

Extracellular Amylases Produced by *Bacillus thuringiensis*

Brenda Román-Ponce^{1*}, Ramón Cruz-Camarillo¹, En Tao Wang¹,
Otto Raúl Leyva-Ovalle² and Luz Irene Rojas-Avelizapa^{2*}

¹Instituto Politécnico Nacional, Escuela Nacional de Ciencias Biológicas, Prolongación Carpio y Plan de Ayala s/n, Col. Santo Tomás, Del. Miguel Hidalgo, C.P. 11340. Ciudad de México, México.

²School of Biological and Agricultural Sciences, Universidad Veracruzana, Josefa Ortíz de Domínguez S/N. Col. Centro, C.P. 94945. Peñuela, Municipio de Amatlán de los Reyes, Veracruz, México.

Original Research Article

ABSTRACT

Bacillus thuringiensis has shown a great biotechnological potential due to its insecticidal Cry-proteins production. Also, this bacterium produces different extracellular enzymes. The study aimed to evaluate the amylolytic system of *B. thuringiensis* strain Bt LBIT 418 cultured in a liquid medium, with starch as a carbon source, where the bacteria secreted the maximum amount of endoamylase after 10h of incubation, in the middle of the exponential phase of growth. Its' cleaves action-released dextrans composed for 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 40h 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.

*Corresponding author: E-mail: luzirenerojas@gmail.com, romanpbrenda@yahoo.com.mx;

In contrast, the exoamylase could be purified 98 times with a recovering of 14%, by fractionating lyophilised supernatants from 13h of 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 pHMB. The results suggest that Bt LBIT 418 produced at least three different amylases and it is the first report concerning about characterisation of the amylolytic system of *B. thuringiensis*. This supports the hypothesis that it 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.

Keywords: Amylases; *Bacillus thuringiensis*; starch; extracellular enzymes.

1. INTRODUCTION

Industrial enzymes will form a market of about 6.2 USD billions by 2020 [1]. Amylases are a large group of enzymes degrading starch and related oligosaccharides and polysaccharide. Amylases have been utilised in breweries, and in diverse areas, such as textile, distilling [2,3], pharmaceutical [4] and 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 can 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 dextrans, 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 -glucosidase (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 hydrolyses 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 extracellular 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 hydrolyse colloidal chitosan to produce different oligosaccharides with potential pharmaceutical applications [16]. In addition, the amylase of Bt was recovered as a byproduct from supernatant released during the harvest of δ -endotoxin using biphasic fermentation strategy and then was purified and characterised [17,18]. Additionally, amylase from Bt was isolated from tapioca waste [19].

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

2. MATERIALS AND METHODS

2.1 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, lyophilised spores were kept at -15°C.

2.2 Culture Medium

The basal medium (BM) (g L^{-1} , pH 6.5) consisted of: 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]. Basal medium complemented with 1% glucose (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.

2.3 Culture Conditions

For kinetic experiment, a fresh colony was inoculated in 25 mL of the test medium and cultured for 12h (180 rpm) at 28°C, 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 serial of 125 mL Erlenmeyer flask containing 25 mL of basal medium. The flasks were incubated for 48h, a flask was sampled every 60 min during the first 14h of incubation, and then at intervals of 2-3h. Bacterial growth of each collected culture 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 cultured samples were centrifuged at 600 rpm for 15 min at 5°C (Sorval centrifuge RC-SB), and supernatants were filtered and then stored in vials at 5°C for further analysis.

2.4 Enzymatic Assays

2.4.1 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°C 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-hydrolysed starch with lugol's method [22]. Also, the amylase activity was 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 was incubated at 50°C for 60

min. The reaction was stopped by heating the tubes for 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 the amount of enzyme produces an increment of 0.01 OD_{600} in 1h, under the test conditions.

2.4.2 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 1h.

2.4.3 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°C 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 1h.

2.4.4 α -glucosidase

The enzyme was evaluated with chromogenic analogue substrate *p*-nitrophenyl- α -D-glucopyranoside (pNGP). The reaction mixture composed of 0.5 mL of enzyme, 0.3 mL of substrate and 0.2 mL of the same buffer, was incubated at 37°C for 120 min. The reaction was terminated by adding 3.0 mL of 0.1 M Na_2CO_3 . The *p*-nitrophenyl produced was determined by reading at 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 1h [10].

2.4.5 Protease

The protocol used [25], a 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°C for 60 min. The reaction was ended by addition of cold 13 % trichloroacetic acid (TCA) (w/v) and then stored at 5°C for 30 min. The precipitate formed was discarded by centrifugation at 10,000 rpm for 5 min, then aliquots of 1.0 mL were neutralised

with 1.0 mL of 1 N NaOH, and OD₄₄₀ was measured. One protease unit was expressed as the amount of enzyme required for an increase of 0.01 OD₄₄₀ per hour, under the reaction conditions employed.

2.5 Products and Substrate Evaluations

The global accumulation of reduced sugars in the culture was determined with DNS method [21]. The results were interpolated in calibration 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] by using seroalbumin as a standard. The residual starch was evaluated with the standard protocol of Wilson & Ingledew [22]. The absorbance data were interpolated in a calibration curve of starch from 1 to 10 mg mL⁻¹.

2.6 TLC Analysis of Carbohydrates

For the identification of oligosaccharides accumulated in the culture during the amylase biosynthesis and produced by hydrolysing the starch *in vitro*, the samples (10 mL) samples were lyophilised 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 24h. After that, drying chromatograms were stained with a methanolic solution of 4% trifenyltetrazolium chloride and heated at 110°C [27].

2.7 The α-amylase Purification

The supernatant (125 mL) was filtrated and then concentrated by lyophilisation (Labanco 45F1) for about 12h. 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. 55 fractions of 2.0 mL were collected using a Spectra/Chrom Mod. CF automatic fraction collector. Amylase and protease activities were determined at an absorbance of 280 nm 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 were collected. The specific amylase and protease activities were measured simultaneously.

