

Extracellular amylases produced by *Bacillus thuringiensis*

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

Abstract

Bacillus thuringiensis has showed a great biotechnological potential due to its insecticidal Cry-proteins production. Besides this bacterium produces different extracellular enzymes. The aim of this work was evaluate the amylolytic system of *B. thuringiensis* strain Bt LBIT 418 cultured in a liquid medium, with starch as carbon source, where the bacteria secreted the maximum amount of endoamylase after 10 h of incubation, in the middle of the exponential phase of growth. Its cleaves action-released dextrans composed by five of more glucose residues. An exoamylase able to convert these dextrans in maltotriose, maltose and glucose appeared at once, given rise to a new increment of the bacterial growth. An α -glucosidase was also secreted, but covering the 40 h of the 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 lyophilized supernatants from 13 h cultures, 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 °C, while the enzyme stability was higher at pH 6-7.5 and 55 °C. The enzyme was inactivated with 0.01 M EDTA and reactivated with 0.01 M CaCl₂, showed that this amylase was a metalloenzyme. Furthermore, its thiol-dependent characteristic was unclear, because the enzyme was inhibited by 0.01M HgCl₂, but did not by 0.01 M pMB. The results suggest that Bt LBIT 418 produced at least three different amylases and it is the first report concerning to the characterization of the amylolytic system of *B. thuringiensis*. This supports hypothesis that is possible to obtain in a sole fermentation process the insecticidal Bt microcrystals, as well as extracellular enzymes with potential biotechnological applications as the amylases.

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

27

28 **Introduction**

29 Industrial enzymes have formed a market of about 6.2 USD billions by 2020 [1]. Amylases are a large group of
30 enzymes degrading starch and related oligosaccharides and polysaccharide. Amylases have been utilized in
31 breweries, and in diverse areas, such as textile, distilling [2,3], pharmaceutical industries [4] and in baking
32 industries [5]. Since starch is one of the important sources of energy for organisms, amylases could be produced
33 by a great variety of living systems [6]. According to their functions, four groups of amylases are recorded:
34 endoamylases, exoamylases, debranching enzymes, and transferases.

35 Endoamylases are able to cleave α , 1-4 glycosidic bonds present in the inner part (endo) of amylose or
36 amylopectin chain. The enzyme α -amylase (EC 3.2.1.1) is a well-known endoamylase [2]. The end products of
37 α -amylase action are oligosaccharides with varying length, such as the α -configuration and α -limit dextrins,
38 which are branched oligosaccharides [7]. Enzymes belonging to the group exoamylases either exclusively
39 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
40 like amyloglucosidase or glucoamylase (EC 3.2.1.3) and α -glucosidase (EC 3.2.1.20). Exoamylases act on the
41 external glucose residues of amylose or amylopectin and thus produce only glucose (glucoamylase and α -
42 glucosidase), or maltose and β -limit dextrin (β -amylase). β -amylase and glucoamylase also convert the anomeric
43 configuration of the liberated maltose from α - to β -figuration. Glucoamylase and α -glucosidase differ in their
44 substrate preference: α -glucosidase acts best on short malto-oligosaccharides and liberates glucose with α -
45 configuration while glucoamylase hydrolyzes long chain polysaccharides best [2]. The endoamylases and
46 exoamylases are mostly produced by bacteria and the genus of *Bacillus* have been extensively studied for their
47 production of extracellular amylases [8,9,10,11].

48 The species *B. thuringiensis* (Bt) are sporulating Gram-positive bacterium, known mainly for its production of
49 crystal-like bodies comprised of one or more Cry-proteins, which can be toxic for insects, nematodes and cancer
50 cells [12]. Also Bt secreted extracellular enzymes as chitinase [13], chitosanase [14], protease [15]. Recently,
51 crude preparations of the chitinase from Bt were used to hydrolyze colloidal chitosan to produce different
52 oligosaccharides with potential pharmaceutical applications [16]. In addition the amylase of Bt was recovered as

53 byproduct from supernatant released during the harvest of δ -endotoxin using biphasic fermentation strategy and
54 then was purified and characterized [17,18]. Also amylase from Bt was isolate from tapioca waste [19].

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

58

59 **Materials and Methods**

60 *Bacterial strain*

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

64 *Culture medium*

65 The basal medium (BM) consisted of (g L⁻¹, pH 6.5): 0.375 K₂HPO₄; 0.250 NaCl; 0.275; 0.275MgSO₄ and 0.375
66 Na₂CO₃; 1.0 casaaminoacids and 1.0 yeast extract. BM served to assay the constitutive production of amylase
67 [20]. **Basal medium** complemented with glucose 1 % (w/v) to repress the amylase synthesis, and casamino acids
68 were substituted by 1% (w/v) of NH₄NO₃ to explore the protease synthesis inhibition.

69 *Culture conditions*

70 For kinetic experiment, a fresh colony was inoculated in 25 mL of the test medium and cultured for 12 h (180
71 rpm) at 28°C, and this cultivation was repeated two times by transferring 0.1 mL of the culture in the subsequent
72 fresh medium. Aliquots of 0.1 mL of the last culture were inoculated in serial of 125 mL Erlenmeyer flask
73 containing 25 mL of **basal medium**. The flasks were incubated for 48h, a flask was sampled every 60 min during
74 the first 14 h of incubation, and then at intervals of 2-3 h. Bacterial growth of each **collected cultures was**
75 **determined either by reading the absorbance at 600 nm (OD₆₀₀) or by counting the colony forming units (CFU**
76 **mL⁻¹). For analyses of enzyme activities, the culture samples were centrifuged at 600 rpm for 15 min at 5 °C**
77 **(Sorval centrifuge RC-SB) and supernatants were filtered and then stored in vials at 5 °C for further use.**

78 ***Enzymatic assays***

79 *Amylase*

80 Preliminary evaluations were achieved by using two methods. In the first, 0.5 mL of 1 % (w/v) soluble starch in
81 0.2 M phosphate buffer (pH 7.0), and 0.5 mL of the culture supernatant were mixed and incubated at 50 °C for
82 60 min. Then, reduced sugars released in the mixture were quantified with DNS method [21]. The second
83 method was based on the evaluation of non-hydrolyzed starch with lugol [22]. Also the amylase activity was
84 evaluated with amylopectin azure and amylose azure [20]. Briefly, 0.5 mL of amylose azure (20 mg mL⁻¹ in 0.2
85 M phosphate buffer pH 7.0), and 1 mL of the culture supernatant at 50 °C for 60 min. Reaction was stopped by
86 heating the tubes 15 min in boiling water. After cooling, the tubes were centrifuged at 10,000 rpm for 5 min, and
87 OD₆₀₀ was measured for the supernatant. Blank control was the same mixture but the culture supernatant
88 (enzyme) was previously inactivated by heat. One amylase unit is defined as amount of enzyme that produces an
89 increment of 0.01 OD₆₀₀ in 1 h, under the test conditions.

