Aladejana OM *et al.* (2020)
Notulae Scientia Biologicae 12(1):154-171
DOI:10.15835/nsb12110521
Research Article

Production, purification and characterization of thermostable alpha amylase from *Bacillus subtilis* Y25 isolated from decaying yam (*Dioscorea rotundata*) tuber

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Abstract

Amylases have wide biotechnological potentials for applications in various industries. An α-amylase-producing bacterium was isolated from deteriorating yam tubers. Molecular characterization using the 16S rRNA gene sequencing was used to confirm the identity of the bacterium as *Bacillus subtilis* Y25. The effect of some cultural and nutritional factors such as pH, temperature, carbon and nitrogen sources on α-amylase production from the bacterium was determined. Maximum α-amylase production was observed using starch and peptone as carbon and nitrogen sources, respectively, with an initial medium pH of 8.0 and incubation at 45 °C for 36 h. The enzyme was purified by ion exchange chromatography on CM Sepharose CL-6B. The kinetic parameters K_m and V_{max} of the enzyme, as well as the effect of pH, temperature, metal ions and ethylenediaminetetra acetic acid (EDTA) on the activity of the purified enzyme were studied. The specific activity of the partially purified enzyme was determined to be 15.21 Units/mg protein with a purification fold of 3.80. The molecular weight of the purified enzyme was estimated to be 58.0 kDa. The V_{max} and K_m values obtained with soluble starch for *Bacillus subtilis* Y25 α-amylase were 314.10 ± 23.30 Units/mg protein and 53.98 ± 12.03 mg/ml, respectively. The enzyme exhibited optimum activity at a temperature of 60 °C and pH 8.0. The metal ion Ca^{2+} had no effect on the enzyme at 20 mM concentration, whereas Na^{+} and Mg^{2+}, as well as EDTA inhibited the enzyme at the same concentration. The characteristics of the α-amylase from *Bacillus subtilis* Y25 revealed it to be a thermostable and an alkaline metalloenzyme with potential for applications in the detergent and saccharification industries.

Keywords: α-amylase; *Bacillus subtilis*; characterization; optimization; starch; thermostable

Introduction

Amylases are industrially important enzymes representing about 65% of the world enzyme market (Reddy *et al*., 2003; Azad *et al*., 2009; Elmansy *et al*., 2018). They are important enzymes that catalyse the breakdown of starch molecules, to give diverse products including dextrin and progressively smaller polymers

Received: 24 Jun 2019. Received in revised form: 20 Mar 2020. Accepted: 26 Mar 2020. Published online: 31 Mar 2020.

composed of glucose units (Gupta *et al*., 2003). Two important groups of amylases are α-amylases [EC 3.2.1.1] which catalyse the endo-hydrolysis of 1,4-α-d-glycosidic linkages between adjacent glucose units inside the linear amylose units (Castro *et al*., 2010) and β-amylases [EC 3.2.1.2], which catalyse the hydrolysis of the second α-1,4-glycosidic linkages in polysaccharides, thereby removing successive maltose (two glucose units) units from the non-reducing ends of the chains (Bijttebier *et al*., 2008).

The α-amylases are widely distributed in nature and can be derived from various sources, such as plants, animals and microorganisms (Omemu *et al*., 2005). However, fungi and bacteria are used for commercial production of amylases because of their reliability, ease of manipulation and economic bulk production capacity (Khan and Yadav, 2011; Mathew *et al*., 2016; Singh *et al*., 2016). They also involve the expenditure of lesser time, space and cost for production. They have potential for several biotechnological applications in diverse industries such as the food, pharmaceutical, cosmetics, fermentation, textile and paper industries (Pandey *et al*., 2000; Konsoula and Liakopoulou-Kyriakides, 2007). Other applications are in detergent formulations in detergent industries for removal of tough stains (Mitidieri *et al*., 2006; Souza and Magalhaes, 2010) and in medicine for clinical diagnosis of several medical conditions (Das *et al*., 2011).

The performance, economics and feasibility of each α-amylase application is influenced by important enzyme characteristics including specificity, stability and optimal temperature and pH value dependence (Gupta *et al*., 2003). The selection of suitable microbial strains which produce enzymes exhibiting good activity and stability at industrially desirable conditions is therefore important (Wanderley *et al*., 2004). Among the bacteria species, the genus *Bacillus* have been explored for commercial production of α-amylases (Konsoula and Liakopoulou-Kyriakides, 2007). These include *B. subtilis*, *B. licheniformis*, *B. stearothermophillus* and *B. amyloliquefaciens*. *Bacillus* sp. are the organisms of choice because of their ubiquitous nature, non-fastidious nutritional requirement and ability to overproduce amylase (Sivaramakrishnan *et al*., 2006).

As most industrial processes are carried out under harsh physicochemical conditions which may not be specifically adjusted to the optimal points required for the activity of the available enzymes, the development of enzymes exhibiting optimal activities at various ranges of salt concentrations, pH and temperature is of significance. For instance, thermostability is a desirable characteristic of amylases because enzymatic liquefaction and saccharification of starch are performed at high temperatures. Therefore, thermostable amylolytic enzymes are being investigated as agents to improve industrial processes of starch degradation for production of valuable products such as glucose, dextrose syrup, maltose and maltodextrins (Gomes *et al*., 2003; Asgher *et al*., 2007).

The present study was therefore designed to explore for amylolytic bacteria from decaying yam (*Dioscorea rotundata*) tubers and evaluate α-amylase production from the selected bacterium under different nutritional and environmental conditions. Subsequent purification and characterization of the enzyme were then carried out to determine its potential for biotechnological and industrial applications.