2.8 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 for 1h and 95V for 3.5h in a cold room (4°C). The gels were stained with Coomassie brilliant blue [29] or silver nitrate method [30].

The zymograms for visualising *in situ* amylase activity were performed in native-PAGE in two separated polyacrylamide gels (8% w/v), kept 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 as used in SDS-PAGE. The gel with the protein markers was stained with Coomassie brilliant blue, and the amylase in gel was revealed by immersion firstly in a solution of 2 % starch (w/v) 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 for 60 min. Finally, the gel was stained with 0.04 N Lugol's solution for 25 min. The enzyme was visualised as a clear band in a blue background.

2.9 Amylase Characterisation

2.9.1 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 (V_e) were determined. The void volume (V_o) 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 V_e/V_o , 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 (R_f) was interpolated in a calibration curve obtained by plotting the R_f values of the protein markers again the logarithm of their corresponding MW [32].

2.9.2 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 an interval of 5 °C. The test was carried out by incubating for 1h and the enzyme mixed with the substrate amylose azure (20 mg mL⁻¹) dissolved in 0.2 M phosphate buffer (pH 7.0). Thermostability was determined by pre-incubating the enzyme at selective temperature for 60 min. The residual activity was evaluated at the optimal conditions (pH 7.0 and 55°C).

2.9.3 Effects of chelating substrates

The pure amylase was dialysed against 0.01 M of ethylene diamine tetraacetic acid (EDTA) during 3h at room temperature. The samples were collected at 15, 30, 60, 90, 120, 150 and 180 min, and the remained amylase activity was determined by 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 for 3h, then the amylase activity was tested.

2.9.4 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 and SDS at (0.1, 0.5 and 1 % v/v) were tested. The remained enzyme activity was evaluated in the standards conditions. In 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 determining the residual activity.

3. RESULTS AND DISCUSSION

3.1 Amylolytic System Evaluation

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

The growth kinetics of Bt LBIT-418 showed a very short adaptation phase, and an exponential phase from 3h to 9h, reaching to 1.5 of OD₆₀₀ and 1×10⁹ CFU mL⁻¹. The starch concentration decreased from 8.6 mg mL⁻¹ at 3h of incubation to almost zero after the further incubation of 6h (Fig. 1c). The stationary growth phase started at 12h and lasted to the end of the culture (51h), maintaining the bacterial population at 1 × 10⁸ (2.0 of OD₆₀₀) and 1 × 10⁹ CFU mL⁻¹ (2.3 of OD₆₀₀) by consuming the reducing sugars accumulated in the culture medium. The first peak of reducing sugars was detected in the culture after 3h of incubation, coinciding with α-glucosidase synthesis (180 U mL⁻¹) (Fig. 1b). As a result of α-amylase activity, a second peak of reducing sugars occurred after 12h 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 12h of incubation (Fig. 1a and 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.

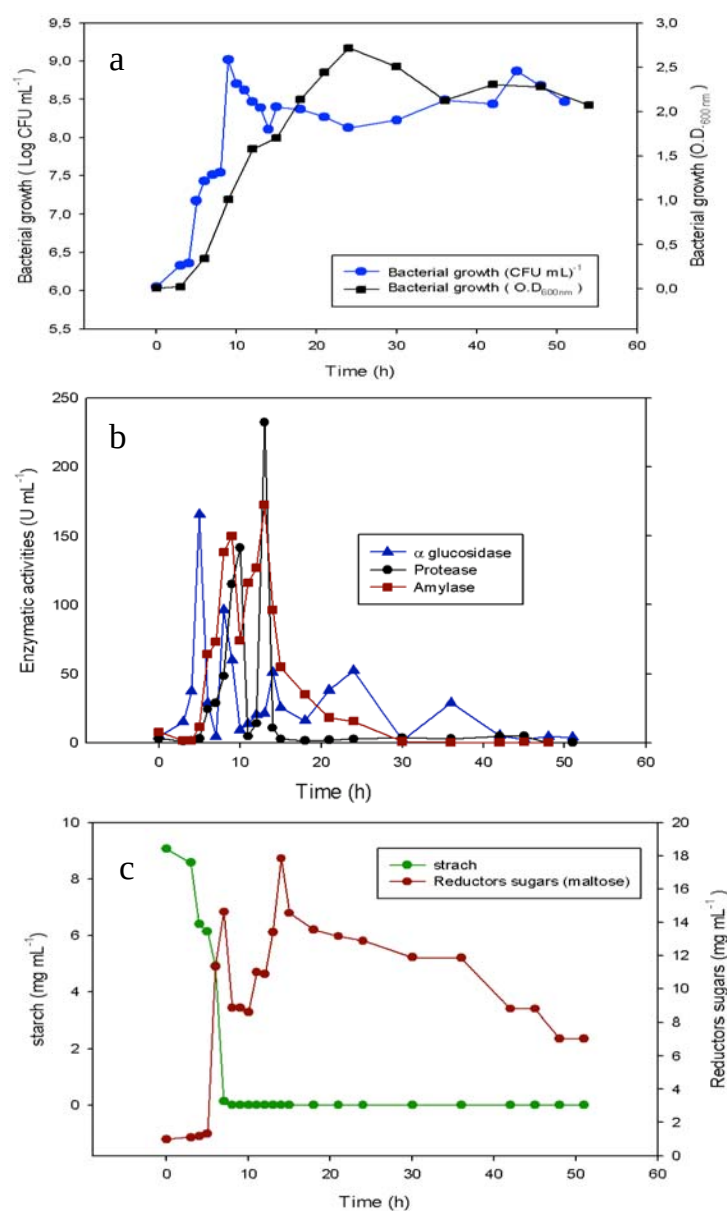


Fig. 1. Kinetics of amylase production of strain *B. thuringiensis* (Bt) LBIT 418 in medium MB with 1% (w/v) soluble starch at room temperature, 180 rpm and pH 6.5. (a) bacterial growth (log CFU mL⁻¹) and optical density at 600 nm. (b) Enzymes production (c) residual starch and reducing sugar accumulation

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 36h (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 compared with the DNS and Lugol's methods. In 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 extracted 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.