90 *Glucoamylase*

91 Exoamylase releases glucose from the non-reduced ends of amylose and amylopectin; therefore this evaluation
92 is based on the quantification of glucose with glucose oxidase, a specific enzyme that transforms glucose in
93 gluconic acid and H₂O₂ [23,24]. One unit of glucoamylase was the amount of enzyme that produces an
94 increment of 0.01 OD₅₄₀ in 1 h.

95 *Maltase*

96 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
97 incubated at 28 °C for 60 min. Then, the glucose produced was determined by the glucose oxidase reaction
98 [23,24]. One unit of maltase produces an increase of 0.01 OD₅₄₀ in 1 h.

99 *α-glucosidase*

100 The enzyme was evaluated with chromogenic analogue substrate *p*-nitrophenyl- α -D-glucopyranoside (pNGP).
101 The reaction mixture composed by 0.5 mL of enzyme, 0.3 mL of substrate and 0.2 mL of the same buffer was
102 incubated at 37 °C for 120 min. The reaction was finished by adding 3.0 mL of 0.1 M Na₂CO₃. The *p*-
103 nitrophenyl produced was determined by reading OD₄₁₀. Blanks were prepared by using the enzyme previously
104 inactivated with Na₂CO₃. One unit of α -glucosidase produces an increase of 0.01 OD₄₁₀ nm in 1 h [10].

105 *Protease*

106 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
107 glycine–NaOH buffer (pH 9.0) was incubated at 37 °C for 60 min. The reaction was ended by addition of cold 13 %

(w/v) of trichloroacetic acid (TCA), then stored for 30 min at 5 °C. The precipitate formed was discarded 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 OD₄₄₀ was measured. One protease unit was expressed as amount of enzyme required for an increase of 0.01 OD₄₄₀ per hour, under the reaction conditions employed.

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 was 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.

Electrophoretic Analysis and Zymograms

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

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

Amylase characterization

Molecular weight (MW)

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

The electrophoretic determination of the MW was achieved with 10 % (w/v) SDS-PAGE (pH 8.3), amylase and

a set of six protein markers with known MW; after running the gel was stained with colloidal Coomassie blue solution. The relative migration distance of amylase (Rf) was interpolated in a calibration curve obtained by plotting the Rf values of the protein markers again the logarithm of their corresponding MW [32].

Kinetic characterization

The effect of pH on activity and stability of amylase was assayed at 50 °C for 60 min with amylose azure at pH 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 to 11.0). 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 °C with intervals of 5 °C. 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 °C).

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: Zn²⁺, Ca²⁺, Mg²⁺ and Co²⁺ 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 HgCl₂, *p*-hydroxymercuribenzoate (*p*-HMB), urea and detergents were tested. The amylase was pre-incubated for 60 min at 55 °C in presence of HgCl₂ or *p*-HMB at 0.0001 or 0.01 M; urea at 0.1, 0.5, 1 M. Also Tween 20 or SDS at (0.1, 0.5 and 1 % v/v) were tested. 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.

191 **Results and discussion**

192 *Amylolytic system evaluation*

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

195 The growth kinetics of Bt LBIT-418 showed a very short adaptation phase, and a exponential phase from 3 h to
196 9 h, reaching to 1.5 of OD₆₀₀ and 1×10⁹ CFU mL⁻¹. The starch concentration decreased from 8.6 mg mL⁻¹ at 3 h
197 of incubation to almost zero after the further incubation of six hours (Fig. 1c). The stationary growth phase
198 started at 12 h and lasted to the end of the culture (51 h), maintaining the bacterial population at 1 × 10⁸ (2.0 of
199 OD₆₀₀) and 1 × 10⁹ CFU mL⁻¹ (2.3 of OD₆₀₀) by consuming the reducing sugars accumulated in the culture
200 medium. The first peak of reducing sugars was detected in the culture after 3 h of incubation, coinciding with
201 α-glucosidase synthesis (180 U mL⁻¹) (Fig. 1b). As result of α-amylase activity, a second peak of reducing
202 sugars occurred after 12 h of incubation (Fig 1c). The amylase and protease activities were detected from early
203 stages in the growth of the strain and the values increased up to 180 U mL⁻¹ and 220 U mL⁻¹, respectively, at
204 first logarithmic phase from 3 to 12 h of incubation (Fig. 1a and Fig. 1b). The simultaneous production of
205 amylase and protease was reported previously for some bacterial and filamentous fungi cultures [33,34,35,36],
206 which could be highly advantageous for large scale extracellular enzyme production.

207 The reducing sugars were rapidly accumulated in the culture medium at 9th and 13th h that coincided with the
208 expression of amylase. Although, the amylase synthesis was not recovered, the accumulation of reducing sugars
209 remained almost constant from 20 to 36 h (Fig 1c). The reducing sugar concentration was consistent with the
210 second exponential growth phase of the test strain. These results suggest that the strain Bt LBIT-418 produces at
211 least two amylolytic enzymes, one in the beginning and another one in the ending of the potential phase of
212 growth. The enzymatic measures with different protocols also evidence the presence of different types of
213 amylolytic enzymes. The results showed that the methods using chromogenic substrates had greater sensibility
214 [37] and they were not interfered by other substances in the enzymatic extract, in comparison with the DNS and
215 Lugol methods. In the case of Lugol's method, the components in the culture medium, such as residual starch,
216 tryptone, peptone and corn steep liquor, could interfere in the iodine-starch complex formation [38]. The high
217 concentration of reducing sugars presented in the extract enzyme interferes the results of DNS method, for this
218 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 [39, 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 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 than 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.