Materials and Methods

Collection of decaying yam tubers

Yam (*Dioscorea rotundata*) tubers, at different stages of decay, were collected from the yam stalls at the Central Market, Ile-Ife, Osun State, Nigeria. They were placed in sealed cellophane bags and transported to the laboratory, where bacterial isolations were carried out immediately.

Isolation and maintenance of bacteria

A sterile scalpel was used to cut into, and remove decayed portions of the yam, which were crushed with sterile mortar and pestle. Five grams of the sample were reconstituted in 45 ml sterile distilled water and serially diluted up to 10^{-6}. One millilitre of the dilution was plated on nutrient agar and the culture was incubated at 30 °C for 24 h. Distinct bacterial colonies were purified twice on sterile nutrient agar plates by resuspension in sterile distilled water and plating on the medium. Colonies were then subculture on sterile nutrient agar slants and maintained in the refrigerator at 4 °C.

Screening of bacteria for α-amylase production

The bacterial isolates were screened for amylolytic activity using the starch hydrolysis test on starch agar plate. Each of the bacteria strains was streaked on separate starch agar plate and incubated for 30 °C for 48 h. After incubation, the starch agar plates were flooded with Grams' iodine solution. The clear zones around the colonies were suggestive of amylase production. The diameter of the zones of clearing was measured and the bacterium with the largest zone of clearance was selected for further studies.

Molecular characterization and identification of bacterium

An overnight culture of the selected bacterium was used for the preparation of genomic DNA. The DNA was isolated using the cetytrimethyl ammonium bromide (CTAB) method. Molecular characterization and identification of the bacterium was carried out by the 16S rRNA gene sequencing using universal primers (El-Helow, 2001). The forward and reverse primers were of the following sequences, respectively: 27f (5'-AGAGTTTGATCMTGGCTCAG-3') and 1492R (5'-ACCTTGTTACGACTT-3'). The PCR was carried out for 94 °C for 3 min, followed by 30 cycles of 94 °C for 60 sec, 56 °C for 60 sec, 72 °C for 120 sec and final extension temperature of 72 °C for 5 min and the 4 °C hold forever. After completion, a fraction of the PCR mixture was examined using agarose gel electrophoresis and the DNA was visualized using the ultraviolet transilluminator. The 16S rRNA gene fragment of the bacterium was sequenced in both direction and Blast program (*https://blast.ncbi.nlm.nih.gov/Blast.cgi*) was used to assess the DNA similarities. Phylogenetic tree for the bacterium was constructed using the Neighbor-joining method with 1000 bootsrap replication (Tamura *et al*., 2013) and *Bacillus anthracis* 2000031650 was used as the outgroup. Evolutionary relationship with other *Bacillus* sp. in the GenBank was conducted in Molecular Evolutionary Genetics Analysis (MEGA) version 6.0.

Enzyme production conditions

Amylase production was carried out in 250 ml Erlenmeyer flask containing 100 ml medium using 1 ml 0.5 McFarland standard inoculum. One liter of the medium contained 1 g soluble starch, 0.1 g KH_2PO_4, 0.25 g Na_2HPO_4, 0.1 g NaCl, 0.005 g $MgSO_4.7H_2O$, 0.005 g $CaCl_2$, 0.2 g $(NH_4)_2SO_4$ and 0.2 g peptone; at pH 8.0. The culture was incubated at 45 °C for 48 h with a steady agitation at 150 rpm. After incubation, the culture was centrifuged at 6,000 rpm for 30 min and the cell free supernatant used for α-amylase activity determination.

Determination of α-amylase activity

α-Amylase activity was determined by using the method of Somogyi (1952). One milliliter reaction mixture contained 0.l ml of 1.0% (w/v) gelatinized starch in 0.85 ml of 10 mM tris buffer, pH 8.0 and 0.05 ml of crude enzyme. The mixture was shaken and then incubated at 50 °C for 20 min. The reaction was terminated by the addition of 1.0 ml combined copper reagent. The reaction mixture was boiled for 20 min and then cooled. This was followed by addition of 1.0 ml of arsenomolybdate reagent while shaking. The solution was made up to 10 ml with 7.0 ml distilled water and the absorbance read at 540 nm against the blank which consisted of all the assay components except the enzyme. One unit of α-amylase is defined as the

amount of enzyme that released 1.0 µg of glucose equivalence from the substrate per millilitre per minute under the specified assay condition. Protein concentration was determined by the method of Bradford (1976), using bovine serum albumin as the standard protein.

Growth pattern and enzyme production

The bacterium growth pattern and α-amylase production were determined by inoculating a 250 ml enzyme production medium with 10 ml standard inoculum of 0.5 McFarland standard in an Erlenmeyer flask. This was incubated at 45 °C for 48 h with agitation at 150 rpm. At 2 h intervals, 5 ml samples were aseptically sampled for a period of 48 h and the growth was checked at 680 nm using Spectrumlab 23A spectrophotometer and recorded as the cell optical density. Also, the α-amylase activity of each cell-free supernatant sample was determined.

Influence of nutritional and environmental factors on α-amylase production

Effect of pH on α-amylase production

The effect of pH, in the range of 6.0-8.5, on α-amylase production, in 50 ml of production medium was determined. Standardized inoculum (0.5 ml) of bacterium was used and incubation was at 45 °C for 48 h with agitation at 150 rpm. After incubation, the cells were removed by centrifugation and the cell free supernatant tested for α-amylase activity.

Effect of temperature on α-amylase production

The optimum temperature for α-amylase production was determined by varying incubation temperature of the culture medium from 30 to 50 °C, at pH 8.0 for 48 h with agitation at 150 rpm. The cells were then removed by centrifugation and the amount of α-amylase present in supernatant was quantified.