Please mention ref. [43] after ref. [42]

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 the 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 shown). This might be due to the fact that the enzyme had a 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 13-21h. The enzymatic activity determined with Lugol's 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 shown).

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 (5h) and the activity decreased at 13h; some others very small increments of enzymes were detected in the stationary growth phase (Fig. 1b). Production of intracellular α -glucosidase was determined by 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 the 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 20h in the growth curve (Fig. 1a).

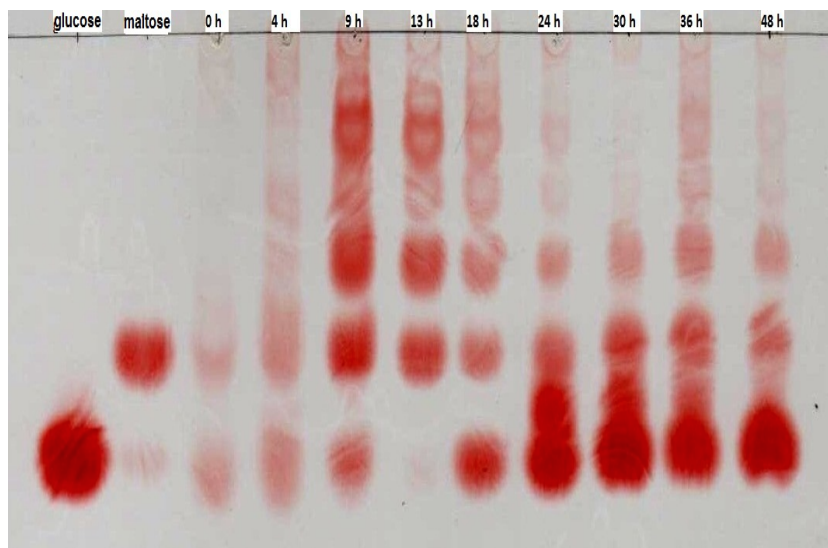


Fig. 2. Reducing sugars accumulated in the culture medium during amylase production identified by TLC

3.2 Kinetics Studies of Amylase Repression

The cell growth pattern is 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 by 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]. The 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. S1c).

3.3 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 50 %. It was showed that the exponential phase started after 5h of incubation, with bacterial population between 1×10^5 and $1 \times 10^7 \text{ CFU mL}^{-1}$. The strain presented a stationary phase from 10th h to 25th h (supplementary Fig. S2a), that was very long in compared with the standard medium. The amylase and protease activities were decreased

by 90% and 42%, respectively, in the medium supplemented with 1% of NH_4NO_3 (Figs. 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 the 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 the basic metabolism of the bacterium. Conversely, the amylase decreased by 90% this value is greater than expected as a result of the cellular concentration in the kinetics growth.

3.4 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 a 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 summarised in Table 1 and Fig. 4.

3.5 Properties of Amylase

The properties of enzyme are summarised in Table 2. The activity *in situ* of the purified amylase from Bt LBIT-418 analysed 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 within 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. thuringiensis*: 35 kDa from Btk [20] and 51 kDa from Bt [17,18]; as well as greater than that of the commonly detected

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

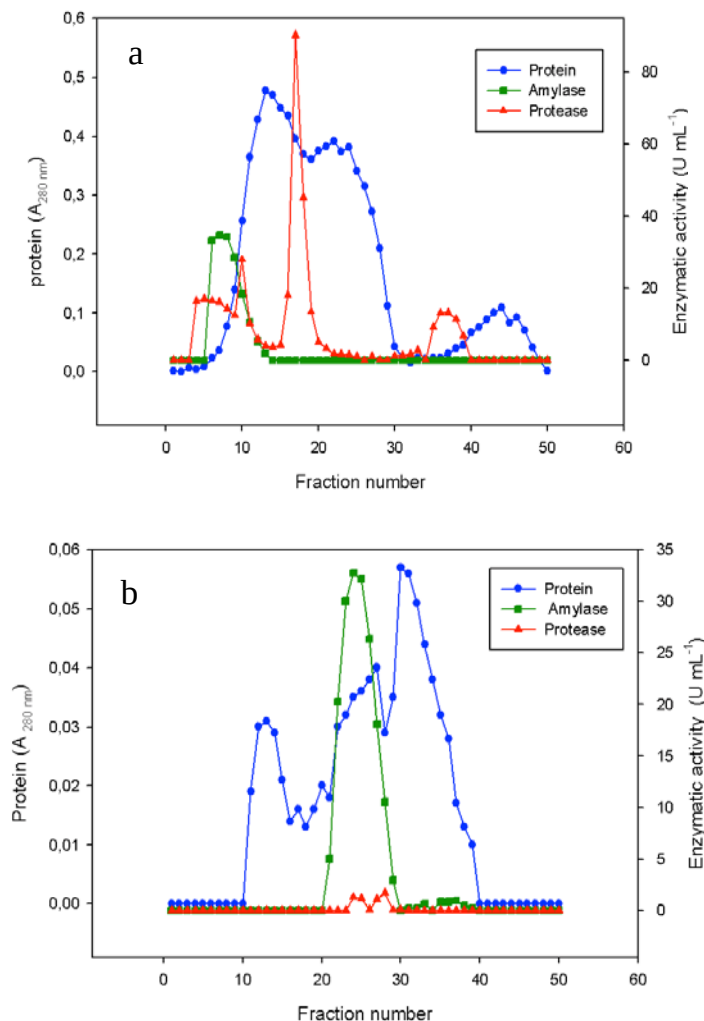


Fig. 3. Purification profile of the extracellular amylase from Bt LBIT 418 obtained in medium MB with 1% (w/v) soluble starch at room temperature, 180 rpm and pH 6.5 after 13h of incubation (a) Profile obtained with CM Sephadex C-50 column. (b) Chromatography pattern with Sephadex G-100

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 [56,57,58,59], the molecular mass of Bt 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 extracted enzyme. Glucose and maltose monomers were also detected at very low amounts (Fig. 6). These

results were consistent 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, proving that the enzyme is α -amylase [60]. 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* [61] and *C. acetobutylicum* [62] while the α -amylase of *B. cereus* NY-14 end product was dextrin of five glucose residues [63].

