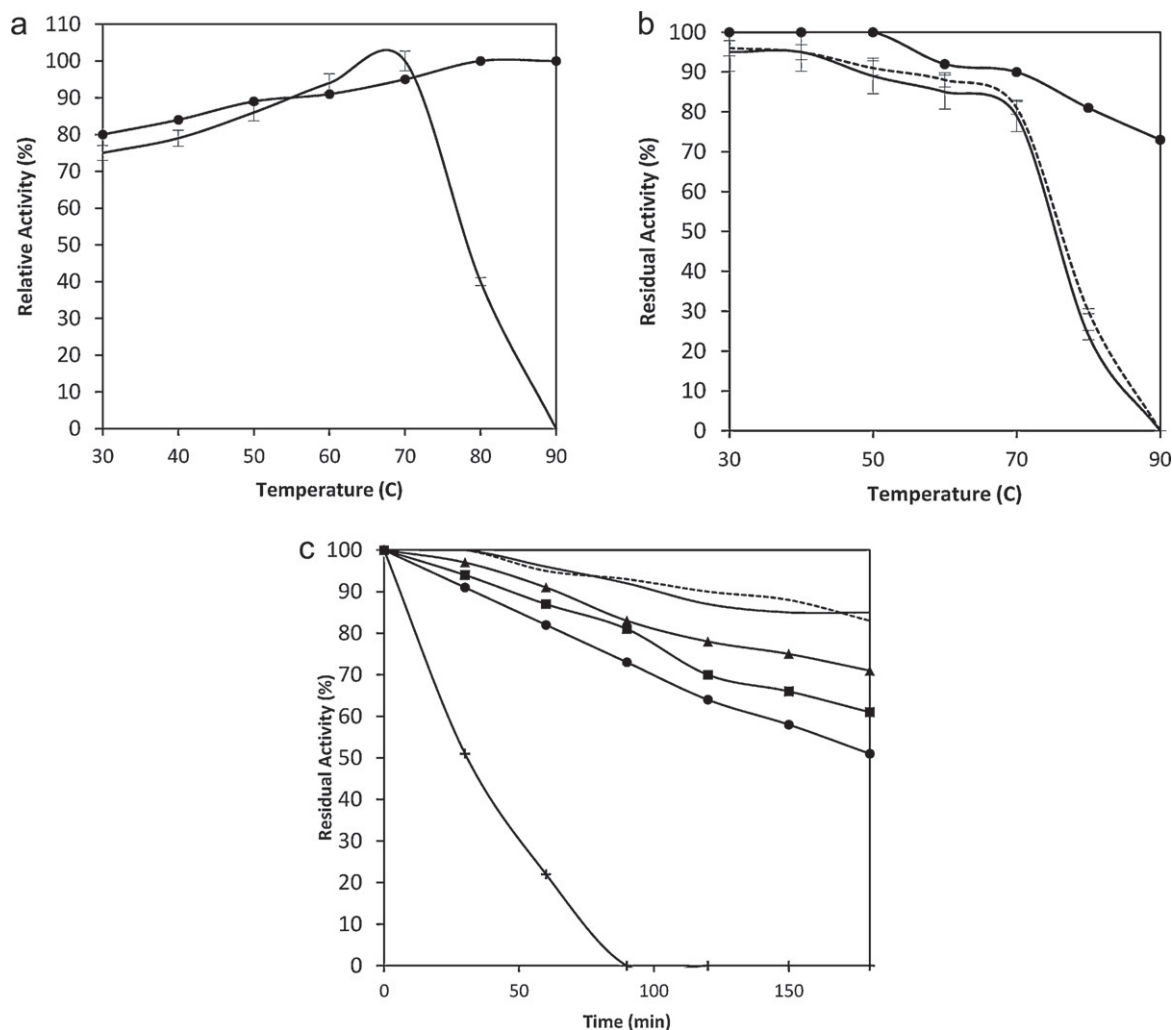
Fig. 7. Effect of various temperatures on the enzyme activity (a) and stability (b). The best temperature for the enzyme activity was at 70 °C. The stability of the enzyme was also investigated in the presence and absence of CaCl₂. The result demonstrated no significant enhancement in the stability of the enzyme in the presence of CaCl₂. (c) The half life of the enzyme at various temperatures and different times. For parts a and b, the purified enzyme with and without CaCl₂ is shown by (—·-), (—) and BLA by (—•—) and for part c legends are as follows: Temp (°C) 30 (—), 40 (—·-), 50 (—▲—), 60 (—■—), 70 (—•—) and 80 (—+—). Each data point represents the mean of three independent assays (the standard errors were less than 5% of the means).