Effect of carbon sources on α-amylase production

The effect of carbon sources on enzyme production was studied by replacing soluble starch (0.1% w/v) with different sugars: glucose, maltose and lactose, while other media components were kept constant. The fermentation medium, previously adjusted to pH 8.0, was inoculated with 0.5 ml cell suspension and the culture incubated for 48 h at 50 °C with agitation at 150 rpm. The supernatant obtained was then tested for amylolytic activity.

Effect of soluble starch concentration on α-amylase production

The influence of different concentrations of soluble starch (0.5, 1.0, 1.5, 2.0 and 2.5%, w/v) on α-amylase production from bacterium was studied. The culture was incubated for 28 h at 45 °C with agitation at 150 rpm. The supernatant was obtained and α-amylase production determined.

Effect of nitrogen sources on α-amylase production

The production medium (50 ml) was composed with different nitrogen sources which included Ca $(NO_3)_2$, $(NH_4)_2SO_4$, peptone and tryptic soy broth (TSB). The medium was incubated for 48 h at 45 °C with agitation at 150 rpm. After incubation, the supernatant obtained was tested for α-amylase activity.

Purification of α-amylase

Purification using ion-exchange chromatography on CM -Sepharose CL-6B

α-Amylase was purified from the cell-free supernatant. Ten milliliters of the supernatant obtained from a batch culture was layered on a 1.0 cm x 10 cm column of CM Sepharose CL-6B which had previously been equilibrated with 10 mM phosphate buffer, pH 8.0. One milliliter fraction was collected at a flow rate of 12 ml/h and bound proteins were eluted with a linear gradient of 0 to 1.0 M NaCl. The fractions were

assayed for α-amylase activities and the protein profile was determined. Active fractions in the peak were pooled and used for further studies.

Determination of α-amylase molecular mass

The native molecular mass of α-amylase from bacterium was determined by gel filtration in sephadex G-100 chromatographic column. Bovine serum albumin (66 kDa), α-chromotrypsinogen A (25 kDa) and Iysozyme (15 kDa) were used as standard proteins.

Determination of kinetic parameter

Kinetic parameters K_m and V_{max} for the purified α-amylase were estimated with soluble starch as substrate, at concentrations 0-200 mg/ml, using the non-linear regressionanalysis software (Graph pad Prism 5).

Effect of temperature on the activity of α-amylase

The effect of temperature on α-amylase activity was studied by incubating an aliquot of the enzyme with the substrate at temperatures ranging from 30 to 90 °C, for 20 mins. Unincubated purified α-amylase was used as the control. The residual activities of the enzyme were determined and plotted against the different temperatures.

Thermal stability of purified α-amylase from Bacillus subtilis

Thermal stability of the purified enzyme was examined by incubating the enzyme preparation for 60 min at different temperatures (50 to 65 °C), in the absence of the substrate. Aliquots were withdrawn at 10 min intervals and the residual enzyme activities were measured under standard assay conditions earlier stated. The residual activities were expressed as a percentage of the activity at zero time which was taken to be 100%. The percentage residual activity was plotted against incubation period.

Effect of pH on the activity of α-amylase

The effect of pH on α-amylase activity was performed in the pH range of 4.0 to 9.0, at 45 °C. The following buffer systems at the indicated pH values were used: 10 mM acetate buffer, pH 4.0-5.5; 10 mM phosphate buffer, pH 6.0-7.5 and 10-mM tris buffer, pH 8.0 - 9.0.

Effect of cations and ethylenediamine tetracetic acid (EDTA) on α-amylase activity

The effect of metal ions on α-amylase activity was determined by incubating the enzyme with NaCl, $MgCl_2$ and $CaCl_2$ separately dissolved in the assay buffer at a final concentration of 20 mM each and pH 8.0. The control did not contain the metal ions. The enzyme activity was expressed as a percentage of the control which was taken to be 100%. The effect of ethylenediamine tetraacetic acid (EDTA) on the activity of α-amylase was determined by assaying the enzyme in the presence of the 20 mM EDTA. The residual activity was also expressed as a percentage of the control which was taken as 100%.

Results

Isolation and screening of amylolytic bacteria

Out of the thirty-six isolates obtained from decaying yam, seven of the isolates showed a zone of clearance around the colonies on starch agar plate. The isolates were labelled Y4, Y22, Y23, Y25, Y31, Y35 and Y36. These isolates were screened for their amylolytic activities under submerged fermentation condition

using soluble starch as sole source of carbon. Isolate Y25 exhibited the highest activity and was therefore selected for further studies.

Molecular characterization and identification of amylolytic bacterium

In order to identify the isolate on molecular basis, the 16S rRNA gene from the strain Y25 was amplified and sequenced. The sequence was analyzed using the BLAST analysis and was found to have 100% homology with several *B. subtilis* reported from different parts of the world in the data bank of the NCBI (Altschul *et al*., 1990). Based on the molecular characterization, the strain Y25 was identified as *B. subtilis*. Phylogenetic analysis revealed a close relationship between the selected bacterial specie and other *Bacillus* species in the GenBank. However, there was a distant relationship with *Bacillus anthracis* (Figure 1).

Growth pattern and α-amylase production

The bacterial growth was observed to increase with increase in aamylase activity. The lag phase lasted for about 2 h, while the exponential phase of the growth lasted for 32 h. The α-amylase activity of *Bacillus subtilis* Y25 reached its maximum level after 36 h incubation. Thereafter, there was a decline in the amylase production (Figure 2).

Influence of nutritional and environmental factors on bacterium α-amylase production

The effect of various nutritional and environmental parameters on α-amylase production from *B. subtilis* Y25 was determined.