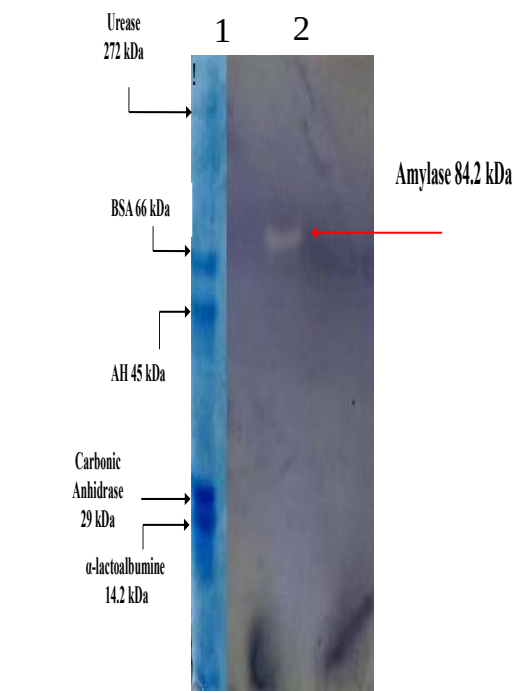


Fig. 4. Electrophoretic analysis of extracellular α -amylase of strain Bt LBIT 418. Zymogram. Line 1, molecular mass markers. Lane 2, native-PAGE obtained from α -amylase obtained Sephadex G-100 ultrafine

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

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 by 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* [64].

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
p-HMB	
0.0001	97
0.01	95
HgCl₂	
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

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 shown 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 1h (Fig. 7a). Statistical analysis ($p < 0.001$) demonstrated that the amylase was severely affected by the pH change, which coincided with several other reports [65,66,67]. However, the amylase from *B. cereus* NY-14 [63] and *B. cereus* GA6 [68] 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

dialysed 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 the activity of the studied amylase. So, the α -amylase of Bt LBIT-418 is a Ca^{2+} -dependent

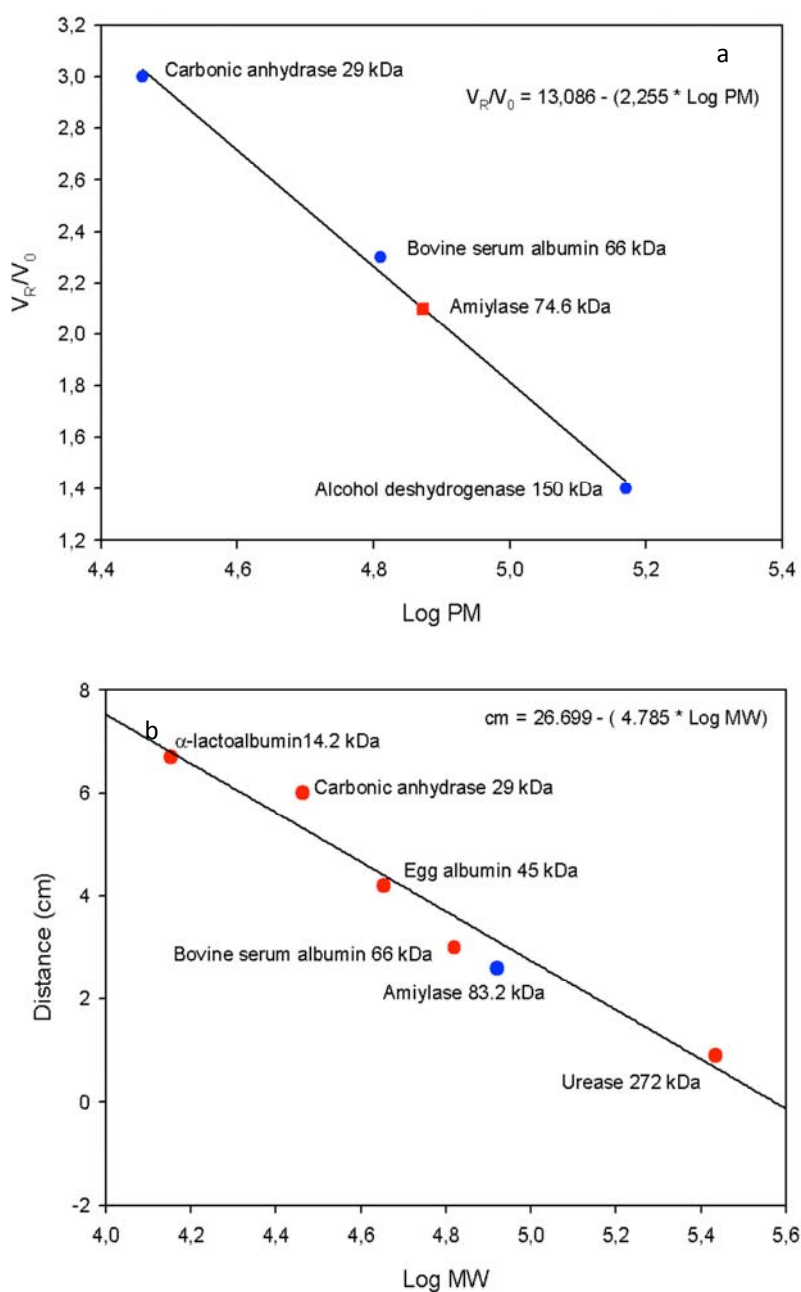


Fig. 5. Determination of MW of amylase. (a) Calibration curve of Sephadex G-100 profile. (b) Calibration curve of SDS-PAGE assay