Furthermore the chromatogram obtained using TLC showed that after 18 h of incubation, amount of dextrans 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 an induced. Therefore, the amylolytic system of Bt LBIT-418 is inducible and repressible, similar to the amylases in other bacteria or fungi [39,47,48,49,50]. Our results were different from those in *A. oryzae* DSM 63303 [45], *Bacillus* sp. MD 124 [51], *Penicillium fellatum* [52], *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. S1e).

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 chromatography 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 $377,142.8 \text{ U mg}^{-1}$ (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. thuringensis*: 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. thuringiensis* are genetically similar, their amylases might have similar features. Table 4 showed the comparison between amylases from *B. cereus* and *B. thuringiensis*. 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 dextrans, 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 dextrans suggested that the purified α -amylase contains trace amounts of endoamylase. Similar results were reported for α -amylases of *B. subtilis* [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 °C 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 °C. The enzyme was stable at 10 °C through 60 °C. The activity of amylase decreased 50% at 70 °C and was completely inactivated at 80 °C (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 °C 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. 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 metallo-enzyme, 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. subtilis* 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 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 three 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.

References

- [1]Metha D & Satyanarayana T. 2016. Bacterial and Archaeal α -Amylases: Diversity and Amelioration of the Desirable Characteristics for Industrial Applications. *Front Microbiol.* doi: 10.3389/fmicb. 2016. 01129.
- [2]Pandey A, Nigam P, Scool CR, Scool VT, Sight D & Mohan R. 2000. Advances in microbial amylases. *Biotechnol Appl Biochem.* 31: 135-152.
- [3]Goyal N, Gupta JK, & Soni SK. 2005. A novel raw starch digesting thermostable α -amylase from *Bacillus* sp. I-3 and its use in the direct hydrolysis of raw potato starch. *Enzyme Microb Technol.* 37: 723-734.
- [4]Oudjeriouat N, Moreau Y, Santimone M, Svensson B, Marchis-Mouren G, Desseaux V. 2003. Mechanism of barley α -amylase. Inhibition of amylose, maltodextrin and maltoheptaose hydrolysis by acarbose and cyclodextrin. *Eur J Biochem* 270: 3871-3879.
- [5]Synowiecki J, Polaina J, & Mac CA. 2007. The use of starch processing enzymes in the food industry. *Industrial Enzymes, Structure, Function and Applications.* 19-34.

- 358 [6]Horvathova V, Janecek S, & Sturdik E. 2001. Amylolytic enzymes: molecular aspects of their properties.
359 *Gen Physiol Biophys.* 20: 7-32.
- 360 [7]Van der Maarel MJEC, van der Veen B, Uitdehaag JCM, Leemhuis H & Dijkhuizen L. 2002. Properties and
361 applications of starch-converting enzymes of the α -amylase family. *J Biotech.* 94: 137-155.
- 362 [8]Chávez- Camarillo GM 1995. Estudios de la X- prolil dipeptidil aminopeptidasa de levaduras. Tesis Doctoral
363 en microbiología, ENCB-IPN (Dissertation in Spanish).
- 364 [9]Ogunremi OR, & Sanni AI. 2011. Occurrence of amylolytic and/or bacteriocin-producing lactic acid bacteria
365 in Ogi and Fufu. *Ann Food Sci Technol.* 12: 71-77.
- 366 [10]Yang H, Liu L, Li J, Du G, & Chen J. 2011. Heterologous expression, biochemical characterization, and
367 overproduction of alkaline α -amylase from *Bacillus alcalophilus* in *Bacillus subtilis*. *Microb Cell Fac.* 10: 1.
- 368 [11]Li X, & Yu HY. 2012. Purification and characterization of novel organic-solvent-tolerant β -amylase and
369 serine protease from a newly isolated *Salimicrobium halophilum* strain LY20. *FEMS Microbiol Lett.*
370 329: 204-211.
- 371 [12]Ventura-Suárez A, Cruz-Camarillo, R, Rampersad J, Ammons DR, López-Villegas EO, Ibarra JE & Rojas-
372 Avelizapa LI. 2011. Characterization of a novel *Bacillus thuringiensis* phenotype possessing multiple
373 appendages attached to a parasporal body. *Curr Microbiol.* 62: 307-312.
- 374 [13]Barbosa-Corona JE, Contreras JC, Velazquez-Robledo R, Bautista-Justo M, Gomez RM, Cruz-Camarillo R
375 & Ibarra JE. 1999. Selection of chitinolytic strains of *Bacillus thuringiensis*. *Biotechnol Lett.* 21: 1125-
376 1129.
- 377 [14]Camarillo RC, Perez OS, Avelizapa NR, Ramirez MG & Avelizapa LR 2004. Chitosanase activity in
378 *Bacillus thuringiensis*. *Folia Microbiologica.* 49: 94.
- 379 [15]Rojas-Avelizapa LI, Cruz-Camarillo R, Guerrero MI, Rodríguez-Vázquez R & Ibarra JE. 1999. Selection
380 and characterization of a proteo-chitinolytic strain of *Bacillus thuringiensis*, able to grow in shrimp
381 waste media. *World J Microbiol Biotechnol.* 15: 299-308.
- 382 [16]Olicón-Hernández DR, Vázquez-Landaverde PA, Cruz-Camarillo R & Rojas-Avelizapa LI. 2017.
383 Comparison of chito-oligosaccharide production from three different colloidal chitosans using the
384 endochitosanolytic system of *Bacillus thuringiensis*. *Prep Biochem Biotechnol.* 47: 116-122.

385 [17]Smitha RB, Jisha VN, Sajith S & Benjamin S. 2013. 403401. Dual production of amylase and δ -endotoxin
386 by *Bacillus thuringiensis* subsp. *kurstaki* during biphasic fermentation. *Microbioly*. 82: 794-800.

387 [18]Smitha RB, Sajith S, Priji P, Unni KNN, Roy TAN & Benjamin S. 2015. Purification and characterization
388 of amylase from *Bacillus thuringiensis* subsp. *kurstaki*. *Bt Research*. 6.

389 [19]Zusfahair Z, Ningsih DR, Kartika D & Fatoni A 2016. Amylase from *Bacillus thuringiensis* isolated from
390 tapioca waste: isolation, partial purification and characterization. *Malaysian J Fundam Appl Sci*. 12.