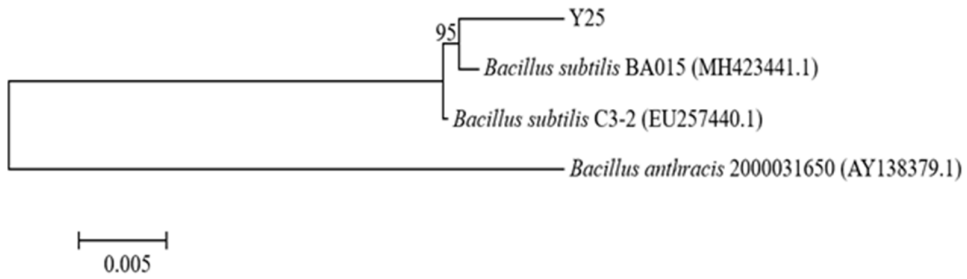

Figure 1. Molecular phylogenetic analysis of 16S rRNA gene of *B. subtilis* Y25. The tree was constructed with MEGA 6 (Neighbour Joining method with 1000 bootstrap replication)

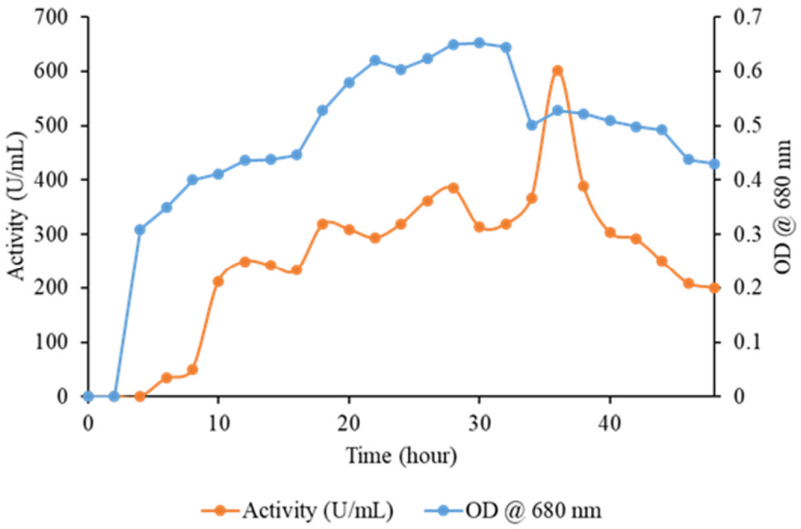

Figure 2. Growth and enzyme production from *B. subtilis* Y25 at 45 °C and pH 8.0 for 48 h.

Effect of pH on α-amylase production

The influence of pH, in the range 6.0 to 9.0, on α-amylase production and from *Bacillus subtilis* Y25 was determined. Maximum α-amylase production was achieved at pH 8.0 (Figure 3). Thus, the pH 8.0 was selected for maximum production of enzyme.

Effect of temperature on α-amylase production

Figure 4 shows the effect of various incubation temperature on α-amylase production from *Bacillus subtilis* Y25. Optimum production of α-amylase was observed at 45 °C (Figure 4). Beyond this temperature, enzyme production decreased drastically.

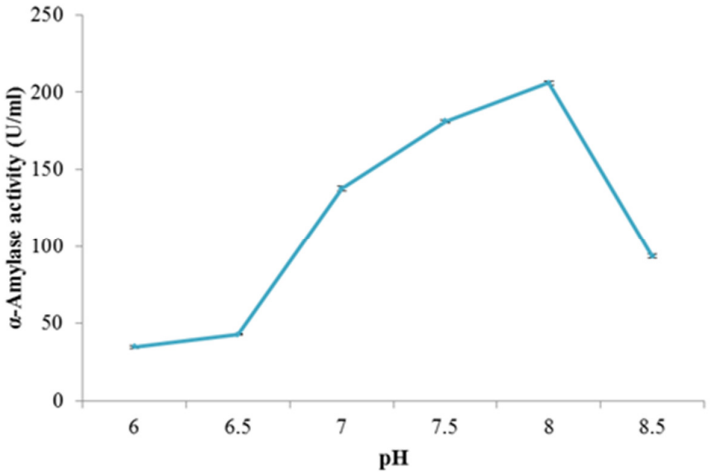

Figure 3. Effect of pH on α-amylase production from *B. subtilis* Y25

Values are means ± standard deviation of three independent experiments used in study

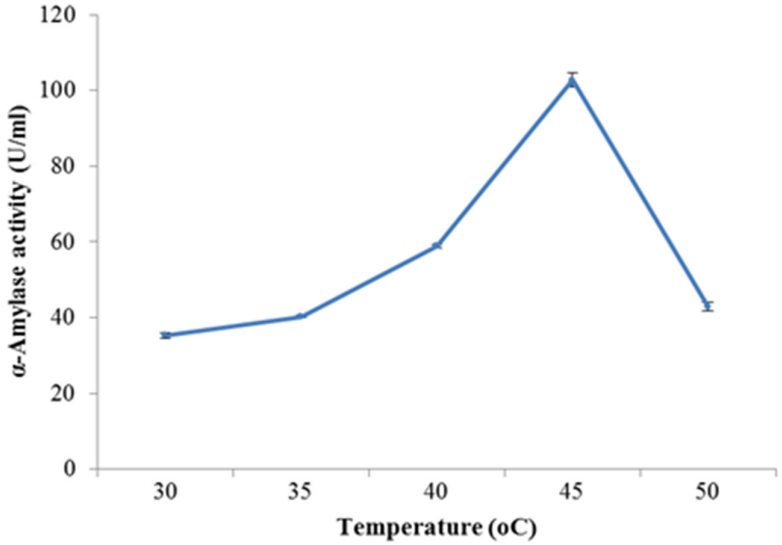

Figure 4. Effect of temperature on α-amylase production from *B. subtilis* Y25
Values are means ± standard deviation of three independent experiments used in study

Effect of different carbon and nitrogen sources on α-amylase production

α-Amylase production from *Bacillus subtilis* Y25 was maximum with the use of starch as the source of carbon (95.25±2.03 U/ml). This was followed by the use of lactose (21.29±1.57 U/ml), glucose (19.03±1.36 U/ml) and maltose (9.48±0.93 U/ml) as carbon sources, respectively (Figure 5). Maximum production of α-amylase from *B. subtilis* Y25 was observed with the use of peptone as the nitrogen source in the production medium (49.97±1.35 U/ml). This was followed by the use of calcium nitrate (16.65±1.20 U/ml), tryptic soy broth (18.02±0.60 U/ml) and ammonium sulphate (37.22±1.09 U/ml) respectively, as sources of nitrogen (Figure 6).

