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Original Research Article

Extracellular amylases produced by *Bacillus thuringiensis*

Short Title: Amylases of *Bacillus thuringiensis* (Bt)

Abstract

The present study revealed that *B. thuringiensis* strain Bt LBIT 418 cultured in a liquid medium, with starch as carbon source, secreted the maximum amount of endoamylase after 10 h of incubation, in the middle of the exponential phase of growth. It cleaves action-released dextrins composed by five of more glucose residues. An exoamylase able to convert these dextrins in maltotriose, maltose and glucose appeared at once, given rise to a new increment of bacterial growth. An α-glucosidase was also secreted, but covering the 40 h of incubation period. Other related hydrolases as glucoamylase and maltase were not found, but proteases whose synthesis profile correlated with the endoamylase were detected, although both activities presented independent synthesis regulations.

On the other hand, the exoamylase could be purified 98 times with at recovering of 14 %, by fractionating supernatants from 13 h cultures, previously concentrated by liophilization, in columns of CM-Sephadex G-50 and G-100. The enzyme showed a MW of 83.2 kDa and maximum activity at pH 7-8 and 50 ºC, while the enzyme stability was higher at pH 6-7.5 and 55 ºC. The enzyme was inactivated with 0.01 M of EDTA and reactivated with 0.01 M $CaCl_2$, showed that the amylase was a metalloenzyme. Furthermore, the thiol-dependent characteristic was unclear, because the enzyme was inhibited by 0.01M $HgCl_2$, but did not by 0.01 M of pHMB. This is the first report concerning to the characterization of the amylolytic system of *B. thuringiensis*.

Keywords: amylases, *Bacillus thuringiensis*, starch, extracellular enzymes.

Introduction

Industrial enzymes have formed a market with about 6.2 billions of USD by 2020 [1]. Amylases as a large group of enzymes degrading starch and related oligosaccharides and polysaccharides, amylases have been utilized in

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breweries, and in diverse areas, such as textile, distilling [2,3], pharmaceutical industries [4] and in baking industries [5]. Since starch is one of the important sources of energy for organisms, amylases could be produced by a great variety of living systems [6]. According to their functions, four groups of amylases are recorded: endoamylases, exoamylases, debranching enzymes, and transferases.

Endoamylases are able to cleave α, 1-4 glycosidic bonds present in the inner part (endo) of amylose or amylopectin chain. The enzyme α-amylase (EC 3.2.1.1) is a well-known endoamylase [2]. The end products of α-amylase action are oligosaccharides with varying length, such as the α-configuration and α-limit dextrins, which are branched oligosaccharides [7]. Enzymes belonging to the group exoamylases either exclusively cleave α-1,4 glycosidic bonds such as β-amylase (EC 3.2.1.2) or cleave both α-1,4 and α-1,6 glycosidic bonds like amyloglucosidase or glucoamylase (EC 3.2.1.3) and α-glycosidase (EC 3.2.1.20). Exoamylases act on the external glucose residues of amylose or amylopectin and thus produce only glucose (glucoamylase and α-glucosidase), or maltose and β-limit dextrin (β-amylase). β-amylase and glucoamylase also convert the anomeric configuration of the liberated maltose from α- to β-figuration. Glucoamylase and α-glucosidase differ in their substrate preference: α-glucosidase acts best on short malto-oligosaccharides and liberates glucose with α-configuration while glucoamylase hydrolyzes long chain polysaccharides best [2]. The endoamylases and exoamylases are mostly produced by bacteria and the genus of *Bacillus* have been extensively studied for their production of extracelular amylases [8,9,10,11].

The species *B. thuringiensis* (Bt) are sporulating Gram-positive bacterium, known mainly for its production of crystal-like bodies comprised of one or more Cry-proteins, which can be toxic for insects, nematodes and cancer cells [12]. Also Bt secreted extracellular enzymes as chitinase [13], chitosanase [14], protease [15]. Recently, crude preparations of the chitinase from Bt were used to hydrolyze colloidal chitosan to produce different oligosaccharides with potential pharmaceutical applications [16]. In addition the amylase of Bt was recovered as byproduct from supernatant released during the harvest of δ-endotoxin using biphasic fermentation strategy and then was purified and characterized [17,18]. Also amylase from Bt was isolate from tapioca waste [19].

Considering that the biotechnological potential of Bt is still insufficiently explored, the present study was performed to investigate the production and properties of amylases from Bt, because the available information of these enzymes from Bt is limited, in spite of amylases are among the more important industrial enzyme.

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Materials and Methods

Bacterial strain

The strain Bt-LBIT-418 was provided by Dr. Jorge E. Ibarra in CINVESTAV-IPN, Irapuato, Mexico. This strain produces bipyramidal crystals highly toxic for *Manduca sexta* and *Spodoptera fugiperda*. For strain long-term maintenance lyophilized spores were kept at -15 ºC.

Culture medium

The basal medium (BM) consisted of (g L^{-1}, pH 6.5): 0.375 K_2HPO_4; 0.250 NaCl; 0.275; 0.275$MgSO_4$ and 0.375 Na_2CO_3; 1.0 casaaminoacids and 1.0 yeast extract. BM served to assay the constitutive production of amylase [20]. BM complemented with glucose 1 % (w/v) to repress the amylase synthesis, and casamino acids were substituted by 1% (w/v) of NH_4NO_3 to explore the protease synthesis inhibition .

Culture conditions

For kinetic experiments, a fresh colony was inoculated in 25 mL of the test medium and cultured for 12 h (180 rpm) at 28 ºC, and this cultivation was repeated two times by transferring 0.1 mL of the culture in the subsequent fresh medium. Aliquots of 0.1 mL of the last culture were inoculated in a serial of 125 mL Erlenmeyer flask containing 25 mL of BM medium. The flasks were incubated for 48 h, a flask was sampled every 60 min during the first 14 h of incubation, and then at intervals of 2-3 h. Bacterial growth of each collected cultures was determined either by reading the absorbance at 600 nm (OD_{600}) or by counting the colony forming units (CFU mL^{-1}). For analyses of enzyme activities, the culture samples were centrifuged at 6000 rpm for 15 min at 5 ºC (Sorvall centrifuge RC-SB) and the supernatants were filtrated and then stored in vials at 5 ºC for further use.