391 [20]Salazar Carrillo, M, S. 2005. Actividad amilolítica extracelular en *Bacillus thuringiensis*. Tesis profesional
392 de IBQ, ENCB-IPN (Disertation in Spanish).

393 [21]Miller GL. 1959. Use of dinitrosalicylic acid reagent for determination of reducing sugars. *Anal Chem*. 31:
394 426-428.

395 [22]Wilson JJ & Ingledew WM. 1982. Isolation and characterization of *Schwanniomyces alluvius* amylolytic
396 enzymes. *Appl Environ Microbiol*. 44: 301-307.

397 [23]Dahlqvist A. 1961. Determination of maltase and isomaltase activities with a glucose oxidase reagent. *J*
398 *Biochem*. 80: 547-551.

399 [24]Worthington Biochemical Corporation 1972. Worthington Enzymes Manual. Enzymes, enzyme reagents
400 and related biochemicals. Freehold. New Jersey. USA. pag. 19-20.

401 [25]Pérez García, G. 2002. Relación entre las actividades de quitinasa y caseinasa en *Bacillus thuringiensis*.
402 Tesis de Maestría en CQB, ENCB-IPN. (Dissertation in Spanish).

403 [26]Bradford MN. 1976. A rapid and sensitive method for the quantitation of micrograms quantities of protein
404 utilizing the principle of protein-dye binding. *Anal Biochem*. 72: 248-254.

405 [27]Pacheco-Hernández MA. 2008. Purificación y caracterización de la endoquitinasa de la cepa Bt -132 de
406 *Bacillus thuringiensis*. Tesis de Maestría en CQB. ENCB-IPN. (Dissertation in Spanish).

407 [28]Laemmli UK & Favre M. 1973. Maturation of the head of bacteriophage T4: I. DNA packaging events. *J*
408 *Mol Biol*. 80: 575-599.

409 [29]Wang W, Lio X & Li Y. 2007. A modified Coomassie brilliant blue staining method at nanogram sensitivity
410 compatible with proteomic analysis. *Biotechnol Lett*. 29: 1599-1603.

- 411 [30] Wray W, Bouliskas T, Wray VP & Hancock R. 1981. Silver staining of proteins in polyacrylamide gels.
412 *Anal Biochem.* 118: 197-203.
- 413 [31]Whitaker JR. 1963. Determination of Molecular Weights of Proteins by Gel Filtration of Sephadex. *Anal*
414 *Chem.* 35: 1950-1953.
- 415 [32]Shapiro AL, Viñuela E & Maizel JV. 1967. Molecular weight estimation of polypeptide chains by
416 electrophoresis in SDS-polyacrylamide gels. *Biochem Biophys Res C.* 28: 815-820.
- 417 [33]Kim EY, Kim YH, Rhee MH, Song JC, Lee KW, Kim KS, Lee SP, Lee IS .& Park, S. C. 2007. Selection of
418 *Lactobacillus* sp. PSC101 that produces active dietary enzymes such as amylase, lipase, phytase and
419 protease in pigs. *J Gen Appl Microbiol.* 53: 111-117.
- 420 [34]Imanaka H, Tanaka S, Feng B, Imamura K, & Nakanishi K. 2010. Cultivation characteristics and gene
421 expression profiles of *Aspergillus oryzae* by membrane-surface liquid culture, shaking-flask culture, and
422 agar-plate culture. *J Biosci Bioeng.* 109: 267-273.
- 423 [35]Corrêa TLR, Moutinho SKDS, Martins MLL, & Martins MA. 2011. Simultaneous α -amylase and protease
424 production by the soil bacterium *Bacillus* sp. SMIA-2 under submerged culture using whey protein
425 concentrate and corn steep liquor: compatibility of enzymes with commercial detergents. *Food Sci*
426 *Technol.* (Campinas) 31: 843-848.
- 427 [36]Kalaichelvan PT. 2012. Production, purification and characterization of α -amylase and alkaline protease by
428 *Bacillus* sp. HPE 10 in a concomitant production medium. *Asian J Plant Sci Res.* 2: 376-382.
- 429 [37]Wong DWS, Batt SB & Robertson GH 2000. Microassay for rapid screening of alpha-amylase activity. *J*
430 *Agric Food Chem.* 48: 4540-4543.
- 431 [38]Manonmani HK. & Kunhi AM 1999. Interference of thiol compounds with dextrinizing activity assay of α -
432 amylase by starch-iodine color reaction: modification of the method to eliminate this interference. *World J*
433 *Microbiol Biotechnol.* 15: 485-487.
- 434 [39]Najafi MF, Deobagkar D & Deobagkar D 2005. Purification and characterization of an extracellular α -
435 amylase from *Bacillus subtilis* AX20. *Protein Expr Purif.* 41: 349-354.
- 436 [40]Hernández-Montañez Z, Juárez-Montiel M, Velázquez-Ávila M, Cristiani-Urbina E, Hernández-Rodríguez
437 C, Villa-Tanaca L, & Chávez-Camarillo G. 2012. Production and characterization of extracellular α -
438 amylase produced by *Wickerhamia* sp. X-Fep. *Appl Biochem Biotechnol.* 167: 2117-2129.

439 [41]Bello-López JM, Navarro-Noya YE, Gómez-Acata S, Hernández-Montañez Z & Dendooven L. 2014.
440 Identification of α -amylase by random and specific mutagenesis of *Texcoconibacillus texcoconensis*
441 13CCT strain isolated from extreme alkaline-saline soil of the former Lake Texcoco (Mexico). *Folia*
442 *Microbiologica*. 59: 235-240.

443 [42]Kocabay S, Çetinkaya S, Akkaya B, & Yenidünya AF. 2016. Characterization of thermostable β -amylase
444 isozymes from *Lactobacillus fermentum*. *Int J Biol Macrom*. 93: 195-202.

445 [43]Soto-Padilla MY, Gortáres-Moroyoqui P, Cira-Chávez LA, Levasseur A, Dendooven L, & Estrada-
446 Alvarado MI. 2016. Characterization of extracellular amylase produced by haloalkalophilic strain *Kocuria*
447 sp. HJ014. *Inter J Environ Health Res*. 1-9.