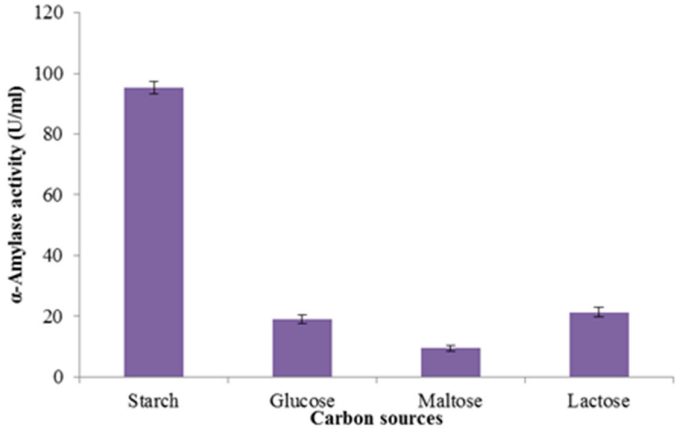

Figure 5. Effect of carbon sources on α-amylase production from *B. subtilis* Y25
Values are means ± standard deviation of three independent experiments used in study

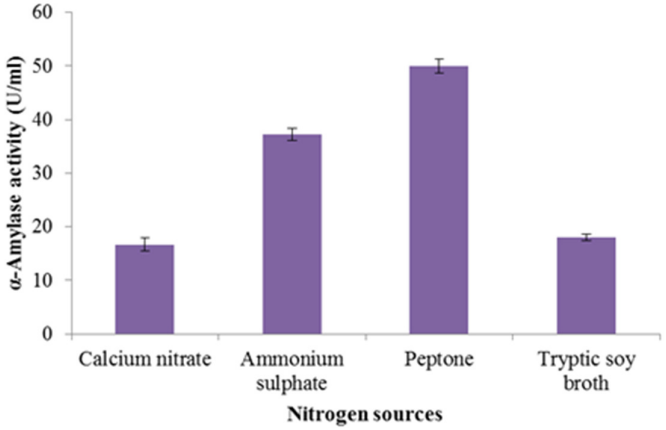

Figure 6. Effect of nitrogen sources on α-amylase production from *B. subtilis* Y25
Values are means ± standard deviation of three independent experiments used in study

Effect of starch concentration on α-amylase production

The influence of different concentration of starch (0.5% to 2.5%$^{w}/_{v}$) in the production medium, on α-amylase production from *B. subtilis* Y25, was studied. Maximum production of α-amylase was observed at 0.5%$^{w}/_{v}$ starch concentration with activity of 97.85±1.11 U/ml. However, the enzyme production decreased with increase in starch concentration beyond 0.5%$^{w}/_{v}$ (Figure 7).

Enzyme purification and characterization

Purification of enzyme using ion exchange column chromatography on CM-Sepharose CL-6B

After ion exchange chromatography on CM-Sepharose CL-6B column in which adsorbed proteins were eluted with a linear gradient of 0 to 1.0 M NaCl, active fractions of α-amylase from *B. subtilis* Y25 were pooled. The elution profile is as shown in Figure 8. The pooled fractions had a specific activity of A single peak of α-amylase activity was observed with a yield of 69.36%, specific activity of 112.37 U/mg protein and purification fold of 3.80 (Figure 8 and Table 1).

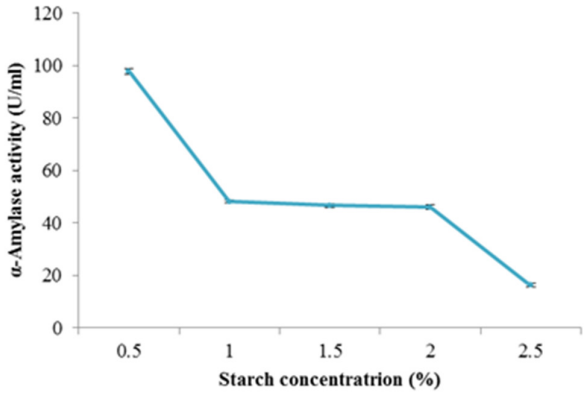

Figure 7. Effect of starch concentration on α-amylase production from *B. subtilis* Y25
Values are means ± standard deviation of three independent experiments used in study

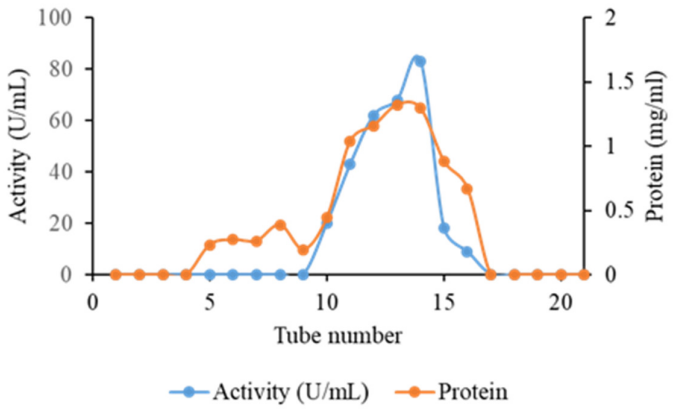

Figure 8. Elution profile of α-amylase from *B. subtilis* Y25 on CM Sepharose CL-6B. The flow rate was 12 ml/h. Bound proteins were eluted with a 0-1 M NaCl gradient dissolved in the elution buffer. Fractions were assayed for amylase activity and protein profile was assayed