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. [63]
<i>B. cereus</i> MTCC 10205	55	5.5	ND	55	ND	EDTA	Ca ²⁺	Kumari et al. [59]
<i>B. cereus</i> GA6	55	9	7 to 11	22	4- 37	Fe, Zn, Cu	Ca ²⁺	Roohi et al. [68]
<i>B. thuringiensis</i> (Btk)	51	ND	ND	ND	ND	ND	ND	Smitha et al. [17]
<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⁺	Smitha et al. [18]
<i>B. thuringiensis</i>	32	4.0	3 to 4	35	ND	ND	ND	Zusfahair et al. [19]
<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

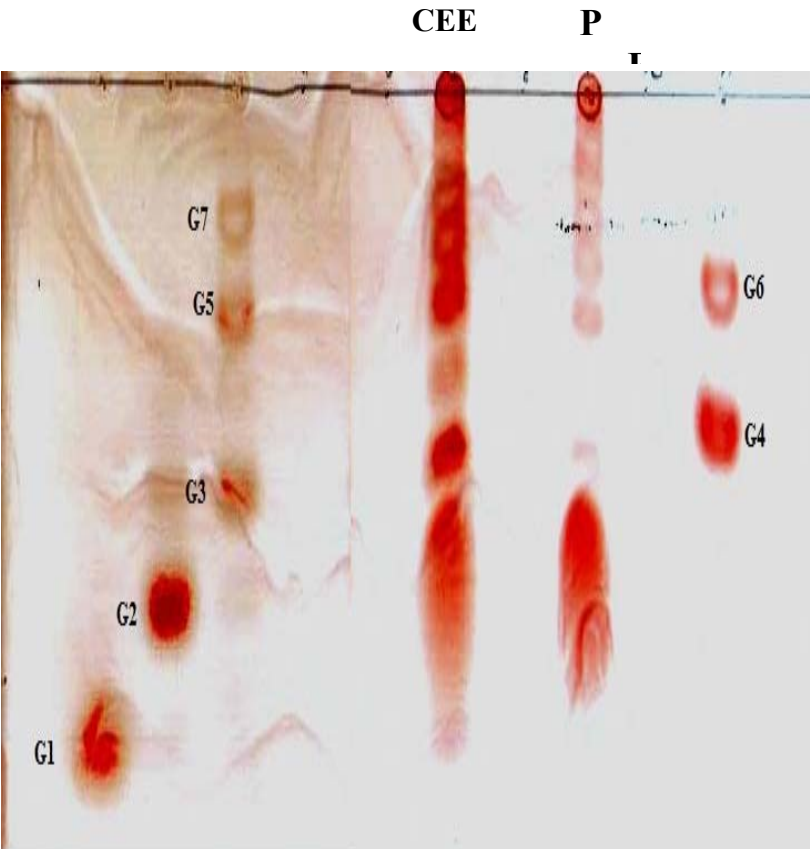


Fig. 6. TLC analysis of reducing sugars from *in vitro* hydrolysis of starch using crude extract of amylase (EC) and pure amylase (EP). Glucose (G1), maltose (G2), maltotriose (G3), maltotetraose (G4), maltopentaose (G5), maltohexaose (G6) and maltoheptaose (G7)