448 [44]Jin F, Li Y, Zhang, C & Yu H. 2001. Thermostable α -amylase and galactosidase production from the
449 thermophilic and aerobic *Bacillus* sp. JF strain. *Process Biochem*. 36: 559-564.

450 [45]Rowe GE & Margaritis A. 2004. Enzyme kinetic properties of α -1, 4-glucosidase in *Bacillus thuringiensis*.
451 *Biochem Eng J*. 17: 121-128.

452 [46]Sheehan H & McCleary BV. 1988. A new procedure for the measurement of fungal and bacterial α -amylase.
453 *Biotechnol Tech*. 2: 289-92.

454 [47]Lachmund A, Urmann U, Minol K, Wirsal S & Ruttkowski E. 1993. Regulation of α -amylase formation in
455 *Aspergillus oryzae* and *Aspergillus nidulans* transformants. *Curr Microbiol*. 26: 47-51.

456 [48]Morkeberg R, Carlsen M & Nielsen J. 1995. Induction and repression of α -amylase production in batch and
457 continuous cultures of *Aspergillus oryzae*. *Microbiology* 141: 2449-2454.

458 [49]Gupta R, Grigas P, Mohapatra H, Goswami VK & Chauhan B. 2003 Microbial amylases: a
459 biotechnological perspective. *Proc. Biochem*. 38: 1599-1616

460 [50]Esfahanibolandbalaie Z, Rostami K, & Mirdamadi SS. 2008. Some studies of alpha-amylase production
461 using *Aspergillus oryzae*. *Pakistan J Biol Sci: PJBS*. 11: 2553-2559.

462 [51]Jana M, Chattopadhyay DJ, & Pati BR. 1997. Effect of different carbon sources on the production of
463 amylase by *Bacillus* sp. MD 124. *Acta Microbiol Immunol Hung*. 45: 229-237.

464 [52]Kathiresan K & Manivannan S. 2006. α -Amylase production by *Penicillium fellutanum* isolated from
465 mangrove rhizosphere soil. *Afr J Biotechnol*. 5:10.

466 [53]Amadioha AC. 1999. Effect of cultural conditions on the growth and amylolytic enzyme production by
467 *rhizophus oryzae*. *Dis Inn*. 11: 69-73.

468 [54]Saha BC & Silman RW. 1993. Amylolytic enzymes produced by a color variant strain of *Aureobasidium*
469 *pullulans*. *Current Microbiol.* 26: 267-273.

470 [55]Vihinen M & Mäntsälä P. 1989. Microbial amylolytic enzymes. *Crit Rev Biochem Mol Biol.* 24: 329-418.

471 [56]Shinke R, Nishira H. & Mugibayshi N. 1974. Isolation of β -amylase producing micro-organisms. *Agric Biol*
472 *Chem.* 38: 665.

473 [57]Shinke R, Kunimi Y & Nishira H. 1975. Production and some properties of β -amylase of *Bacillus* sp BQ10.
474 *J Ferment Technol.* 53: 693.

475 [58]Nanmori T, Shinke R, Aoki K & Nishira H. 1983. Purification and characterization of β -amylase from
476 *Bacillus cereus* BQ10-S1 Spo II. *Agric Biol Chem.* 47: 941-947.

477 [59]Ray RR. 2000. Purification and characterization of extracellular beta-amylase of *Bacillus megaterium* B(6).
478 *Acta Microbiol Immunol Hung.* 47: 29-40.

479 [60]Matsuzaki H, Yamane K, Yamaguchi K, Nagata Y & Maruo B. 1974. Hybrid α -amylases produced by
480 transformants of *Bacillus subtilis*. I. Purification and characterization of extracellular α -amylases produced
481 by the parental strains and transformants. *Biochimica et Biophysica Acta (BBA)-Protein Structure.* 365:
482 235-247.

483 [61]Paquet V, Croux C, Goma G & Soucaille P. 1991. Purification and characterization of the extracellular
484 alpha-amylase from *Clostridium acetobutylicum* ATCC 824. *Appl Environmen Microbiol.* 57:212-218.

485 [62]Yoshigi N, Chikano T & Kamimura M. 1985. Purification and properties of an amylase from *Bacillus*
486 *cereus* NY-14. *Agric Biol Chem.* 49: 3369-3376.

487 [63]Sun H, Zhao P, Ge X, Xia Y, Hao Z, Liu J & Peng M. 2010. Recent advances in microbial raw starch
488 degrading enzymes. *Appl Biochem Biotechnol.* 160: 988-1003.

489 [64]Robyt JF & Ackerman RJ. 1971. Isolation, purification and characterization of maltotetraose- producing
490 amylase from *Pseudomonas stutzeri*. *Arch Biochem Biophys* 145: 105-114.

491 [65]Krishnan T & Chandra AK. 1983. Purification and characterization of α – amylase from *Bacillus*
492 *licheniformis* CUMC305. *Appl Environ Microbiol.* 46: 430-437.

493 [66]Coronado MJ, Vargas C, Hofemeister J, Ventosa A. & Nieto JJ. 2000. Production and biochemical
494 characterization of an α -amylase from the moderate halophile *Halomonas meridiana*. *FEMS. Microbiol*
495 *Lett* 183: 67-71.

- 496 [67]Roohi R, Kuddus M & Saima S. 2013. Cold-active detergent-stable extracellular α -amylase from *Bacillus*
497 *cereus* GA6: Biochemical characteristics and its perspectives in laundry detergent formulation. *J Biochem*
498 *Tech.* 4: 636-644.
- 499 [68]Dey G, Palit S, Banerjee R, & Maiti BR. 2002. Purification and characterization of maltooligosaccharide-
500 forming amylase from *Bacillus circulans* GRS 313. *J Ind Microbiol Biotechnol.* 28: 193-200.
- 501 [69]Tobey, T. F. & Yousten, A. A. 1976. Factors affecting the production of amylase by *Bacillus thuringiensis*.
502 En Developments in Industrial Microbiology. (Ed). Leland Unterkofler. Jekyll Island, Georgia, USA. 18:
503 499-510.
- 504 [70]Weeb J. 1963. Enzymes and Metabolic Inhibitors: General principles of inhibition (Vol. 1). Academic.
- 505 [71]Kiran KK & Chandra TS. 2008. Production of surfactant and detergent-stable, halophilic, and alkalitolerant
506 alpha-amylase by a moderately halophilic *Bacillus* sp. strain TSCVKK. *Appl Microbiol Biotechnol.* 77:
507 1023-1031.
- 508 [72]Kumari N, Jain V & Malhotra S. 2013. Purification and characterization of extracellular acidophilic α -
509 amylase from *Bacillus cereus* MTCC 10205 isolated from soil. *Global J Microbol Res.* 1:1-9