Table 1. Summary of purification steps α-amylase obtained from *Bacillus subtilis* Y25

Purification step	Volume (ml)	Activity (Units/ml)	Total activity (Units)	Protein (mg/ml)	Total protein (mg)	Specific activity (U/mg)	Yield (%)	Purification fold
Crude enzyme	135	12	1620	3	405	4	100	1
CM Sepharose CL-6B	10	112.37	1123.7	7.39	70.39	15.21	69.36	3.8

Molecular mass of α-amylase

The native molecular weight of the purified α-amylase from *Bacillus subtilis* Y25 was determined by gel permeation chromatography on Sephadex G-100. The molecular weight was estimated to be 58 KDa.

Kinetic parameter

The Michaelis-Menten constants (K_m and V_{max}) of the purified *Bacillus subtilis* Y25 α-amylase for hydrolysis of soluble starch were estimated using Graphpad prism 5 Computer Software Programme to be 53.98±12.03 mg/ml and 314.10±23.30 Units/mg protein, respectively (Figure 9).

Optimum temperature of α-amylase from B. subtilis

The effect of temperature on the activity of purified α-amylase, in the range 30 to 100 °C, was studied. The enzyme activity increased gradually from 30 °C to reach the maximum at a temperature of 60 °C. Beyond this optimum temperature, the activity reduced drastically (Figure 10).

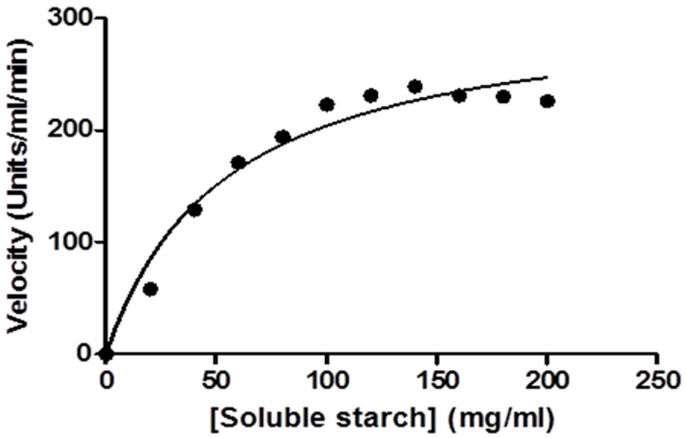

Figure 9. Non-linear regression plot for kinetic parameter of α-amylase from B. subtilis Y25 using Graph Pad Prism. Incubation was for 20 min at temperature of 60 °C and pH of 8.0

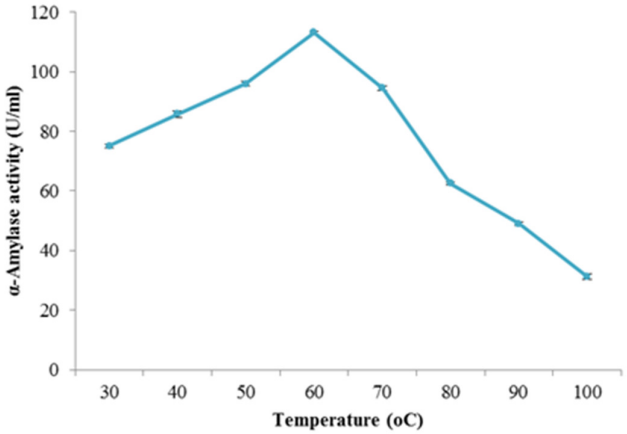

Figure 10. Effect of temperature on the activity of purified α-amylase from *B. subtilis* Y25
Values are means ± standard deviation of three independent experiments used in study

Thermal stability of α-amylase from B. subtilis

The enzyme showed appreciable thermal stability up to 60 °C retaining 70.39%, 56.89% and 50.44% of its activity after 30 min, 40 min and 50 min, respectively. However, it was rapidly inactivated at longer periods and at higher temperature (Figure 11).

Optimum pH of α-amylase from B. subtilis

The activity of α-amylase from *B. subtilis* increased as the pH increased reaching the optimum at pH 8.0. The enzyme was observed to exhibit appreciable activity at pH range 6.0 to 7.5 and very good activity at pH range 7.0 to 9.0 (Figure 12).

Influence of metal ions and EDTA on α-amylase from B. subtilis Y25

The effect of metal ions and EDTA on the activity of purified α-amylase from *Bacillus subtilis* Y25 was studied and shown in Table 2. The enzyme showed great stability against all the tested metal ions (Na^{+}, Mg^{2+},

Ca^{2+}), at 20 mM concentration. However, EDTA produced an inhibition effect of 37.0% on the enzyme activity (Table 2).

