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Expression and characterization of α-Amylases from *penicillium citrinum* with bread as growth substrate

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ABSTRACT

In an attempt to enhance the industrial production of α-amylases in the tropics, sterile fresh bread was inoculated with spore suspensions of *Penicillium citrinum* at 25 °C. Extracellular α-amylases were produced and subjected to partial purification by ammonium sulphate precipitation and dialysis. Further purification by gel filtration and ion-exchange chromatography was engaged. The molecular weights of the α-amylase fractions obtained and estimated by gel filtration using Sephadex G-100 were approximately 56,234 Daltons, 53,089 Daltons and 11,885 Daltons. The apparent Michalis-Menten constant (K_m) values for the hydrolysis of starch by the purified α-amylase fractions were approximately 8.3 mg/ml, 10 mg/ml and 7.14 mg/ml respectively. Optimum activities were at 30 °C for one of the fractions and 35 °C for the other two fractions and were at pH 5.5 and pH 6.0. The activities of the α-amylase fractions produced by the fungus were stimulated at varying degrees by NaCl, KCl, $CaCl_2$ and $MgCl_2$ but inhibited by Ethylene Diamine Tetraacetic Acid (EDTA), mercuric chloride ($HgCl_2$) and 2,4-dinitrophenol (DNP). The α-amylase fractions were sensitive to heat, losing all their activities within twenty minutes of heating at 80 °C. The industrial production of α-amylases should be encouraged in the tropics using bread as a cheap source of substrate.
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Keywords: α-Amylase, expression, bread, purification, characterization.

INTRODUCTION

α-Amylases are starch degrading enzymes that catalyze the hydrolysis of α-1, 4-O-glycosidic bonds in polysaccharides with the retention of α-anomeric configuration in the products. Most of the α-amylases are metalloenzymes which require calcium ions (Ca^{2+}) for their activity, structural integrity

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and stability (Bordbar et al., 2005). Amylases are one of the most important industrial enzymes that have a wide variety of applications ranging from conversion of starch to sugar syrups, to the production of cyclodextrins for the pharmaceutical industry. These enzymes account for about 30% of the world's enzyme production (van der Maarel et al., 2002). The α-amylase family can roughly be divided into two groups: the starch hydrolyzing enzymes and the starch modifying or transglycosylating enzymes (Satyanarayana et al., 2005). Ajayi et al. (2014) reported the production of amylase by *Aspergillus chavilieri* with optimum activity observed at 35 ^{o}C and at pH 6.5. Starch as carbon source of growth with ammonium chloride as nitrogen source are good supports for production of α-amylase by *Aspergillus niger* (Adejuwon et al., 2015a). However, starch as carbon source of growth urea, tryptone or peptone as nitrogen source support good expression of amylase by *Penicillium rubrum* within ten days (Adejuwon et al., 2015b). Maltose, lactose or sucrose as carbon source for growth with potassium nitrate as nitrogen source seem not support much early expression of α-amylase in *Penicillium citrinum* (Adejuwon, 2015b).

Bread is made up of dough, leavened using baker's yeast, *Saccharomyces cerevisiae* (Otero et al., 2010). It contains iron, thiamine, riboflavin, niacin, vitamin B_6, folate, fibre, magnesium and zinc (Sizer and Whitney, 2000; Willey et al., 2008). This makes it a good source of microbial growth (Adejuwon, 2013a).

The production of α-amylases, which are industrially useful enzymes, can be encouraged using microbial physiological applications. In this current study, bread was inoculated with spore suspensions of *Penicillium citrinum* at a particular temperature and observed daily for deterioration. This was with the view to enhancing the industrial production of α-amylases in the tropics.