Enzymatic assays

Amylase

Preliminary evaluations were achieved by using two methods. In the first, 0.5 mL of 1 % (w/v) soluble starch in 0.2 M phosphate buffer (pH 7.0), and 0.5 mL of the culture supernatant were mixed and incubated at 50 ºC for 60 min. Then, reduced sugars released in the mixture were quantified with DNS method [21]. The second method was based on the evaluation of non-hydrolyzed starch with lugol [22]. Also the amylase activity was

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evaluated with amylopectin azure and amylose azure [20]. Briefly, 0.5 mL of amylose azure (20 mg mL^{-1} in 0.2 M phosphate buffer pH 7.0), and 1 mL of the culture supernatant at 50 ºC for 60 min. Reaction was stopped by heating the tubes 15 min in boiling water. After cooling, the tubes were centrifuged at 10,000 rpm for 5 min, and OD_{600} was measured for the supernatant. Blank control was the same mixture but the culture supernatant (enzyme) was previously inactivated by heat. One amylase unit is defined as amount of enzyme that produces an increment of 0.01 OD_{600} in 1 h, under the test conditions.

Glucoamylase

Exoamylase releases glucose from the non-reduced ends of amylose and amylopectin; therefore this evaluation is based on the quantification of glucose with glucose oxidase, a specific enzyme that transforms glucose in gluconic acid and H_2O_2 [23,24]. One unit of glucoamylase was the amount of enzyme that produces an increment of 0.01 OD_{540} in 1 h.

Maltase

For this assay, a mixture of 1.0 mL of enzyme and 19.7 % (w/v) maltase in 0.1 M phosphate buffer (pH 6.8) was incubated at 28 ºC for 60 min. Then, the glucose produced was determined by the glucose oxidase reaction [23,24]. One unit of maltase produces an increase of 0.01 OD_{540} in 1 h.

α-glucosidase

The enzyme was evaluated with chromogenic analogue substrate *p*-nitrophenyl-α-D-glucopyranoside (pNGP). The reaction mixture composed by 0.5 mL of enzyme, 0.3 mL of substrate and 0.2 mL of the same buffer was incubated at 37 ºC for 120 min. The reaction was finished by adding 3.0 mL of 0.1 M Na_2CO_3. The p-nitrophenyl produced was determined by reading OD_{410}. Blanks were prepared by using the enzyme previously inactivated with Na_2CO_3. One unit of α-glucosidase produces an increase of 0.01 OD_{410} nm in 1 h [10].

Protease

The protocol used [25], mixture of 0.2 mL of enzyme with 0.3 mL of 2% (w/v) azocasein dissolved in 0.2 M glycine–NaOH buffer (pH 9.0) was incubated at 37 ºC for 60 min. The reaction was ended by adition of cold 13 % (w/v) of trichloroacetic acid (TCA), then stored for 30 min at 5 ºC. The precipitate formed was discarted by centrifugation at 10,000 rpm for 5 min, then aliquots of 1.0 mL were neutralized with 1.0 mL of 1 N NaOH and OD_{440} was measured. One protease unit was expressed as amount of enzyme required for an increase of 0.01 OD_{440} per hour, under the reaction conditions employed.

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Products and substrate evaluations

The global accumulation of reduced sugars in the culture was determined with DNS method [21]. The results were interpolated in calibrations curves prepared with glucose or maltose as the standards. The glucose accumulated was measured with the glucose oxidase method describe above. The soluble proteins were calculated with the Bradford method [26] used seroalbumin as a standard. The residual starch was evaluated with Wilson & Ingledew [22] protocol. The absorbance data were interpolated in a calibration curve of starch from 1 to 10 mg mL^{-1}.

TLC analysis of carbohydrates

For the identification of oligosaccharides accumulated in the culture during the amylase biosynthesis and produced by hydrolyzing the starch *in vitro*, the samples (10 mL) samples were lyophilized and then dissolved in 1.0 mL of 0.1 M phosphate buffer (pH 7.0). The total volume applied were 8 µL and 20 µL for saccharification process with the crude amylase and the pure amylase, respectively. Glucose, maltose and six glucose-composed oligosaccharides were placed as standards. The chromatogram was developed with a solvent composed by n-butanol/methanol/ammonium hydroxide/water (50:40:20:10) at room temperature for 24 h. After that, drying chromatograms were stained with a methanolic solution of 4% trifenyltetrazolium chloride and heated at 110 °C [27].

The α-amylase purification

The supernatant (125 mL) was filtrated and then concentrated by lyophilization (Labanco 45F1) for about 12 h. The dried residue was dissolved in 12.5 mL of 0.2 M phosphate buffer (pH 7.0). In all the purification steps the activities of amylase, protease and protein content were evaluated to determine the specific activities (enzymatic units per mg protein), to obtain the purification factors.

The first column fractionation of amylase was carried out by loading 4 mL of enzyme solution into a CM-Sephadex G-50 column (1.5 x 23 cm), previously equilibrated with 0.05 M phosphate buffer (pH 7.0) and eluted with stepwise a discontinuous concentration gradient of NaCl from 0 to 0.5 M, with a flow rate of 0.5 mL min^{-1}. Fifty-five fractions of 2.0 mL were collected using a Spectra/Chrom Mod. CF automatic fraction collector. The absorbance at 280 nm, amylase and protease activities were determined in each fraction. The fractions showed higher amylase activity were pooled, concentrated and dissolved in 500 mL of 0.2 M phosphate buffer (pH 7.0).

The second fractionation started by loading the above sample into a Shepadex G-100 ultrafine column (1.5 × 60 cm), eluted with 0.2 M phosphate buffer (pH 7.0). Sixty-three fractions of 800 µL each one were collected. The specific amylase and protease activities were measured.

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138 *Electrophoretic Analysis and Zymograms*

139 Samples containing 6 µg of proteins were suffered to SDS-PAGE [28]. The stacking and running gels were 3
140 and 10 % (w/v), respectively and the mini-Protean II Slab cell (Bio Rad) was used for the electrophoresis. The
141 electrophoresis was performed at 45V during 1 h and 95V for 3.5 h in a cold room (4 °C). The gels were stained
142 with Coomassie brillant blue [29] or silver nitrate method [30] .