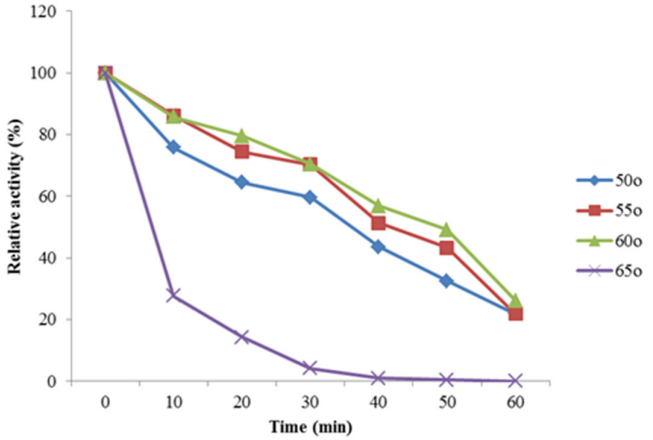

Figure 11. Thermal stability of purified α-amylase from *Bacillus subtilis* Y25
Values are means ± standard deviation of three independent experiments used in study

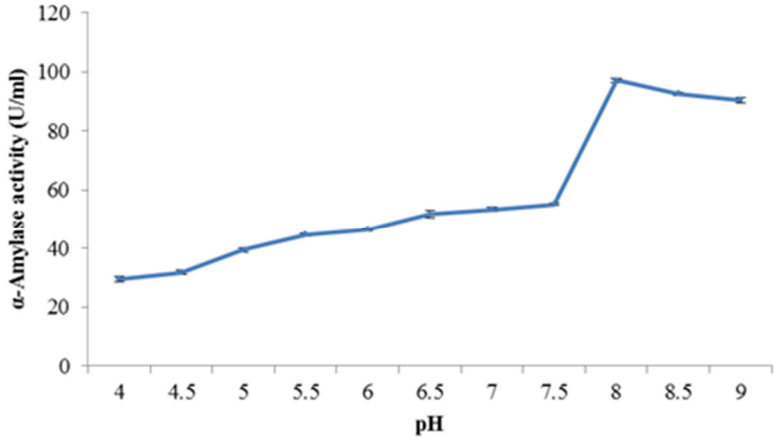

Figure 12. Effect of pH on the activity of purified α-amylase from *Bacillus subtilis* Y25
Values are means ± standard deviation of three independent experiments used in study

Table 2. Effect of metal ions and EDTA on the activity of α-amylase from *B. subtilis* Y25

S/No.	Addition	Relative activity (%)
1	None (control)	100
2	NaCl	83
3	$CaCl_2$	100
4	$MgCl_2$	97
5	EDTA	63

Discussion

In the present study, thirty-six bacterial isolates were obtained from decaying yam tubers and screened for potential α-amylase production on starch agar plates. Seven of the isolates expressed α-amylase production

by showing zones of clearance around their colonies. Subjection of these to enzyme production and quantitation of their α-amylase activities revealed the isolate Y25 as the best enzyme producer. This bacterium was selected for subsequent studies. It was characterized and identified by molecular method, based on the 16S rRNA gene sequencing, as a strain of of *B. subtilis* with maximum identity of 100% to other *B. subtilis*. Several species of bacteria belonging to the genera *Bacillus* are reported to exhibit the ability for α-amylase production (Shalini and Solanki, 2014; Ayansina *et al*., 2017; Demirkan *et al*., 2017; Ozdemir *et al*., 2018).

The production of enzymes such as α-amylase is often dependent on growth of the producing bacterium in the appropriate culture medium. In the present study, the α-amylase production increased with increase in the incubation period reaching a maximum at 32 h incubation after which enzyme production declined. The growth pattern followed a similar trend with *B. subtilis* Y25 having a lag phase of 2 h and then growing logarithmically from 4 h to 32 h with a corresponding increase in enzyme production. The growth peaked at 36 h of incubation period beyond which a steady decline occurred. A similar pattern of bacterium growth and α-amylase production, in relation to incubation period, was reported by Prakash *et al*. (2008).

The optimum pH for *B. subtilis* Y25 α-amylase production was observed to be 8.0. Anupama and Jayaraman (2011) reported a similar pH for α-amylase production from *B. aquimaris* VITP4. However, pH of 7.0 was reported for α-amylase production from strains of several *Bacillus* species (Mohammed *et al*., 2011; Singh *et al*., 2012; Omoboye *et al*., 2014; Demirkan *et al*., 2017). Saxena *et al*. (2007) had reported that α-amylase production from *Bacillus* species mostly occur in alkaline conditions (pH 7.5 to 11.0). The optimum pH influences the way the shape of the active site is most complementary to the shape of the substrate. Any change above or below the optimum will quickly cause a decrease in the rate of reaction, since more of the enzyme molecules will have active sites whose shapes are not or at least are less complementary to the shape of their substrate (Demirkan *et al*., 2017).

Maximum production of α-amylase occurred at a temperature of 45 °C beyond which there was decline in enzyme production. A similar incubation temperature was best for the enzyme production from *B. licheniformis* RD24 (Omoboye *et al*., 2014) and *B. cereus* Ms6 (Mohammed *et al*., 2011). However, optimum temperature of 40 °C was reported for α-amylase production from several other *Bacillus* species (Konsoula and Liakopoulou-Kyriakides, 2004; Mukhtar and Ikram, 2012; Singh *et al*., 2015). At high temperatures, enzyme production decreased which might be due to growth inhibition and hence enzyme inactivation (Goes and Sheppard, 1999).