Legends of the figures

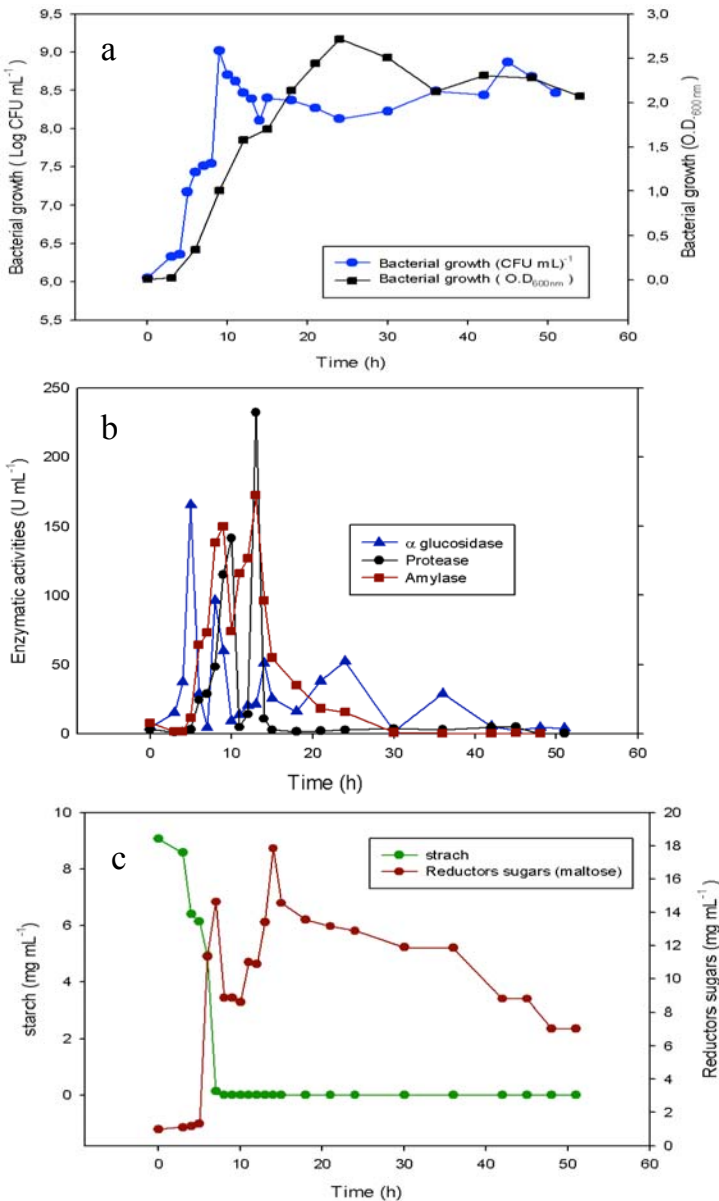


Fig. 1. Kinetics of amylase production of strain *B. thuringiensis* (Bt) LBIT 418 in medium MB with 1% (w/v) soluble starch at room temperature, 180 rpm and pH 6.5. (a) bacterial growth (log CFU mL⁻¹) and optical density at 600 nm. (b) Enzymes production (c) residual starch and reducing sugar accumulation.

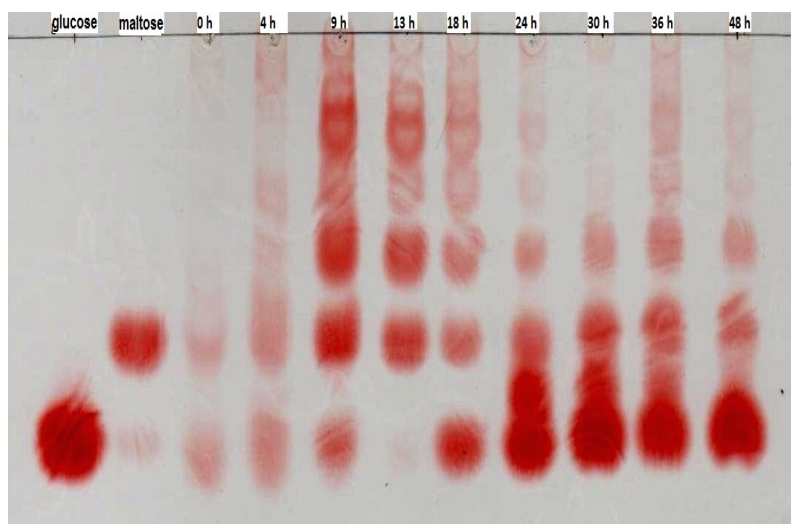


Fig. 2. Reducing sugars accumulated in the culture medium during amylase production identified by TLC.