143 The zymograms for visualize *in situ* amylase activity were performed in native-PAGE in two separated
144 polyacrylamide gels (8%, w/v), placing in one case 10 µL (9 ng) of the purified enzyme, and in the other one a
145 mixture of five protein markers with known MW. The running conditions were the same used in SDS-PAGE.
146 The gel with the protein markers was stained with Coomassie brilliant blue, and the amylase in gel was reveled
147 by immersion firstly in a solution of 2 % (w/v) starch in 0.2 M phosphate buffer (pH 7.0) for 60 min, followed
148 by a new immersion step in the same buffer but incubated at 37 ºC during 60 min. Finally, the gel was stained
149 with 0.04 N lugol solution for 25 min. The enzyme was visualized as a clear band in a blue background.

150 *Amylase characterization*

151 *Molecular weight (MW)*

152 The amylase MW was estimated with gel filtration and electrophoresis. In the first case, five proteins with MW
153 ranged from 14 to 272 kDa were passed through a column (1.5 x 60 cm) of Sephadex G-100 ultrafine, eluted
154 with 0.1 M of phosphate buffer (pH 7.0). The individual elution volumes (Ve) were determined. The void
155 volume (Vo) of the column was evaluated by the elution of blue dextran with a MW of 2000 kDa. The resulted
156 data were used to elaborate a calibration curve where the individual value of Ve/Vo, against the logarithm of
157 MW was obtained, for interpolating the ratio corresponding to the purified enzyme [31].

158 The electrophoretic determination of the MW was achieved with 10 % (w/v) SDS-PAGE (pH 8.3), amylase and
159 a set of six protein markers with known MW; after running the gel was stained with colloidal Coomassie blue
160 solution. The relative migration distance of amylase (Rf) was interpolated in a calibration curve obtained by
161 plotting the Rf values of the protein markers again the logarithm of their corresponding MW [32].

162 *Kinetic characterization*

163 The effect of pH on activity and stability of amylase was assayed at 50 ºC for 60 min with amylose azure at pH

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ranged from 4 to 11 with intervals of 0.5 units: the reaction system was buffered by 0.2 M of citrate (pH 4.0 to 5.5); phosphate (pH 6.0 to 7.5), and glycine-NaOH (pH 8.0 to11.0). For enzyme stability assay, the enzyme was pre-incubated for 60 min at the tested pH value and then the remaining activity was determined as mentioned above.

The temperature effect on amylase activity and stability was investigated between 10 to 70 ºC with intervals of 5 ºC. The test was carried out by incubating for 1 h the enzyme mixed with the substrate amylose azure (20 mg mL^{-1}) dissolved in 0.2 M phosphate buffer (pH 7.0). Thermo stability was determined by pre-incubating the enzyme at chosen temperature for 60 min. The residual activity was evaluated at the optimal conditions (pH 7.0 and 55 ºC).

Effects of chelating substrates

The pure amylase was dialyzed against 0.01 M of ethylenediaminetetraacetic acid (EDTA) during 3 h at room temperature. The samples were collected at 15, 30, 60, 90, 120, 150 and 180 min, the remained amylase activity was determined using the standard protocol. The reactivation of the EDTA-inactivated enzyme was studied by placing in the presence of chloride salts of the following divalent ions: Zn^{2+}, Ca^{2+}, Mg^{2+} and Co^{2+} at concentrations from 0.0001 or 0.01 M during 3h, then the amylase activity was tested.

Effect of specific and non-specific inhibitors

The actions of substances as $HgCl_2$, *p*-hydroxymercuribenzoate (*p*-HMB), urea and detergents were tested. The amylase was pre-incubated for 60 min at 55 ºC in presence of $HgCl_2$ and *p*-HMB at 0.0001 or 0.01 M; urea at 0.1 0.1, 0.5,1 M; and Tween 20 and SDS, respectively, at (0.1, 0.5 and 1 % v/v). The remained enzyme activity was evaluated in the standards conditions. In the case of enzyme treated with thiol-specific reagents, a new incubation process was carried out with the reducing agent β-mercaptoethanol (0.01 M) before determined the residual activity.

Results and discussion

Amylolytic system evaluation

The strain Bt LBIT-418 showed at least three amylolytic enzymes and morphological characterization indicated that the strain produces bipyramidal crystals after 96 h of incubation.

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The growth kinetics of Bt LBIT-418 showed a very short adaptation phase, and a exponential phase from 3 h to 9 h, reaching to 1.5 of OD_{600} and 1×10^9 CFU mL^{-1}. The starch concentration decreased from 8.6 mg mL^{-1} at 3 h of incubation to almost zero after the further incubation of six hours (Fig. 1c). The stationary growth phase started at 12 h and lasted to the end of the culture (51 h), maintaining the bacterial population at 1×10^8 (2.0 of OD_{600}) and 1×10^9 CFU mL^{-1} (2.3 of OD_{600}) by consuming the reducing sugars accumulated in the culture medium. The first peak of reducing sugars was detected in the culture after 3 h of incubatation, coinciding with α-glucosidase synthesis (180 U mL^{-1}) (Fig. 1b). As result of α-amylase activity, a second peak of reducing sugars occurred after 12 h of incubation (Fig 1c). The amylase and protease activities were detected from early stages in the growth of the strain and the values increased up to 180 U mL^{-1} and 220 U mL^{-1}, respectively, at first logarithmic phase from 3 to 12 h of incubation (Fig. 1a and Fig. 1b). The simultaneous production of amylase and protease was reported previously for some bacterial and filamentous fungi cultures [33,34,35,36], which could be highly advantageous for large scale extracellular enzyme production.

The reducing sugars were rapidly accumulated in the culture medium at 9th and 13th h that coincided with the expression of amylase. Although, the amylase synthesis was not recovered, the accumulation of reducing sugars remained almost constant from 20 to 36 h (Fig 1c). The reducing sugar concentration was consistent with the second exponential growth phase of the test strain. These results suggest that the strain Bt LBIT-418 produces at least two amylolytic enzymes, one in the beginning and another one in the ending of the potential phase of growth. The enzymatic measures with different protocols also evidence the presence of different types of amylolytic enzymes. The results showed that the methods using chromogenic substrates had greater sensibility [37] and they were not interfered by other substances in the enzymatic extract, in comparison with the DNS and Lugol methods. In the case of Lugol´s method, the components in the culture medium, such as residual starch, tryptone, peptone and corn steep liquor, could interfere in the iodine-starch complex formation [38] . The high concentration of reducing sugars presented in the extract enzyme interferes the results of DNS method, for this reason it was necessary to make dilutions. The DNS method has been used extensively [38, 39, 40, 41,42], but until now interference to the method has not been reported. According to the results in our study, the amylose-azure method was selected for further α-amylase assay.