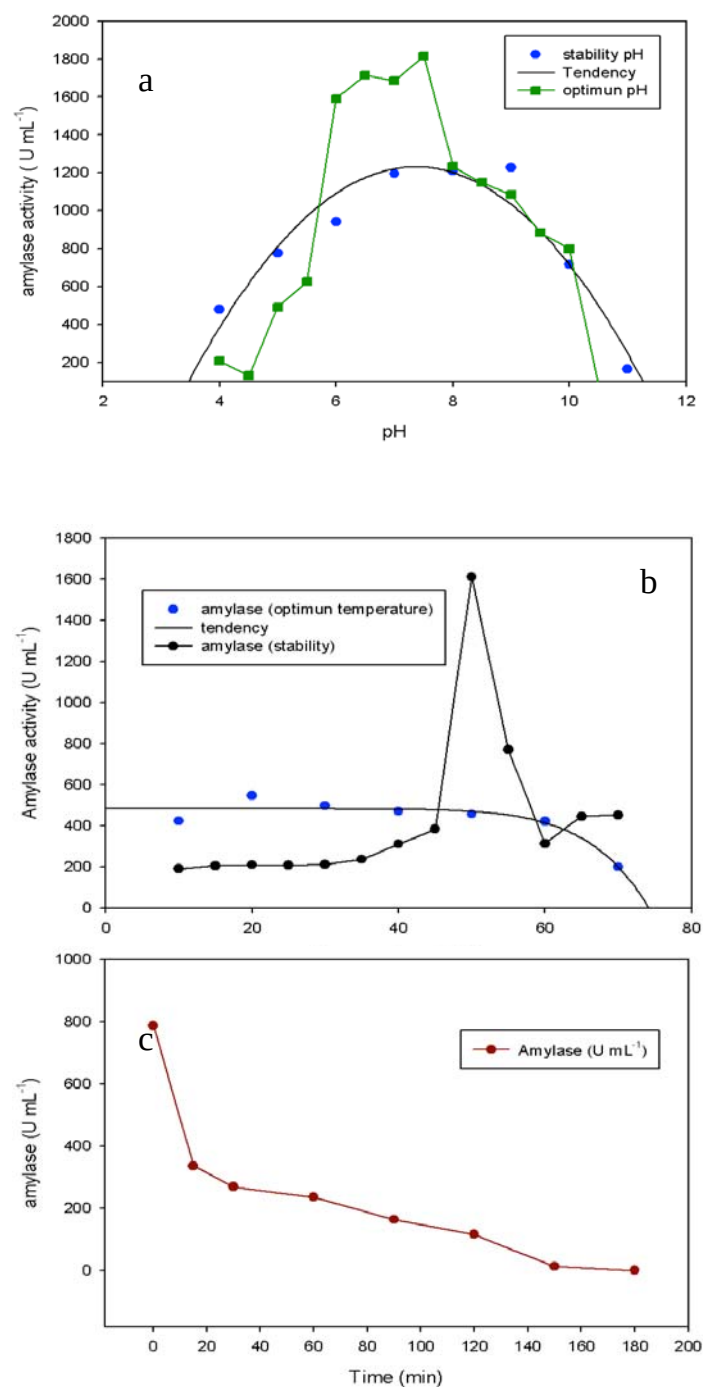


Fig. 7. Characterisation of purified amylase (a) optimum pH of pH stability (b) Effect of temperature on the activity of purified α-amylase and optimum temperature activity under different temperatures at pH 9.0. (c) Effect of EDTA on purified amylase

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 [69] and adversely

affected the stability of amylases, while Ca^{2+} and Mg^{2+} contribute to their stability [66]. 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].

Please mention ref. [70] after ref. [69]

The enzyme activity was decreased by 80% and 3% in presence of HgCl_2 and pHMB, respectively (supplementary Fig. 4a), indicating that the amylase could be depends 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 that 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 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, which might react with ammonium groups present in lysine and arginine but also found in the active site of enzyme [71]. 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 [63], 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 [72].

4. 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 biotechnology of the strain.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

REFERENCES

1. Metha D, Satyanarayana T. Bacterial and archaeal α -amylases: Diversity and amelioration of the desirable characteristics for industrial applications. *Front Microbiol*; 2016. DOI: 10.3389/fmicb.2016.01129
2. Pandey A, Nigam P, Scool CR, Scool VT, Sight D, Mohan R. Advances in microbial amylases. *Biotechnol Appl Biochem*. 2000; 31:135-152.
3. Goyal N, Gupta JK, Soni SK. 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*. 2005;37:723-734.
4. Oudjeriouat N, Moreau Y, Santimone M, Svensson B, Marchis-Mouren G, Desseaux V. Mechanism of barley α -amylase. Inhibition of amylose, maltodextrin and maltoheptaose hydrolysis by acarbose and cyclodextrin. *Eur J Biochem*. 2003;270:3871-3879.
5. Synowiecki J, Polaina J, Mac CA. The use of starch processing enzymes in the food industry. *Industrial Enzymes, Structure, Function and Applications*. 2007;19-34.
6. Horvathova V, Janecek S, Sturdik E. Amylolytic enzymes: Molecular aspects of their properties. *Gen Physiol Biophys*. 2001;20:7-32.
7. Van der Maarel MJEC, van der Veen B, Uitdehaag JCM, Leemhuis H, Dijkhuizen L. Properties and applications of starch-converting enzymes of the α -amylase family. *J Biotech*. 2002;94:137-155.
8. Chávez- Camarillo GM. Estudios de la X-prolil dipeptidil aminopeptidasa de levaduras. Tesis Doctoral en microbiología, ENCB-IPN (Dissertation in Spanish); 1995.
9. Ogunremi OR, Sanni AI. Occurrence of amylolytic and/or bacteriocin-producing lactic acid bacteria in Ogi and Fufu. *Ann Food Sci Technol*. 2011;12:71-77.
10. Yang H, Liu L, Li J, Du G, Chen J. Heterologous expression, biochemical characterization, and overproduction of alkaline α -amylase from *Bacillus alcalophilus* in *Bacillus subtilis*. *Microb Cell Fac*. 2011;10:1.