Alpha amylase production from *Bacillus subtilis* Y25 was maximum with the use of starch as the carbon source, followed by the use of lactose, glucose and maltose, respectively. Elkady *et al*. (2017) and Gangadharan *et al*. (2006) reported that *Bacillus* sp. NRC12017 and *Bacillus amyloliquefaciens*, respectively, gave the highest enzyme yield with soluble starch. A decrease in enzyme production was observed with an increase in starch concentration. This is similar to the report of Elmansy *et al*. (2018). High starch concentrations cause the broth culture to be more viscous, thus interfering with O_2 transfer resulting in restriction of dissolved O_2 required for the microbial growth. Maximum production of α-amylase from *B. subtilis* Y25 was observed with the use of peptone as the nitrogen source in the production medium. This may be due to the fact that peptone is readily available to the bacterium for metabolism during growth and enzyme production as opposed to other nitrogen sources. Nitrogen source type and relative concentration in the growth medium are important for both microbial growth and amylase production (NandLal *et al*., 2016). The nitrogen is metabolized to produce primarily amino acids, nucleic acids, protein, enzymes and other cellular components that play a vital role in metabolism. Purification using ion exchange chromatography through CM-Sepharose CL-6B gave a specific activity 1123.7 U/mg protein and yield of 69.36% with purification fold of 3.80. Abdel-Fattah *et al*. (2012) and Bakare *et al*. (2014) indicated enzyme purification fold and percentage yield of 59.3 and 12.6%, and 4.76 and 47.0%, respectively.

The native molecular weight of the enzyme was estimated to be 58.0 KDa. The molecular weight of α-amylase from *Bacillus* sp. was reported to vary between 50.0 and 60.0 KDa with only a few exceptions (Smitha *et al*., 2019). Liu *et al*. (2008) found that the molecular weight of *B. licheniformis*, a thermostable α-amylase was 53.13 KDa.

The Michaelis-Menten constant (K_m and V_{max}) values of the purified *Bacillus subtilis* Y25 α-amylase for hydrolysis of soluble starch were estimated to be 53.98 ± 12.03 mg/ml and 314.10 ± 23.30 Units/mg protein, respectively. Benjamin *et al*. (2013) stated that smaller values of K_m observed for *Bacillus* spp. indicated that the enzyme and substrate are tightly bound and form the enzyme-substrate complex more quickly, thus more activity. The low K_m value of purified *Bacillus subtilis* Y25 α-amylase is an indication that the enzyme has the potential for effectiveness in the transformation of starch and starch-based substrates.

The purified α-amylase from *Bacillus subtilis* Y25 displayed activity over a wide pH range of 4.5 to 9.0 but with optimum at 8.0. Anupama and Jayaraman (2011) recorded a similar pH of 8.0 for the enzyme from *Bacillus aquimaris* VITP4. α-Amylases from most *Bacillus* sp. have optimum pH in the range 5.0 to 7.0 (Malhotra *et al*., 2000; Gupta *et al*., 2003; Sivaramakrishan *et al*., 2006). Yang *et al*. (2011) reported an alkaline α-amylase with optimum pH of 9.0. The alkaline α-amylase from *Bacillus subtilis* Y25, with optimum activity at pH 8.0, has potential applications for hydrolyzing starch under high pH conditions in the starch and textile industries. It may also be of value as ingredients in detergent formulation for automatic dishwashers and laundries.

The purified α-amylase from *B. subtilis* Y25 exhibited optimal activity at 60 °C. Similar results were obtained for α-amylases from *B. subtilis* (Ozdemir *et al*., 2011) and *B amyloliquefaciens* P-001 (Deb *et al*., 2013). However, Yang *et al*. (2011) obtained an optimum temperature of 50 °C for α-amylase while Bakare *et al*. *(*2014) and Ozdemir *et al*. (2018) recorded optimal temperature of 70 °C for α-amylases from *Bacillus licheniformis* RD24 and *B. mojavensis* SO-B11, respectively. The enzyme exhibited appreciable thermal stability at temperatures up to 60 °C for 50 min. The relatively higher optimum temperature for activity and good thermal stability are properties of great significance for starch saccharification and may make the enzyme suitable for application to several biotechnological and industrial purposes such as in brewing and food processing.

The enzyme showed stability against calcium ion (Ca^{2+}), at 20 mM concentration, but was slightly inhibited by sodium ion (Na+) and magnessium ion (Mg^{2+}), at the same concentration. Also, α-amylase activity was significantly inhibited by EDTA. Similar results were reported by Hmidet *et al*. (2008) for α-amylase from *B. licheniformis* NH1 and Yang *et al*. (2011) for alkaline α-amylase from recombinant *B. subtilis*. However, Ca^{2+} activated α-amylase from *B. mojavensis* SO-10 (Ozdemir *et al*., 2018). Lin *et al*. (1998) suggested that calcium ion acts as a stabilizer and activator for α-amylase and that requirement for Ca^{2+} differ for different α-amylases. EDTA produced an inhibitory effect on the enzyme activity. This suggests that the α-amylase from *B. subtilis* Y25 is a metalloenzyme and that the enzyme contains metallic ions which are removed by the chelating agent, forming an active complex with EDTA and consequent loss of activity (Femi-Ola and Olowe, 2011). Similar results were reported for purified α-amylase by several authors (Hmidet *et al*., 2008; Xie *et al*., 2014; Ozdemir *et al*., 2018). Chelating agents such as EDTA inactivate enzymes by either removing metal ions from the enzyme or by binding inside the enzyme as a ligand (Zeng *et al*., 2014).

Conclusions

α-Amylase-producing *Bacillus subtilis* Y25 was isolated from deteriorating yam (*Dioscorea rotundata*) tubers. Enhanced α-amylase production from the bacterium was achieved at pH and temperature conditions of 8.0 and 45 °C, respectively and with the use of starch and peptone as carbon and nitrogen sources,

respectively. Purified α-amylase from the isolate exhibited optimum activity at pH 8.0 and temperature of 60 °C. As a result of its activity and stability at high temperature and pH, the enzyme has potentials for biotechnological applications for starch hydrolysis in textile industries and as an ingredient in detergents for dishwashers and launderers.

Acknowledgements

This research received no specific grant from any funding agency in the public, commercial, or not-for-profit sectors.

Conflict of Interests

The authors declare that there are no conflicts of interest related to this article.

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