Several studies reported the production of different amounts or types of glycoside hydrolases by the same organism [40, 41, 44]. Further kinetic studies showed that amylolytic system of Bt LBIT-418 was composed of at least three enzymes (Fig. 1b). The amylase activity evaluated with amylose-azure showed two increments of

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enzymatic activity with 150 and 180 U mL^{-1} at 9th and 13th h, respectively. The amylase activity units evaluated with amylopectin-azure was four times lower that that determined with amylose-azure at the same time (data not show). This may be due to that the enzyme had greater specificity for the linear molecules with α-1,4 glycosidic bands in amylose. The amylolytic activity assayed with Miller´s protocol presented an increase up to 5 U mL^{-1} at 13-21 h. The enzymatic activity determined with Lugol method showed the values increased up to 20 U mL^{-1} at the same time, greater than that evaluated with chromogenic dyed substrate (data not show).

The α-glucosidase activity was also observed from early stages in the growth of strain and the values increased up to 180 U mL^{-1} at the first exponential growth phase (5 h) and the activity decreased at 13 h; some other very small increments of enzymes were detected in the stationary growth phase (Fig. 1b). Production of intracellular α-glucosidase was determined using several carbohydrates with the PNPG from *B. thuringiensis* subspecies *kurstaki* HD-1 [45] . Although, the assay using PNPG was simple, reliable and precise, but it was expensive; therefore its application is limited to some specific test of amylase [46] . The glucoamylase and maltase activities were not detected in this study.

Futhermore the TLC chromatogram showed that after 18 h of incubation, amount of dextrins started to decrease, giving place to the formation and accumulation of smaller oligosaccharides such as glucose or maltose (Fig. 2). Smaller oligosaccharides accumulation in the culture extract was caused by activity of exoamylases, which could not be detected by using starch or amylose-azure as a substrate. Also these smaller reduced sugars allowed a second population increase observed between 15 and 20 h in the growth curve (Fig. 1a).

Kinetics studies of amylase repression

The cell growth pattern are very similar in the medium added with different substrates (supplementary Fig. S1a). The statistical analysis showed no significant difference (P=0.407). In the culture medium supplemented with starch (1%, w/v) the maximum amylase activity (123 U mL^{-1}) was observed in the logarithmic growth phase (10 h) while amylase activity decreased 90% in the medium supplemented with 1% (w/v) of both glucose and starch, suggesting that the presence of glucose repressed activity of amylase. Furthermore, amylase synthesis in the medium containing 1% of glucose alone was only 4% of that in medium with starch alone (supplementary Fig. S1b), demonstrating that the amylase synthesis was a induced. Therefore, the amylolytic system of Bt LBIT-418 is inducible and repressible, similar to the amylases in other bacteria or fungi [40,47,48,49,50] . Our results were different from those in *A. oryzae* DSM 63303 [45], *Bacillus* sp. MD 124 [51] , *Penicillium fellatum* [52],

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Rhizopus oryzae [53] and *A. pullulans* NRRL Y-12974 [54], in which no expression effect was observed. The protease synthesis was not significantly affected in any of the tested medium (information available in Fig. S1c).

Determination and repression of protease activity

The protease activity pattern was similar to that of amylase (supplementary Fig. S2). The maximum protease activity (220 U mL^{-1}) was observed at 13th h of incubation, corresponding to the end of the exponential phase of growth. In the protease repression assay, the cell concentration decreased around of 50 %. It was showed that the exponential phase started after 5 h of incubation, with bacterial population between 1×10^5 and 1×10^7 CFU mL^{-1}. The strain presented a stationary phase from 10th h to 25th h (supplementary Fig. S2a), that was very long in comparison with the standard medium. The amylase and protease activities were decreased 90% and 42%, respectively, in the medium supplemented with 1% of NH_4NO_3 (Fig. S2b and S2c). Statistical analysis showed significant differences in cell concentration and amylase activity in the two tested media, but the protease activity was less affected (42%) by presence of ammonia. Meanwhile, the decrease in protease activity was similar to the decrement in cellular concentration in the same medium, demonstrating that the proteases are essential for basic metabolism of the bacterium. Conversely, the amylase decreased 90% this value is greater than expected as result of the cellular concentration value in the kinetics growth.

Purification of amylolytic enzyme and electrophoretic analysis

The enzyme concentrated by freeze-drying and purified by cation exchange cromathography presented specific activity up to 3920.3 U mg^{-1} (Fig. 3a and Table 1). After the filtration gel (Sephadex ultrafine G-100) column, the purified amylase showed specific activity of 377,142.8 U mg^{-1} (Fig. 3b and Table 1) and 98.3–fold of purification with 14.7% yield. Purification results are summarized in Table 1 and Fig. 4.

Properties of amylase

The properties of enzyme are summarized in Table 2. The activity in situ of the purified amylase from Bt LBIT-418 analyzed in zymogram revealed a single amylolytic band against starch (Fig. 4). This affirmation was corroborated with duplicated gel stained with silver (data not shown), in which only one band was observed with the same mobility as in the zymogram. The MW of the native enzyme from Bt LBIT 418 was 74.6 kDa (Fig. 5a) determinate by gel filtration, and that of the purified amylase was 83.2 kDa estimated by native gel (Fig. 5b). Both the values were in the range of amylases: from 10 to 210 kDa [47] . However the molecular weight of amylase from Bt LBIT 418 was apparently greater than those described for other strains of *B.*

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thutringensis: 35 kDa from Btk [20] and 51 kDa from Bt [17,18] ; as well as greater than that of the commonly detected amylases from different microorganisms, which are normally between 50 to 60 kDa [55]. Since *B. cereus* and *B. thuringuiensis* are genetically similar, their amylases might have similar features. Table 4 showed the comparison between amylases from *B. cereus* and *B. thuringuiensis.* The molecular mass of *B. cereus* amylases ranges between 55 to 160 kDa [56,57,58] , the molecular mass of Btk LBIT-418 α-amylase was found in this range.