The stability of enzyme was also studied by incubating the enzyme in the presence of surfactants (SDS, Triton X-100) for 60 min at 42 °C (Table 3). The enzyme was greatly stable in the presence of the non-ionic surfactants such as Triton X-100. In addition, the enzyme was highly persistent in the presence of strong anionic surfactant (SDS), retaining approximately 72% of its initial activity when it was incubated with 10% SDS in reaction buffer. The purified amylase was also relatively stable towards oxidizing agents, retaining 50% of its activity after 60 min incubation in the presence of 1 M (v/v) H_2O_2. These properties of enzyme along with thermostability and pH profiles make it as an appropriate enzyme in laundry industries.

3.7. Hydrolysis of starch

Thin layer chromatography was used for analysis of the hydrolysis products of starch by the enzyme (Fig. 8). When the enzyme was incubated with 1% soluble starch, only short length maltooligosaccharides were produced during the reaction subsequently converted to maltose and mainly glucose. From results depicted in Fig. 7, it could be concluded that the enzyme is an exo-acting enzyme in its mechanism of action, also, it could be considered as glucoamylase since the main product was glucose. Furthermore, this result infers us that the enzyme is suitable for application in the industry, since it is a fast starch as well as other malto-oligosacharid hydrolyzing enzyme (further discussion in the next section).

3.8. Substrate specificty

Among the investigated substrates, DR8806 glucoamylase shows a significant activity toward maltodextrin and pulullan and a lower activity for glycogen compared with starch as the reference saccharide. The measured activities were 73%, 68% and 12% for maltodextrin, pullulan and glycogen respectively. No hydrolytic activity towards α/β cyclodextrin was observed (Fig. 9). Therefore, we conclude that our purified enzyme (*Bacillus subtilis* DR8806) could be considered as a maltodextrinase as well as a glucoamylopulullanase. Purification of amylo-pullulanase has previously been described for *B.stearothermophilus* TS-23 [30], *Bacillus* sp. DSM 405
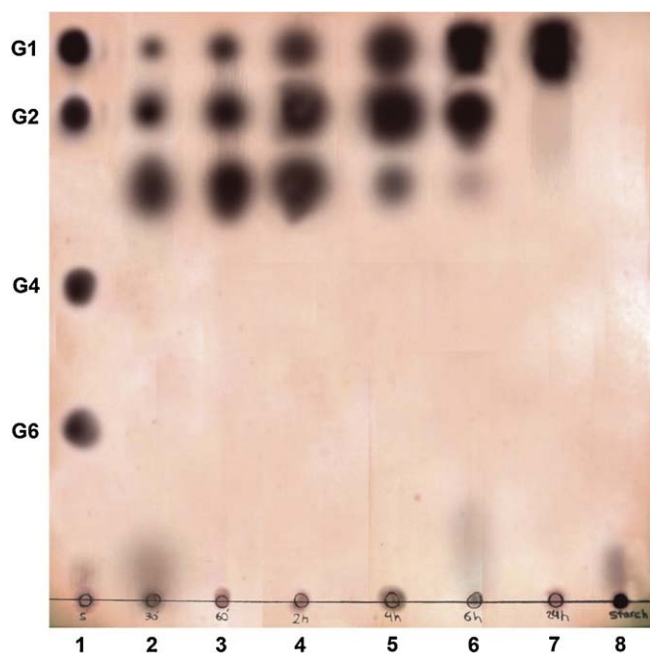
Fig. 8. Thin layer chromatogram of the end products of raw potato starch after hydrolysis with the purified enzyme. Lane 1: Standard malto-oligosaccharides; lane 2: hydrolysis after 30 min; lane 3: 1 h, lane 4: 2 h, lane 5: 6 h, lane 6: 24 h and lane 7: unhydrolyzed soluble starch. The "G" number in the left side, represents the number of C_6 sugar in the malto-oligosaccharides.
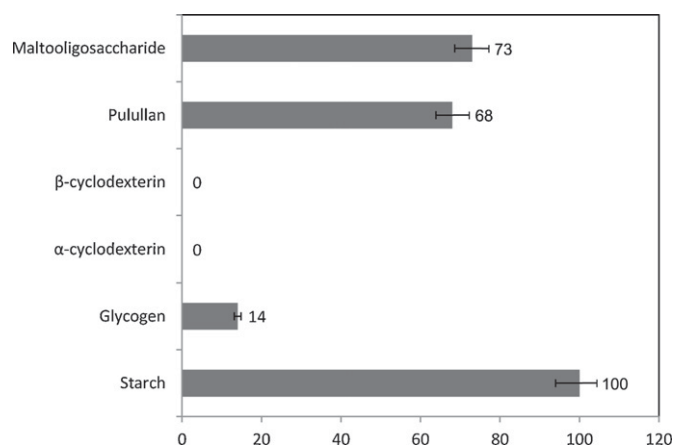

Fig. 9. Substrate specificity of the purified enzyme toward various substrates. Each data point represents the mean of three independent assays (the standard errors were less than 5% of the means).

