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

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A B S T R A C T

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 H2O2. 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 alkaliphilic and thermostable enzyme.

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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.
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 Quagen (Quagen, USA). DNS (3,5-dinitrosalicylic acid), maltotriosaccharide, TLC plate, starch, amylase and amylopectin, were obtained from Sigma (Sigma–Aldrich, USA). Q-Sepharose was purchased from Pharma- cia (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 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 “Berger’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′- GGCTGACTGCTGAGACTTC-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; soluble starch, 20.0; tryptose, 10.0; NaCl, 5.0; medium #2: white soybeans, 100.0; starch, 20.0; medium #3: glucose, 20.0; g/l: -glutamic acid, 4.0; citric acid, 2.0; K 2HPO 4, 0.5; g/l: starch, 20.0; ferric ammonium citrate, 0.5; MgSO 4, 0.5; medium #4: bacteriological peptone, 60.0; MgSO 4, 0.5; KC, 5.0; g/l: starch, 20.0; medium #5: nutrient broth, 32.0; g/l: Fructose, 10.0; peptone, 10.0; g/l: starch, 20.0. 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 × 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 × 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 pre-cipitated 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. Subse- quently, the solution was applied onto a HiTrap Q-Sepharose Fast Flow™ column (3 cm × 15 cm) -equilibrated with the above buffer and washed with three column volumes to remove unbound proteins. Elution of the bound pro- teins 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 frac- tions was concentrated using an Amicon ultrafiltration apparatus equipped with 10kDa 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. Qualification of the protein concentration was performed in all purifi- cation 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 amylose 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 α-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 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 PhastCel™ pH 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 accor- dance 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 buffer- ing systems were as follows: 50 mM sodium acetate/acetate for pH 3–5.5; 50 mM dipotassium hydrogen phosphate/potassium dihydrogen orthophosphate for pH 6–7.5; 50 mM Tris/hydrochloric acid pH 8–9.5 and 50 mM sodium bicarbon- ate/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 deter- mined by measuring the absorbance at 540 nm. BLA (β-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 temper- atures 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 tem- perature 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 CaCl2 and then compared to CaCl2 free condi- tion to determine whether the enzyme thermostability is dependent on CaCl2 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⁺, Al³⁺, Ba²⁺, Ca²⁺, Cu²⁺, Fe³⁺, Co²⁺, Mn²⁺, Mg²⁺, 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-hydroxymercurobenzoate, 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 H₂O₂ as oxidizing agent plus 1% Triton 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 to 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 spots. Buffers 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% H₂PO₄, 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 glycozen, α/β-cyclodextrin, pulullan and maltoligosaccharide 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 the enzyme. The enzyme was mixed with each substrate at 1:5 ratios (v/v), and the activity 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 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 (OD₆₀₀nm) 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 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 B 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
Table 1
Biochemical properties of Bacillus subtilis DR8806.

<table>
<thead>
<tr>
<th>Morphology</th>
<th>Nutrient utilization</th>
<th>Polysaccharide degradation</th>
<th>Physical growth conditions</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gram Staining</td>
<td>Shape</td>
<td>Spore Forming</td>
<td>Citrate</td>
</tr>
<tr>
<td>+</td>
<td>Rod</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>

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.
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 pI 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 pI is one unit lower than the pI described for B. subtilis AX20 by Najafi et al. [15]. No other records exist about pI 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.

<table>
<thead>
<tr>
<th>Purification step</th>
<th>Total protein (mg)</th>
<th>Total enzyme (Unit/ml)</th>
<th>Fold purification</th>
<th>Yield of enzyme</th>
</tr>
</thead>
<tbody>
<tr>
<td>Crude extract</td>
<td>50.9</td>
<td>54.7</td>
<td>1</td>
<td>100%</td>
</tr>
<tr>
<td>Ammonium sulfate precipitation</td>
<td>47.4</td>
<td>50.5</td>
<td>–1</td>
<td>92.3%</td>
</tr>
<tr>
<td>Ion exchange chromatography pH 6</td>
<td>7.2</td>
<td>48.6</td>
<td>6.7</td>
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</tr>
<tr>
<td>Ion exchange chromatography pH 8</td>
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<td>43.5</td>
<td>22</td>
<td>79.5%</td>
</tr>
<tr>
<td>Ion exchange chromatography pH 7.5</td>
<td>1.6</td>
<td>41.0</td>
<td>24</td>
<td>75%</td>
</tr>
</tbody>
</table>

* Only the highest peak was reported here.

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.
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 Ca²⁺ enhances thermal stability of the enzyme or not, the enzyme was incubated at 70 °C in the presence of 10 mM Ca²⁺. As illustrated in Fig. 7b there was no significant enhancement of the thermal stability in the presence of Ca²⁺. 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 Ca²⁺ 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 increase 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 majavensis [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].

![Graph](image)

**Fig. 6.** pH profile for enzyme activity and stability. In (a) the effect of pH on the enzyme activity was shown. The optimal 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 still 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).

### 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.

<table>
<thead>
<tr>
<th>Type</th>
<th>Agent</th>
<th>Concentration</th>
<th>Relative activity (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Surfactant and oxidizing agents</td>
<td>H₂O₂</td>
<td>1 M</td>
<td>50</td>
</tr>
<tr>
<td></td>
<td>Triton X-100</td>
<td>1%</td>
<td>80</td>
</tr>
<tr>
<td></td>
<td>SDS (10%)</td>
<td>10%</td>
<td>72</td>
</tr>
<tr>
<td>Metal ions</td>
<td>Hg²⁺</td>
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<tr>
<td></td>
<td>Al³⁺</td>
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<tr>
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<td>Zn²⁺</td>
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<td>Hydroxymercuribenzoate</td>
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<td>β-</td>
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<tr>
<td></td>
<td>Mercaptoethanol</td>
<td>10 mM</td>
<td>100</td>
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</tbody>
</table>

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 Ca²⁺, Na⁺ and Fe²⁺ did not affect the enzyme activity, Mg²⁺ and Ba²⁺ showed a little decrease in the enzyme activity especially at 10 mM concentration. Hg²⁺ and Zn²⁺ 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 stabilized 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.
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) H₂O₂. 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-oligosaccharid hydrolyzing enzyme (further discussion in the next section).

3.8. Substrate specificity

Among the investigated substrates, DR8806 glucoamylase shows a significant activity toward maltodextrin and pullulan 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 glucoamyllopullulanase. Purification of amyllo-pullulanase has previously been described for B.stearothermophilus TS-23 [30], Bacillus sp. DSM 405
4. Conclusion

In the present study, a novel thermostable alkaline glucoamylopullulanase from \textit{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 pI close to 6. The optimum temperature for amylolytic activity was 70°C that it has not so far been reported for strains of \textit{B. subtilis}. Its thermostability is independent of the presence of Ca²⁺ 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 pullulan, a polysaccharide polymer consisting of maltotriose units. The significance of the present purified enzyme is that there was not any report with respect to amyl-o-pullulanase from \textit{B. subtilis} so far. The former reports with regard to this enzyme are from other \textit{Bacillus} genuses, none of which show ability of strach hydrolysis 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 \textit{B. subtilis} DR8806 glucoamylopullulanase 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 mechanism 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; especially determination of the 3D structure of enzyme until a better illustration of enzyme structure and domains becomes available.

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References