In the TLC analysis, oligosaccharides with five or more glucose residues were detected as major end products from the extract enzyme. Glucose and maltose monomers were also detected at very low amounts (Fig. 6). These results were consisted with the kinetics experiments, that amylolytic system of Bt LBIT 418 possesses endoamylases for producing dextrins, but also contain exoamylases that produce glucose and maltose. Maltose was the major monomers in the end products of the purified amylase (Fig. 6), while glucose monomers were detected at lower amounts, proofing that the enzyme is α-amylase [59] . Also the detection of dextrins suggested that the purified α-amylase contains trace amounts of endoamylase. Similar results were reported for α-amylases of *B. subtillis* [60] and *C. acetobutylicum* [61] while the α-amylase of *B. cereus* NY-14 end product was dextrin of five glucose residues [62].

The amylase activity increased with the temperature to reach an optimum at 50 ºC and decreased at higher temperatures (Fig. 7b). Statistical analysis showed significant differences ($P \leq 0.001$), confirming that the optimum temperature of the tested amylase is 50 ºC. The enzyme was stable at 10 ºC through 60 ºC. The activity of amylase decreased 50% at 70 ºC and was completely inactivated at 80 ºC (Fig. 7b). This feature is also detected in α-amylases produced by other *Bacillus* species such as *B. thermoleovorans*, *B. licheniformis*, and *B. sphaericus* [63] .

The optimum pH of different amylases ranges from 2 to 12 [55] , the amylases of several bacteria and fungi have an optimal pH in acid-neutral region [2] . As show in Fig. 7a, the amylase from Bt LBIT-418 presented high activity from pH 6 to 7.5, the amylolytic activity descended 33% at pH 8.0 and was inactivated at pH 10.5. The enzyme was stable in a range of pH 7-8 at 50 ºC for 1 h (Fig. 7a). Statistical analysis ($P < 0.001$) demonstrated that the amylase was severely affected by the pH change, which coincided with several other reports [64,65,66] However, the amylase from *B. cereus* NY-14 [62] and *B. cereus* GA6 [67] showed stability in a wide range of pH: from 5.5 to 10 and from 7 to 11, respectively. In addition, the amylase from *B.*

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thuringiensis Btk presented stability in a range of 5 to 6.8, whereas the amylase from *B. thuringiensis* showed stability in a range of pH 3 to 4. These results demonstrated that the amylases produced by different *B. thuringiensis* strains presented various features.

The effects of chelating agents and ions on the amylase (Fig. 7c) revealed that the purified amylase was inactivated 50% after 15 min dialyzed against EDTA, indicating that some metal ions contribute to the enzyme activity, maybe by maintaining the molecular conformation; therefore the amylase of Bt LBIT-418 is a metalloenzyme. In enzyme preparations previously dialyzed against EDTA, 80% of the enzyme activity was recovered in presence of 0.001 M Ca^{2+} and 20% with 0.001 M of Co^{2+}, Mg^{2+} and Zn^{2+} (supplementary Fig. S3), demonstrating that the inactivation with EDTA was reversible and calcium ion is essential for activity of the studied amylase. So, the α-amylase of Bt LBIT-418 is a Ca^{2+}-dependent metallienzyme, similar to the amylases in previous report [2]. It has been reported that heavy metal ions, such as Hg^{2+}, Ag^{2+} and Cu^{2+}, inhibited the amylase activity [68] and adversely affected the stability of amylases, while Ca^{2+} and Mg^{2+} contribute to their stability [65]. Regarding, *B. cereus* and *B. thuringiensis*-secreted α-amylases have been reported to be inhibited by EDTA [18,62,69], but this inhibition was not found for the α-amylase from *B. subtillis* AX20 [40].

The enzyme activity was decreased 80% and 3% in presence of $HgCl_2$ and pHMB, respectively (supplementary Fig. 4a), indicating that the amylase could be depend on thiol groups at their active site. The α-amylase recovered the total activity when it was treated with β-MOH (supplementary Fig. 4b). The relative activity range of enzyme appeared to reside between 20 and 103% in the presence of different denaturing reagents (Table 3). Some of these results suggested the presence of thiol groups in the active site of α-amylase from Bt LBIT-418 and possibly the enzyme might have cysteine in the active site as due to the enzyme was inhibited by $HgCl_2$ and reactivated with β-MOH, but not inhibited by pHMB. In this respect, the $HgCl_2$ could react not only with SH-groups, that might react with ammonium groups present in lysine and arginine, but also was found in the active site of enzyme [70] . The *B. cereus* NY-14 amylase was strongly inhibited by some metal ions such as Fe^{2+}, Cu^{2+} and Hg^{2+} suggesting that it was a thiol-enzyme. However, other thiol-specific reagents, 0.1 M pCMB and 1 mM Iodoacetamide, did not inhibit amylase [62], similar to the observation in the amylase from Bt LBIT-418.

The activity decreased between 50 and 80% at different concentrations of the anionic detergent SDS. Tween 20 decreased 2-40% enzymatic activity at 0.1 and 0.5 M. The effects of surfactants on amylase activity were very high; hence the enzyme may be not used in the detergent industries. Finally, the urea did not significantly affect

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the activity; and the amylase retained about 85-99% activity (supplementary Fig. S5), suggesting that the amylase has no hydrophobic amino acid residues [71].

Conclusion

The amylolytic system of Bt LBIT-418 consisted of at least there enzymes: endoamylase, exoamylase and α-glucosidase. Also the strain produces protease at the same time. The purified amylase exhibits MW of 83.2 kDa, is a metalloenzyme depending on Ca^{2+}. The production of amylase is induced by starch and repressed by glucose. In the future studies Bt LBIT-418 could be used to co-produce amylase and protease and thereby increase the potential biotechnological of the strain.