[31] and *Bacillus* sp. KSM-1378 [32] but not for any strains of *B. subtilis*.

4. Conclusion

In the present study, a novel thermostable alkaline glu-coamylopullulanase from *B. subtilis* DR8806 was purified and characterized. The purified enzyme was homogenous on SDS–PAGE and its molecular weight was estimated to be 65.5 kDa with a p*I* close to 6. The optimum temperature for amylolytic activity was 70 °C that it has not so far been reported for strains of *B. subtilis*. Its thermostability is independent of the presence of Ca^{2+} that is more important for industrial usage. Interestingly, the enzyme is highly active and stable over a wide range of pH from 5.0 to 9.0. Another important feature of the enzyme was its ability to digest pullu-lan, a polysaccharide polymer consisting on maltotriose units. The significance of the present purified enzyme is that there was not any report with respect to amylo-pullulanase from *B. subtilis* so far. The former reports with regard to this enzyme are from other *Bacillus* genuses, none of which show ability of strach hydroly-sis as well as pullulan degradation. Considering the high activity and stability for a wide range of pH, temperatures, surfactants and the commercial detergents as well as the final product of the enzyme reaction, the *B. subtilis* DR8806 glucoamylopullulanse is an appropriate candidate for application in laundry detergents and starch processing industries. Moreover, amino acid sequence of the enzyme remains to be elucidated in order to understand the mech-anism of its activity. We speculate that this enzyme has two active sites, one for amylase activity and the other for pullulanase activity; however, this suggestion demands further investigations; espe-cially determination of the 3D structure of enzyme until a better illustration of enzyme structure and domains becomes available.

Acknowledgments

References

[1] Prakash O, Jaiswal N. Alpha-amylase: an ideal representative of thermostable enzymes. Appl Biochem Biotechnol 2010;160:2401–14.

[2] Yeoman CJ, Han Y, Dodd D, Schroeder CM, Mackie RI, Cann IK. Ther-mostable enzymes as biocatalysts in the biofuel industry. Adv Appl Microbiol 2010;70:1–55.

[3] Yano JK, Poulos TL. New understandings of thermostable and peizostable enzymes. Curr Opin Biotechnol 2003;14:360–5.

[4] Synowiecki J, Grzybowska B, Zdzieblo A. Sources, properties and suitabil-ity of new thermostable enzymes in food processing. Crit Rev Food Sci Nutr 2006;46:197–205.

[5] Bergey DH, Holt JG. Bergey's manual of determinative bacteriology. Baltimore: Williams & Wilkins; 1994. p. xviii, 787 p.

[6] Sambrook J, Russell DW. Molecular cloning: a laboratory manual. Cold Spring Harbor, N.Y.: Cold Spring Harbor Laboratory Press; 2001.

[7] Asoodeh A, Chamani J, Lagzian M. A novel thermostable, acidophilic alpha-amylase from a new thermophilic *Bacillus* sp. Ferdowsicous isolated from Ferdows hot mineral spring in Iran: purification and biochemical character-ization. Int J Biol Macromol 2010;46:289–97.

[8] Atlas RM. Handbook of media for environmental microbiology. Boca Raton: Taylor & Francis; 2005.

[9] Bernfeld P. Amylases, α and β. Methods in Enzymology, vol. 1; 1955. p. 149–58.

[10] Bradford MM. A rapid and sensitive method for the quantitation of micro-gram quantities of protein utilizing the principle of protein–dye binding. Anal Biochem 1976;72:248–54.

[11] Simpson RJ. Purifying proteins for proteomics: a laboratory manual. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press; 2004. p. xi, 801 p.

[12] Zhang Z, Xie J, Zhang F, Linhardt RJ. Thin-layer chromatography for the analysis of glycosaminoglycan oligosaccharides. Anal Biochem 2007;371:118–20.

[13] Liu Y, Lu F, Chen G, Snyder CL, Sun J, Li Y, Wang J, Xiao J. High-level expres-sion, purification and characterization of a recombinant medium-temperature alpha-amylase from *Bacillus subtilis*. Biotechnol Lett 2010;32:119–24.

[14] Nagarajan DR, Rajagopalan G, Krishnan C. Purification and characterization of a maltooligosaccharide-forming alpha-amylase from a new *Bacillus subtilis* KCC103. Appl Microbiol Biotechnol 2006;73:591–7.