11. Li X, Yu HY. Purification and characterization of novel organic-solvent-tolerant β -amylase and serine protease from a newly isolated *Salimicrobium halophilum* strain LY20. FEMS Microbiol Lett. 2012;329:204-211.
12. Ventura-Suárez A, Cruz-Camarillo R, Rampersad J, Ammons DR, López-Villegas EO, Ibarra JE & Rojas-Avelizapa LI. Characterization of a novel *Bacillus thuringiensis* phenotype possessing multiple appendages attached to a parasporal body. Curr Microbiol. 2011;62: 307-312.
13. Barbosa-Corona JE, Contreras JC, Velazquez-Robledo R, Bautista-Justo M, Gomez RM, Cruz-Camarillo R, Ibarra JE. Selection of chitinolytic strains of *Bacillus thuringiensis*. Biotechnol Lett. 1999;21: 1125-1129.
14. Camarillo RC, Perez OS, Avelizapa NR, Ramirez MG, Avelizapa LR. Chitosanase activity in *Bacillus thuringiensis*. Folia Microbiologica. 2004;49:94.
15. Rojas-Avelizapa LI, Cruz-Camarillo R, Guerrero MI, Rodríguez-Vázquez R, Ibarra JE. Selection and characterization of a proteo-chitinolytic strain of *Bacillus thuringiensis*, able to grow in shrimp waste media. World J Microbiol Biotechnol. 1999;15:299-308.
16. Olicón-Hernández DR, Vázquez-Landaverde PA, Cruz-Camarillo R, Rojas-Avelizapa LI. Comparison of chito-oligosaccharide production from three different colloidal chitosans using the endochitonsanolytic system of *Bacillus thuringiensis*. Prep Biochem Biotechnol. 2017;47:116-122.
17. Smitha RB, Jisha VN, Sajith S, Benjamin S. Dual production of amylase and δ -endotoxin by *Bacillus thuringiensis* subsp. kurstaki during biphasic fermentation. Microbiol. 2013;82:794-800.
18. Smitha RB, Sajith S, Priji P, Unni KNN, Roy TAN, Benjamin S. Purification and characterization of amylase from *Bacillus thuringiensis* subsp. kurstaki. Bt Research. 2015;6.
19. Zufahair Z, Ningsih DR, Kartika D, Fatoni A. Amylase from *Bacillus thuringiensis* isolated from tapioca waste: Isolation, partial purification and characterization. Malaysian J Fundam Appl Sci. 2016;12.
20. Salazar Carrillo MS. Actividad amilolítica extracelular en *Bacillus thuringiensis*. Tesis profesional de IBQ, ENCB-IPN (Disertation in Spanish); 2005.
21. Miller GL. Use of dinitrosalicylic acid reagent for determination of reducing sugars. Anal Chem. 1959;31:426-428.
22. Wilson JJ, Ingledew WM. Isolation and characterization of *Schwanniomyces alluvius* amyolytic enzymes. Appl Environ Microbiol. 1982;44:301-307.
23. Dahlqvist A. Determination of maltase and isomaltase activities with a glucose oxidase reagent. J Biochem. 1961;80:547-551.
24. Worthington Biochemical Corporation. Worthington Enzymes Manual. Enzymes, enzyme reagents and related biochemicals. Freehold. New Jersey. USA. 1972;19-20.
25. Pérez García G. Relación entre las actividades de quitinasa y caseinasa en *Bacillus thuringiensis*. Tesis de Maestría en CQB, ENCB-IPN. (Dissertation in Spanish); 2002.
26. Bradford MN. A rapid and sensitive method for the quantitation of micrograms quantities of protein utilizing the principle of protein-dye binding. Anal Biochem. 1976; 72:248-254.
27. Pacheco-Hernández MA. Purificación y caracterización de la endoquitinasa de la cepa Bt -132 de *Bacillus thuringiensis*. Tesis de Maestría en CQB. ENCB-IPN. (Dissertation in Spanish); 2008.
28. Laemmli UK, Favre M. Maturation of the head of bacteriophage T4: I. DNA packaging events. J Mol Biol. 1973;80: 575-599.
29. Wang W, Lio X, Li Y. A modified Coomassie brilliant blue staining method at nanogram sensitivity compatible with proteomic analysis. Biotechnol Lett. 2007;29:1599-1603.
30. Wray W, Bouliskas T, Wray VP, Hancock R. Silver staining of proteins in polyacrylamide gels. Anal Biochem. 1981; 118:197-203.
31. Whitaker JR. Determination of molecular weights of proteins by gel filtration of sephadex. Anal Chem. 1963;35:1950-1953.
32. Shapiro AL, Viñuela E, Maizel JV. Molecular weight estimation of polypeptide chains by electrophoresis in SDS-polyacrylamide gels. Biochem Biophys Res C. 1967;28:815-820.
33. Kim EY, Kim YH, Rhee MH, Song JC, Lee KW, Kim KS, Lee SP, Lee IS, Park SC. Selection of *Lactobacillus* sp. PSC101 that produces active dietary enzymes such as

- amylase, lipase, phytase and protease in pigs. *J Gen Appl Microbiol*. 2007;53:111-117.
34. Imanaka H, Tanaka S, Feng B, Imamura K, Nakanishi K. Cultivation characteristics and gene expression profiles of *Aspergillus oryzae* by membrane-surface liquid culture, shaking-flask culture, and agar-plate culture. *J Biosci Bioeng*. 2010;109:267-273.
 35. Corrêa TLR, Moutinho SKDS, Martins MLL, Martins MA. Simultaneous α -amylase and protease production by the soil bacterium *Bacillus* sp. SMIA-2 under submerged culture using whey protein concentrate and corn steep liquor: compatibility of enzymes with commercial detergents. *Food Sci Technol. (Campinas)* 2011;31:843-848.
 36. Kalaichelvan PT. Production, purification and characterization of α -amylase and alkaline protease by *Bacillus* sp. HPE 10 in a concomitant production medium. *Asian J Plant Sci Res*. 2012;2:376-382.
 37. Wong DWS, Batt SB, Robertson GH. Microassay for rapid screening of alpha-amylase activity. *J Agric Food Chem*. 2000;48:4540-4543.
 38. Manonmani HK, Kunhi AM. Interference of thiol compounds with dextrinizing activity assay of α -amylase by starch-iodine color reaction: modification of the method to eliminate this interference. *World J Microbiol Biotechnol*. 1999;15:485-487.
 39. Najafi MF, Deobagkar D, Deobagkar D. Purification and characterization of an extracellular α -amylase from *Bacillus subtilis* AX20. *Protein Expr Purif*. 2005;41:349-354.
 40. Hernández-Montañez Z, Juárez-Montiel M, Velázquez-Ávila M, Cristiani-Urbina E, Hernández-Rodríguez C, Villa-Tanaca L, Chávez-Camarillo G. Production and characterization of extracellular α -amylase produced by *Wickerhamia* sp. X-Fep. *Appl Biochem Biotechnol*. 2012;167:2117-2129.
 41. Bello-López JM, Navarro-Noya YE, Gómez-Acata S, Hernández-Montañez Z, Dendooven L. Identification of α -amylase by random and specific mutagenesis of *Texcoconibacillus texcoconensis* 13CCT strain isolated from extreme alkaline-saline soil of the former Lake Texcoco (Mexico). *Folia Microbiologica*. 2014;59:235-240.
 42. Kocabay S, Çetinkaya S, Akkaya B, Yenidünya AF. Characterization of thermostable β -amylase isozymes from *Lactobacillus fermentum*. *Int J Biol Macrom*. 2016;93:195-202.
 43. Soto-Padilla MY, Gortáres-Moroyoqui P, Cira-Chávez LA, Levasseur A, Dendooven L, Estrada-Alvarado MI. Characterization of extracellular amylase produced by haloalkalophilic strain *Kocuria* sp. HJ014. *Inter J Environ Health Res*. 2016;1-9.
 44. Jin F, Li Y, Zhang, C, Yu H. Thermostable α -amylase and galactosidase production from the thermophilic and aerobic *Bacillus* sp. JF strain. *Process Biochem*. 2001;36:559-564.
 45. Rowe GE, Margaritis A. Enzyme kinetic properties of α -1, 4-glucosidase in *Bacillus thuringiensis*. *Biochem Eng J*. 2004;17:121-128.
 46. Sheehan H, McCleary BV. A new procedure for the measurement of fungal and bacterial α -amylase. *Biotechnol Tech*. 1988;2:289-92.
 47. Lachmund A, Urmann U, Minol K, Wirsal S, Rutkowski E. Regulation of α -amylase formation in *Aspergillus oryzae* and *Aspergillus nidulans* transformants. *Curr Microbiol*. 1993;26:47-51.
 48. Morkeberg R, Carlsen M, Neilsen J. Induction and repression of α -amylase production in batch and continuous cultures of *Aspergillus oryzae*. *Microbiology*. 1995;141:2449-2454.
 49. Gupta R, Grigas P, Mohapatra H, Goswami VK, Chauhan B. Microbial amylases: A biotechnological perspective. *Proc. Biochem*. 2003;38:1599-1616.
 50. Esfahanibolandbalaie Z, Rostami K, Mirdamadi SS. Some studies of alpha-amylase production using *Aspergillus oryzae*. *Pakistan J Biol Sci: PJBS*. 2008;11:2553-2559.
 51. Jana M, Chattopadhyay DJ, Pati BR. Effect of different carbon sources on the production of amylase by *Bacillus* sp. MD 124. *Acta Microbiol Immunol Hung*. 1997;45:229-237.
 52. Kathiresan K, Manivannan S. Amylase production by *Penicillium fellutanum* isolated from mangrove rhizosphere soil. *Afr J Biotechnol*. 2006;5:10.
 53. Amadioha AC. Effect of cultural conditions on the growth and amylolytic enzyme production by *Rhizophus oryzae*. *Dis Inn*. 1999;11:69-73.
 54. Saha BC, Silman RW. Amylolytic enzymes produced by a color variant strain of