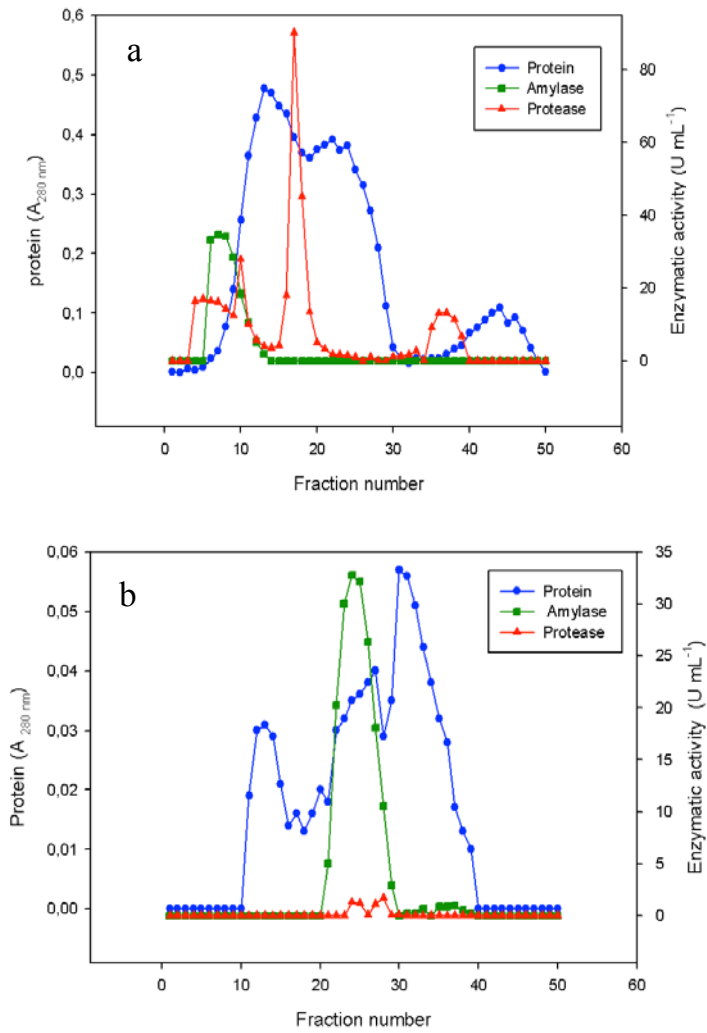
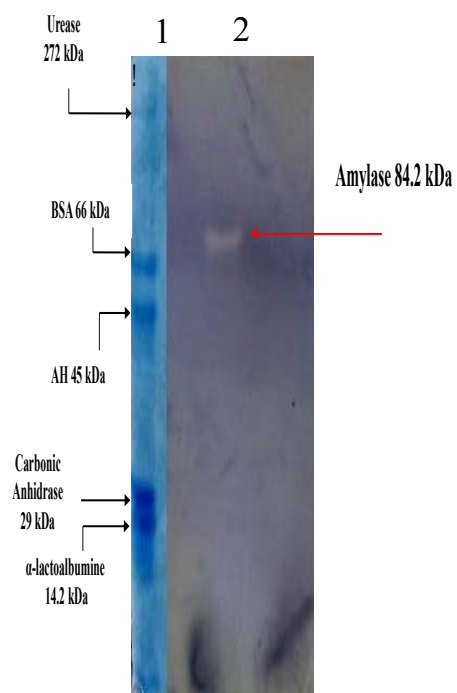


Fig. 3. Purification profile of the extracellular amylase from Bt LBIT 418 obtained in medium MB with 1% (w/v) soluble starch at room temperature, 180 rpm and pH 6.5 after 13 h of incubation. (a) Profile obtained with CM Sephadex C-50 column. (b) Chromatography pattern with Sephadex G-100.



541

542 **Fig. 4.** Electrophoretic analysis of extracellular α -amylase of strain Bt LBIT 418. Zymogram. Line 1,
543 molecular mass markers. Lane 2, native-PAGE obtained from α -amylase obtained Sephadex G-100 ultrafine.

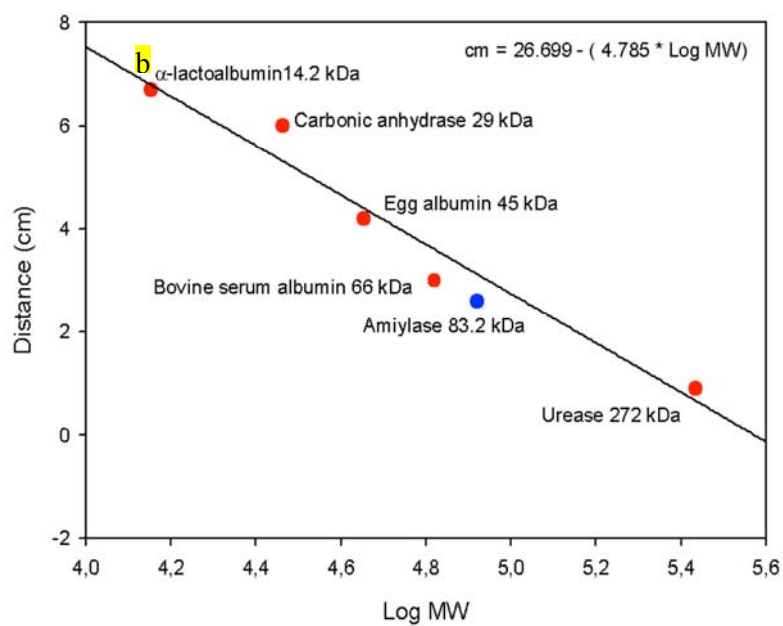
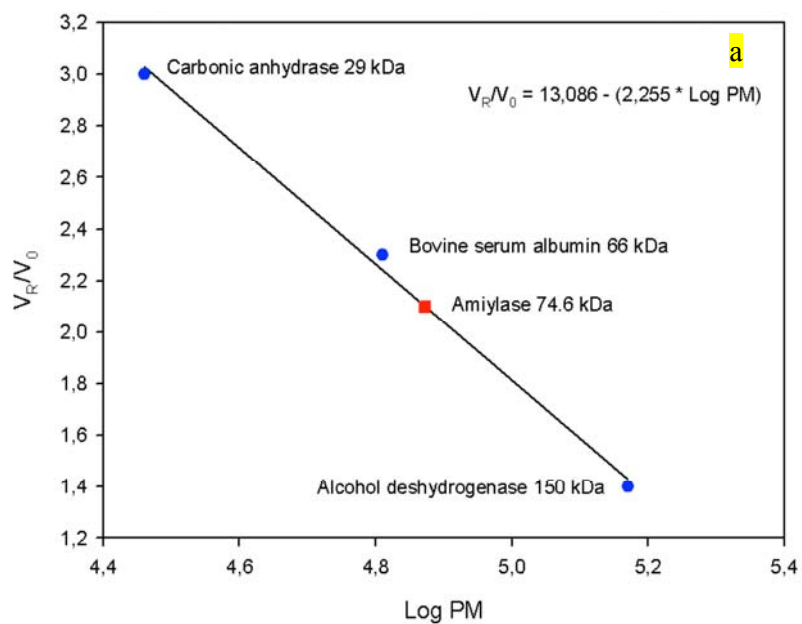


Fig. 5. Determination of MW of amylase. (a) Calibration curve of Sephadex G-100 profile. (b) Calibration curve of SDS-PAGE assay.