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504 **Legends of the figures**

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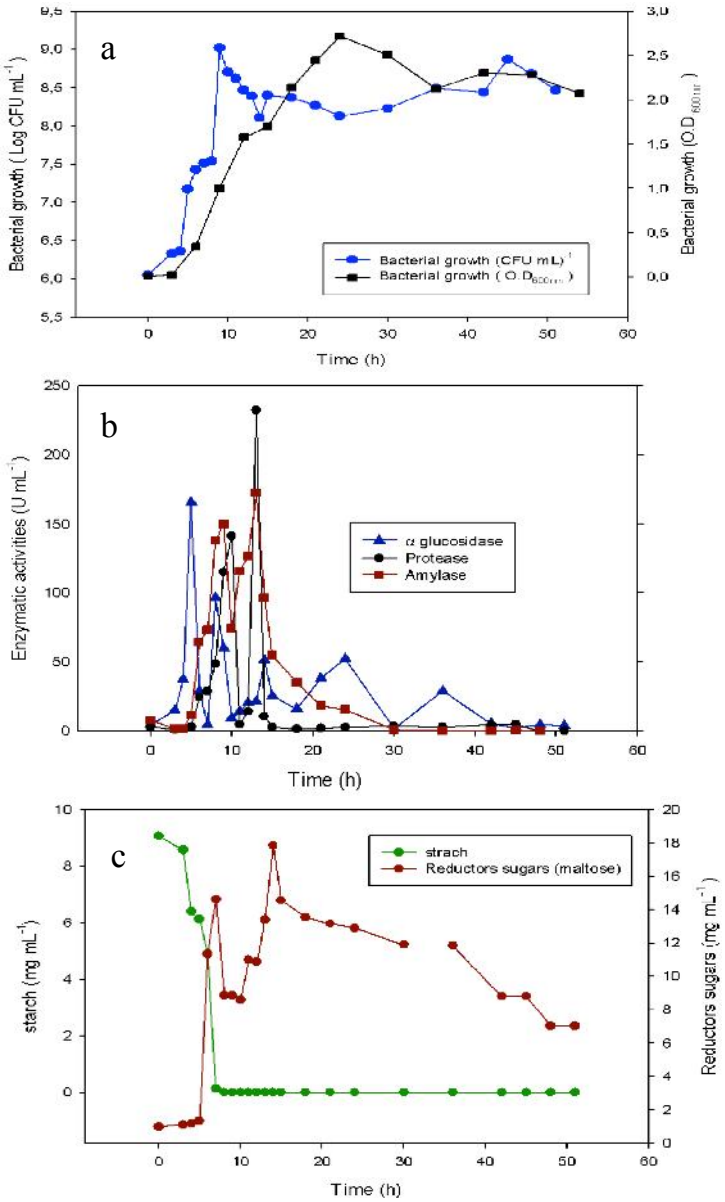

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508 **Fig. 1.** Kinetics of amylase production of strain *B. thuringiensis* (Bt) LBIT 418 in medium MB with 1% (w/v)

509 soluble starch at room temperature, 180 rpm and pH 6.5. (a) bacterial growth (log CFU mL^{-1}) and optical

510 density at 600 nm. (b) Enzymes production (c) residual starch and reducing sugar accumulation.

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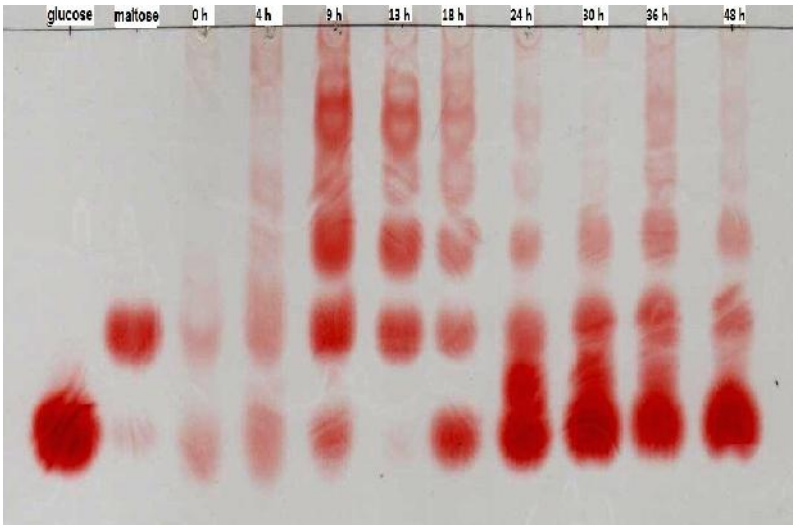

Fig. 2. Reducing sugars accumulated in the culture medium during amylase production identified by TLC.

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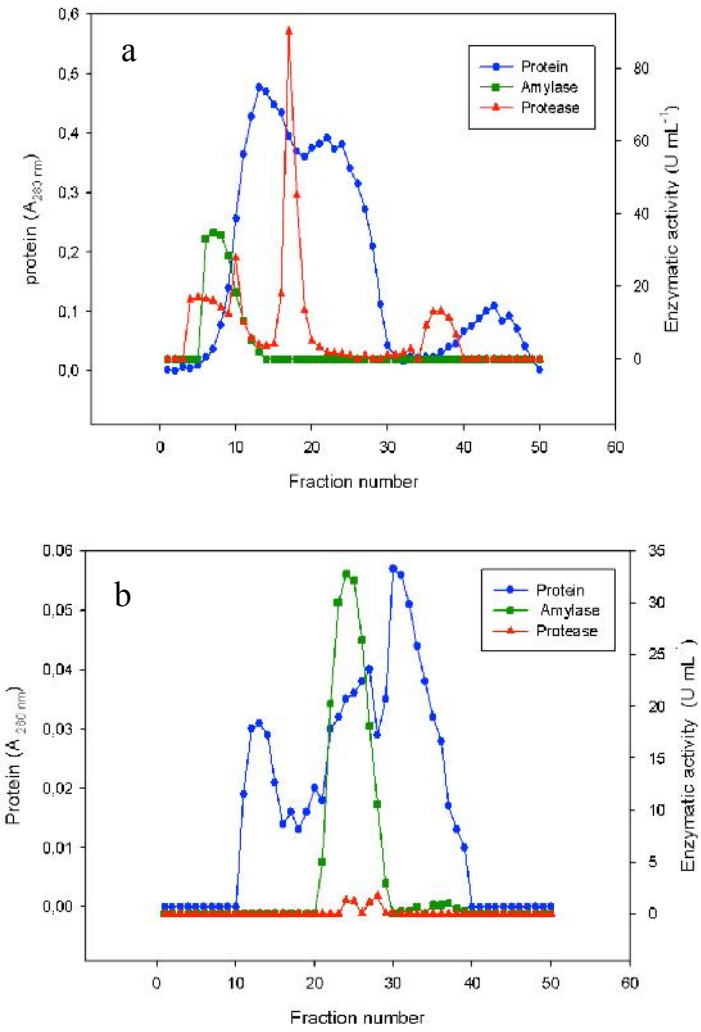

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528 **Fig. 3**. Purification profile of the extracellular amylase from Bt LBIT 418 obtained in medium MB with 1%
529 (w/v) soluble starch at room temperature, 180 rpm and pH 6.5 after 13 h of incubation. (a) Profile obtained with
530 CM Sephadex C-50 column. (b) Chromatography pattern with Sephadex G-100.