- Aureobasidium pullulans*. Current Microbiol. 1993;26:267-273.
55. Vihinen M, Mäntsälä P. Microbial amylolytic enzymes. Crit Rev Biochem Mol Biol. 1989;24:329-418.
 56. Shinke R, Nishira H, Mugibayshi N. Isolation of β -amylase producing micro-organisms. Agric Biol Chem. 1974;38:665.
 57. Shinke R, Kunimi Y, Nishira H. Production and some properties of β -amylase of *Bacillus* sp BQ10. J Ferment Technol. 1975;53:693.
 58. Nanmori T, Shinke R, Aoki K, Nishira H. Purification and characterization of β -amylase from *Bacillus cereus* BQ10-S1 Spo II. Agric Biol Chem. 1983;47:941-947.
 59. Kumari N, Jain V, Malhotra S. Purification and characterization of extracellular acidophilic α -amylase from *Bacillus cereus* MTCC 10205 isolated from soil. Global J Microbiol Res. 2013;1:1-9
 60. Ray RR. Purification and characterization of extracellular beta-amylase of *Bacillus megaterium* B(6). Acta Microbiol Immunol Hung. 2000;47:29-40.
 61. Matsuzaki H, Yamane K, Yamaguchi K, Nagata Y, Maruo B. Hybrid α -amylases produced by transformants of *Bacillus subtilis*. I. Purification and characterization of extracellular α -amylases produced by the parental strains and transformants. Biochimica et Biophysica Acta (BBA)-Protein Structure. 1974 ;365:235-247.
 62. Paquet V, Croux C, Goma G, Soucaille P. Purification and characterization of the extracellular α -amylase from *Clostridium acetobutylicum* ATCC 824. Appl Environ Microbiol. 1991;57:212-218.
 63. Yoshigi N, Chikano T, Kamimura M. Purification and properties of an amylase from *Bacillus cereus* NY-14. Agric Biol Chem. 1985;49:3369-3376.
 64. Sun H, Zhao P, Ge X, Xia Y, Hao Z, Liu J, Peng M. Recent advances in microbial raw starch degrading enzymes. Appl Biochem Biotechnol. 2010;160:988-1003.
 65. Robyt JF, Ackerman RJ. Isolation, purification and characterization of maltotetraose- producing amylase from *Pseudomonas stutzeri*. Arch Biochem Biophys. 1971;145:105-114.
 66. Krishnan T, Chandra AK. Purification and characterization of α – amylase from *Bacillus licheniformis* CUMC305. Appl Environ Microbiol. 1983;46:430-437.
 67. Coronado MJ, Vargas C, Hofemeister J, Ventosa A, Nieto JJ. Production and biochemical characterization of an α -amylase from the moderate halophile *Halomonas meridiana*. FEMS. Microbiol Lett. 2000;183:67-71.
 68. Roohi R, Kuddus M, Saima S. Cold-active detergent-stable extracellular α -amylase from *Bacillus cereus* GA6: Biochemical characteristics and its perspectives in laundry detergent formulation. J Biochem Tech. 2013;4:636-644.
 69. Dey G, Palit S, Banerjee R, Maiti BR. Purification and characterization of maltooligosaccharide-forming amylase from *Bacillus circulans* GRS 313. J Ind Microbiol Biotechnol. 2002;28:193-200.
 70. Tobey TF, Yousten AA. Factors affecting the production of amylase by *Bacillus thuringiensis*. In: Developments in Industrial Microbiology. (Ed). Leland Unterkofer. Jekyll Island, Georgia, USA. 1976;18:499-510.
 71. Weeb J. Enzymes and metabolic inhibitors: General principles of inhibition (Vol. 1). Academic; 1963.
 72. Kiran KK, Chandra TS. Production of surfactant and detergent-stable, halophilic, and alkali-tolerant α -amylase by a moderately halophilic *Bacillus* sp. strain TSCVKK. Appl Microbiol Biotechnol. 2008;77:1023-1031.