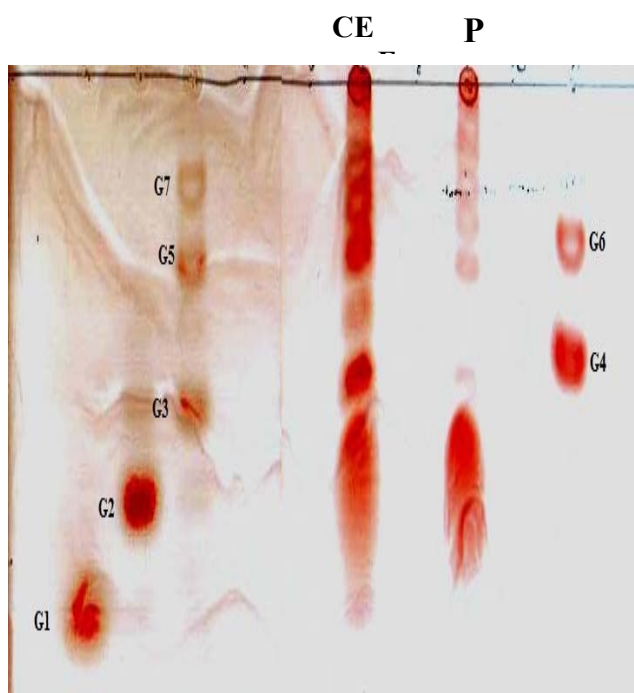


Fig. 6. TLC analysis of reducing sugars from in vitro hydrolysis of starch using crude extract of amylase (EC) and pure amylase (EP). Glucose (G1), maltose (G2), maltotriose (G3), maltotetraose (G4), maltopentaose (G5), maltohexaose (G6) and maltoheptaose (G7).

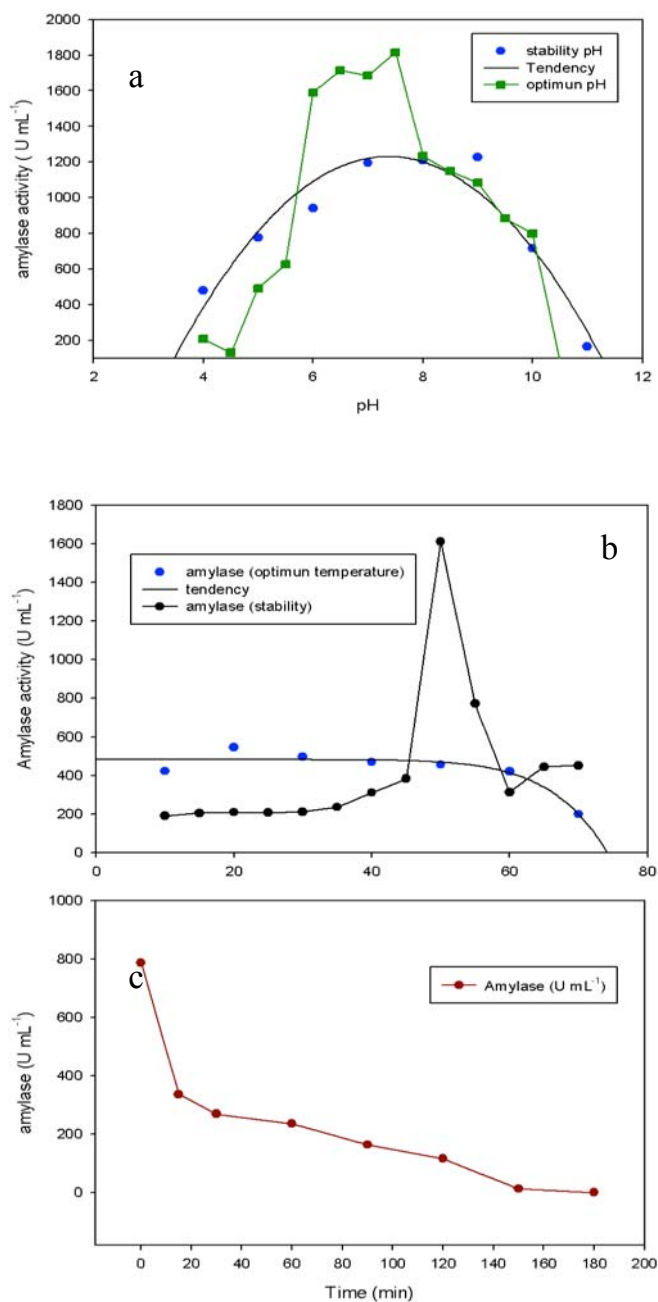


Fig. 7. Characterization of purified amylase (a) optimum pH of pH stability (b) Effect of temperature on the activity of purified α -amylase and optimum temperature activity under different temperatures at pH 9.0. (c) Effect of EDTA on purified amylase.

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

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

Property	Value
Optimum temperature	50 °C
Temperature stability	10-60 °C
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

Table 3. Effect of chemical reagents at optimum conditions

Chemical reagent (M) or *(v/v). %Relative activity (pH: 7.5, 50 °C)	
Absence in chemical reagent % relative activity is 100	
EDTA	
0.001	50
<i>p</i> -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

582 **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 (°C)	Tem. Stability (°C)	Inhibitors	Requirements	References
<i>B. cereus</i> NY-14	55	6.0	5.5 to 10	55	below 35	ND	Ca ²⁺	Yoshigi et al.1985
<i>B. cereus</i> MTCC 10205	55	5.5	ND	55	ND	EDTA	Ca ²⁺	Kumari et al. 2013
<i>B. cereus</i> GA6	55	9	7 to 11	22	4- 37	Fe, Zn, Cu	Ca ²⁺	Roohi et al. 2013
<i>B. thuringiensis</i> (Btk)	51	ND	ND	ND	ND	ND	ND	Smitha et al. 2013
<i>B. thuringiensis</i> (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
<i>B. thuringiensis</i>	32	4.0	3 to 4	35	ND	ND	ND	Zusfahair et al. 2016
<i>B. thuringiensis</i> (Bt) LBIT-418	74.6/83.2	7.5	7-8	50	10-60	EDTA, Hg, pHMB, SDS, Tween 20	Ca ²⁺	This study

583