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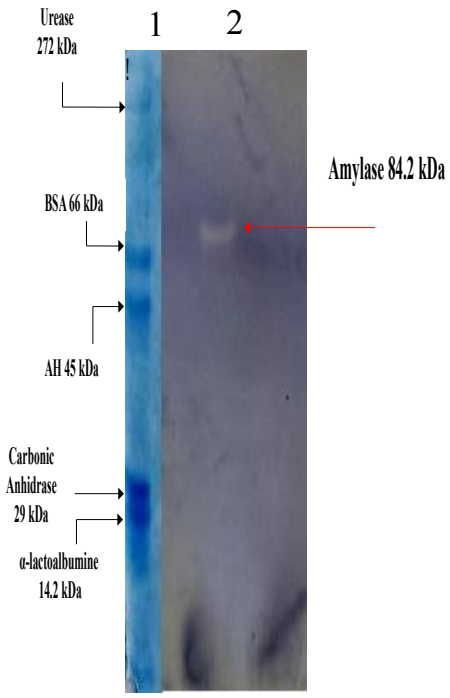

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536 **Fig. 4**. Electrophoretic analysis of extracellular α-amylase of strain Bt LBIT 418. Zymograme. Line 1,

537 molecular mass markers. Lane 2, native-PAGE obtained from α-amylase obtained Sephadex G-100 ultrafine.

UNDER PEER REVIEW

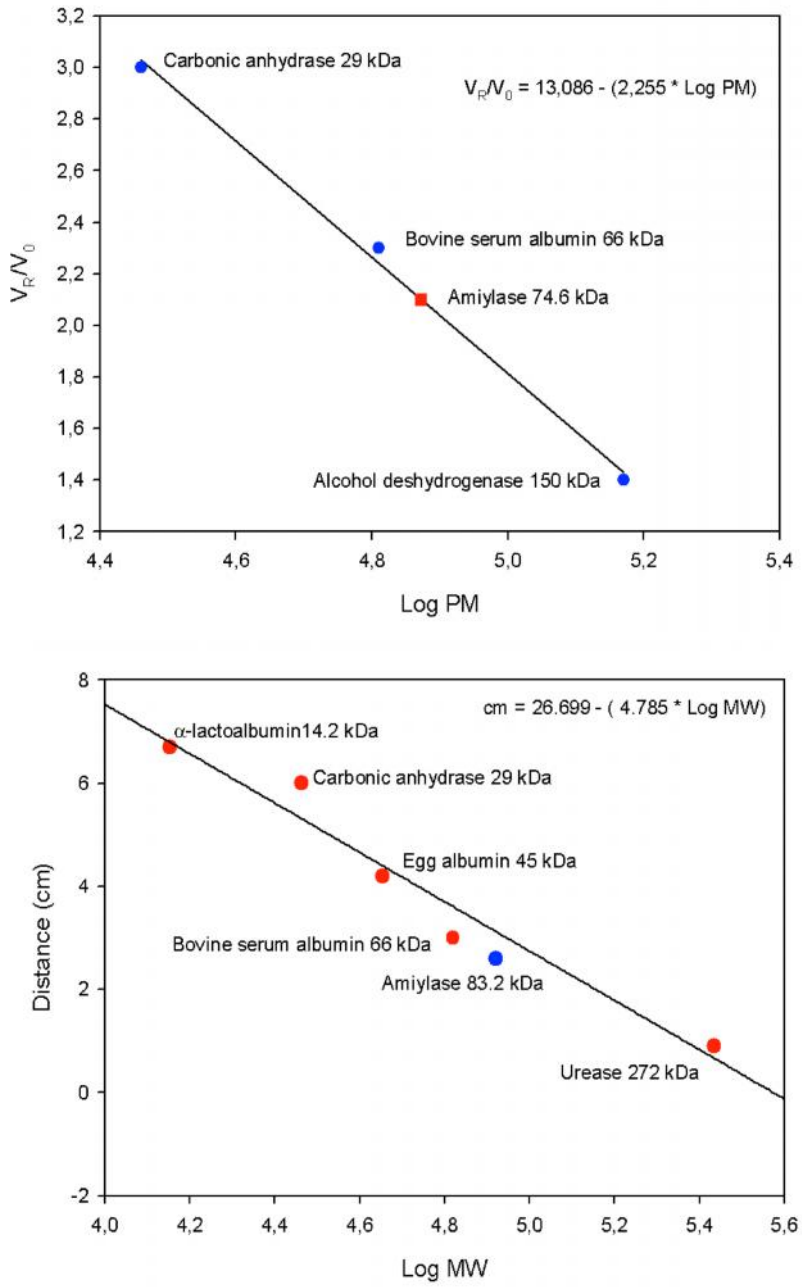

Fig. 5. Determination of MW of amylase. (a) Calibration curve of Sephadex G-100 profile. (b) Calibration curve of SDS-PAGE assay.

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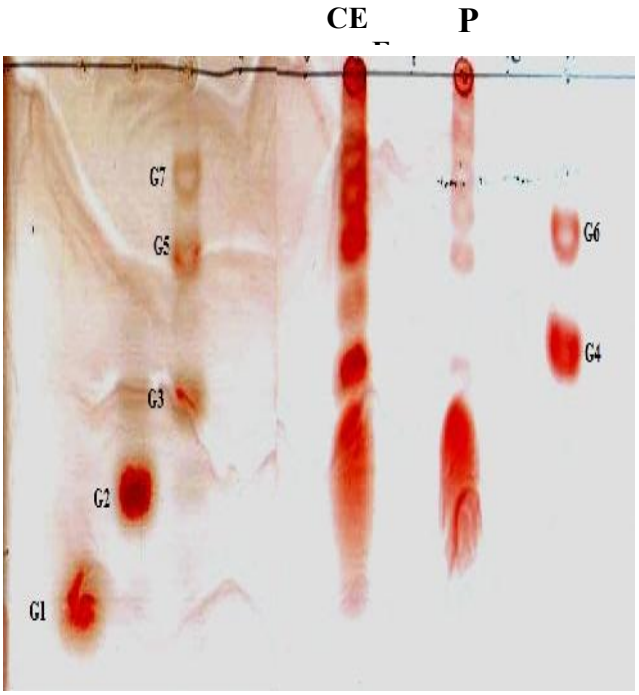

Fig. 6. TLC analysis of reducing sugars from in vitro hydrolysis of starch using crude extract of amylase (EC) and pure amylase (EP). Glucose (G1), maltose (G2), maltotriose (G3), maltotetraose (G4), maltopentaose (G5), maltohexaose (G6) and maltoheptaose (G7).