[15] Najafi MF, Deobagkar D. Purification and characterization of an extracellular alpha-amylase from *Bacillus subtilis* AX20. Protein Expr Purif 2005;41:349–54.

[16] Orlando AR, Ade P, Di Maggio D, Fanelli C, Vittozzi L. The purification of a novel amylase from *Bacillus subtilis* and its inhibition by wheat proteins. Biochem J 1983;209:561–4.

[17] Rajagopalan G, Krishnan C. Hyper-production of alpha-amylase from agro-residual medium with high-glucose in SSF using catabolite derepressed *Bacillus subtilis* KCC103. J Basic Microbiol 2010;50:336–43.

[18] Das K, Doley R, Mukherjee AK. Purification and biochemical characterization of a thermostable, alkaliphilic, extracellular alpha-amylase from *Bacillus subtilis* DM-03, a strain isolated from the traditional fermented food of India. Biotech-nol Appl Biochem 2004;40:291–8.

[19] Dercova K, Augustin J, Krajcova D. Cell growth and alpha-amylase production characteristics of *Bacillus subtilis*. Folia Microbiol (Praha) 1992;37:17–23.

[20] Liu YH, Lu FP, Li Y, Yin XB, Wang Y, Gao C. Characterisation of mutagenised acid-resistant alpha-amylase expressed in *Bacillus subtilis* WB600. Appl Microbiol Biotechnol 2008;78:85–94.

[21] Kindle K. Characteristics and production of thermostable α-amylase. Appl Biochem Biotechnol 1983:153–70.

[22] Hayashida S, Teramoto Y, Inoue T. Production and characteristics of raw-potato–starch–digesting alpha-amylase from *Bacillus subtilis* 65. Appl Environ Microbiol 1988;54:1516–22.

[23] Mitsuiki S, Mukae K, Sakai M, Goto M, Hayashida S, Furukawa K. Comparative characterization of raw starch hydrolyzing [alpha]-amylases from various *Bacillus* strains. Enzyme Microb Technol 2005;37:410–6.

[24] Cho MH, Park SE, Lee MH, Ha SJ, Kim HY, Kim MJ, Lee SJ, Madsen SM, Park CS. Extracellular secretion of a maltogenic amylase from *Lactobacillus gasseri* ATCC33323 in *Lactococcus lactis* MG1363 and its application on the production of branched maltooligosaccharides. J Microbiol Biotechnol 2007;17:1521–6.

[25] Hmidet N, Maalej H, Haddar A, Nasri M. A novel alpha-amylase from *Bacillus mojavensis* A21: purification and biochemical characterization. Appl Biochem Biotechnol 2010;162:1018–30.

[26] Alikhajeh J, Khajeh K, Naderi-Manesh M, Ranjbar B, Sajedi RH, Naderi-Manesh H. Kinetic analysis, structural studies and prediction of pKa values of *Bacillus* KR-8104 [alpha]-amylase: The determinants of pH-activity profile. Enzyme Microb Technol 2007;41:337–45.

[27] Mamo G, Gessesse A. Purification and characterization of two raw-starch-digesting thermostable α-amylases from a thermophilic *Bacillus*. Enzyme Microb Technol 1999;25:433–8.

[28] Lee S, Oneda H, Minoda M, Tanaka A, Inouye K. Comparison of starch hydrolysis activity and thermal stability of two *Bacillus licheniformis* alpha-amylases and insights into engineering alpha-amylase variants active under acidic conditions. J Biochem 2006;139:997–1005.

[29] Khemakhem B, Ali MB, Aghajari N, Juy M, Haser R, Bejar S. Engineering of the alpha-amylase from *Geobacillus stearothermophilus* US100 for detergent incorporation. Biotechnol Bioeng 2009;102:380–9.

[30] Chen JT, Chen MC, Chen LL, Chu WS. Structure and expression of an amylopullulanase gene from *Bacillus stearothermophilus* TS-23. Biotechnol Appl Biochem 2001;33:189–99.

[31] Brunswick JM, Kelly CT, Fogarty WM. The amylopullulanase of *Bacillus* sp. DSM 405. Appl Microbiol Biotechnol 1999;51:170–5.

[32] Hatada Y, Igarashi K, Ozaki K, Ara K, Hitomi J, Kobayashi T, Kawai S, Watabe T, Ito S. Amino acid sequence and molecular structure of an alkaline amylopullulanase from *Bacillus* that hydrolyzes alpha-1,4 and alpha-1,6 linkages in polysaccharides at different active sites. J Biol Chem 1996;271:24075–83.