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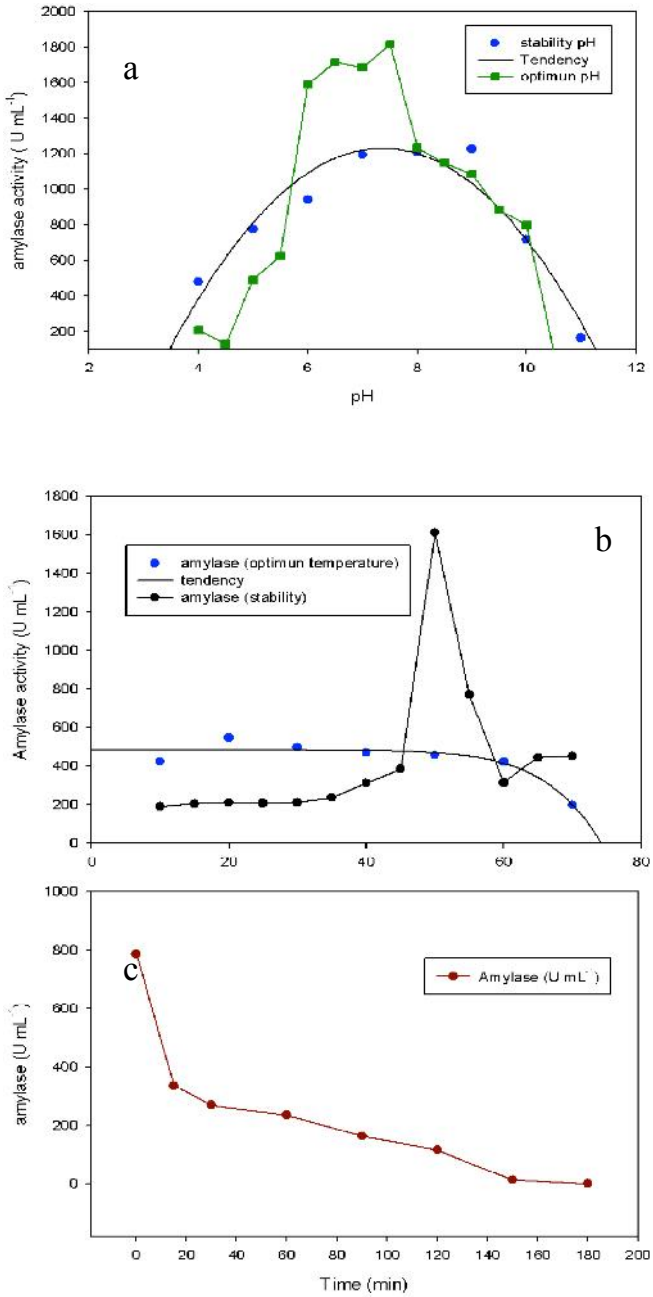

Fig. 7. Characterization of purified amylase (a) optimum pH of pH stability (b) Effect of temperature on the activity of purified α-amylase and optimum temperature activity under different temperatures at pH 9.0. (c) Effect of EDTA on purified amylase.

UNDER PEER REVIEW

562 **Table 1**. Purification process of amylase from *B. thuringiensis* (Bt) LBIT-418

Step	Total activity (U)	Total protein (mg)	Specific activity U mg^{-1}	Yield (%)	Fold
Enzymatic extract	4,845	1.26	3,837.3	100	1
DEAE-CM-Sephadex C-50	758	0.193	3,920.3	15.6	1.02
Sephadex-Ultrafine G-100	712.8	0.0019	377,142.8	14.7	98.3

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566 **Table 2**. Properties of amylase from *B. thuringiensis* (Bt) LBIT-418

Property	Value
Optimum temperature	50 ºC
Temperature stability	10-60 ºC
Optimum pH	7.5
pH stability	7-8
Metal ion requirement	Ca^{2+}
Inhibitors	Hg^{2+}
Mode of action	Endo
M_r by	
Sephadex-Ultrafine G-100	74.6 kDa
Native-PAGE	82.4 kDa

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569 **Table 3**. Effect of chemical reagents at optimum conditions

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Chemical reagent (M) or *(v/v).	%Relative activity (pH: 7.5, 50 ºC)
Absence in chemical reagent % relative activity is 100	
EDTA	
0.001	50
p-HMB	
0.0001	97
0.01	95
$HgCl_2$	
0.0001	25
0.01	20
Urea	
0.1	100
0.5	90
1	85
Tween 20*	
0.1	98
0.5	78
1	60
SDS*	
0.1	50
0.5	43
1	20

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576 **Table 4. Comparison of properties of some amylases from B. cereus with the purified α-amylase from B. thuringiensis.**

Source	M_r (kDa)	opt. pH	pH stability	opt. tem (ºC)	Tem. Stability (ºC)	Inhibitors	Requirements	References
B. cereus NY-14	55	6.0	5.5 to 10	55	below 35	ND	Ca^{2+}	Yoshigi et al.1985
B. cereus MTCC 10205	55	5.5	ND	55	ND	EDTA	Ca^{2+}	Kumari et al. 2013
B. cereus GA6	55	9	7 to 11	22	4- 37	Fe, Zn, Cu	Ca^{2+}	Roohi et al. 2013
B. thuringiensis (Btk)	51	ND	ND	ND	ND	ND	ND	Smitha et al. 2013
B. thuringiensis (Btk)	51	6.0	5 to 6.8	60	50-65	EDTA, SDS	Ag^{2+}, Ca^{2+},Cu^{2+}, Mg^{2+},Mn^{2+}	Smintha et al. 2015
B. thuringiensis	32	4.0	3 to 4	35	ND	ND	ND	Zusfahair et al. 2016
B. thuringiensis (Bt) LBIT-418	74.6/83.2	7.5	7-8	50	10-60	EDTA, Hg, pHMB, SDS, Tween 20	Ca^{2+}	This study

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