MATERIALS AND METHODS

Materials

Starch (Sigma), 2,4-dinitrophenol (DNP), ethylene diamine tetraacetic acid (EDTA), mercuric chloride, sodium chloride, magnesium chloride, potassium chloride, calcium chloride, lactophenol cotton blue, potato dextrose agar (PDA) and bovine serum albumin (BSA) were products of Sigma Chemical Company, Illinois, USA or British Drug House (BDH), Poole, England. Sephadex G-100, CM-Sephadex C-25 and CM-Sephadex C-50 were purchased from Pharmacia Fine Chemicals, Uppsala, Sweden. Ethanol, sodium azide, sodium carbonate, sodium hydroxide, hydrated copper sulphate, potassium sodium tartrate, iodine, potassium iodide, absolute ethanol, diethyl ether, pyridine, benzene, acetic anhydride, hydrochloric acid, citric acid, disodium hydrogen orthophosphate, catalase, glucose oxidase, human haemoglobin, egg albumin, horse myoglobin and cytochrome C were bought from British Drug House (BDH). Glass fibre filter papers were from Whatman and Folin-Ciocalteu's phenol reagent was from Sigma-Aldrich Chemie GmbH, Fluka Biochemika. Freshly baked bread was purchased from the bakery of the Department of Food Science and Technology, Obafemi Awolowo University, Ile-Ife, Nigeria.

Apparatus used included, top load weighing balance (Mettler PB 153), pH meter (Jenway 3015), electric stirrer, cold centrifuge (Optima LE-80K ultracentrifuge, Beckman, USA), UV/VIS spectrophotometer (Cecil 2041), automated LKB fraction collector (700A Ultro Rac), glass tube chromatographic columns (Pharmacia, Sweden), water bath, Neubauer counting chamber (Gallenhamp),

multiple dialyser (Pope Scientific Inc. Model 220, USA), portable autoclave (Express Equipment, Dixon's Surgical Instruments Ltd, USA), heater (Mettler), inoculating chamber (Beckman, USA).

Preparation of buffers and reagents

Preparation of 0.2 M citrate phosphate buffer (pH 6.0) containing 5 mM sodium azide

The buffer was prepared by weighing 21.014 g of citric acid, 28.392 g of disodium hydrogen phosphate and 0.32505 g of sodium azide (NaN_3) into a beaker and dissolving with little amount of distilled water. The total volume of the solution was made up to 1 litre with distilled water after the pH had been checked with a digital pH meter.

Preparation of 0.2% starch in 0.2 M citrate phosphate buffer (pH 6.0) containing 5 mM sodium azide

Exactly 0.2 g of soluble starch was weighed in a conical flask and warmed to dissolve with 10 ml of distilled water. The total volume was made up to 100 ml with 0.2 M citrate phosphate buffer pH 6.0 containing 5 mM sodium azide.

Preparation of iodine solution (0.3% Iodine in 3% KI)

Exactly 3.0 g of potassium iodide (KI) was weighed in a dark bottle and warmed to dissolve in 100 ml of distilled water. Into this, 0.3 g of iodine was thereafter added and allowed to dissolve in the solution by mixing and warming.

Preparation of Lowry's reagents

Lowry's reagents were prepared by weighing 2 g of Na_2CO_3 in conical flask and dissolving with 100 ml of 0.1 N NaOH (Reagent A). Reagent B was prepared by weighing 0.5 g of $CuSO_4.5H_2O$ and dissolving with 100 ml of 1% potassium sodium tatrate solution. Reagent C was 50 ml of Reagent A mixed with 1 ml of Reagent B. Reagent D was Folin-Ciocalteu's phenol reagent diluted with distilled water in the ratio 1:1 (v/v).

Elution buffers

The elution buffer used for fractionation on Sephadex G-100 column was 0.2 M citrate phosphate buffer, pH 6.0 containing 5 mM sodium azide. While the elution buffer used for fractionation on CM-Sephadex C-25 and CM-Sephadex C-50 columns was 0.2 M citrate phosphate buffer, pH 6.0 containing 5 mM sodium azide with linear gradients of 0.1 – 0.5 M NaCl.

Sources and identification of isolates

The isolate of *P. citrinum* for this research was isolated from mouldy bread and identified using techniques contained in the illustrated Handbook of Fungi (Hanlin, 1990). The identification was done by observing cultural and morphological characteristics. The isolate was cultured on Potato Dextrose agar. The nature of growth, rate of growth, colony colour and sporulation patterns were carefully observed. Sporulating mature cultures were used in microscopic examination. Fungal samples were taken from advancing margins and centres of the growth regions with the aid of sterile inoculating needle. The samples were smeared on glass slides and stained with lactophenol cotton blue. Macroscopic and microscopic morphological characteristics like arrangement and shape of spores, type of sporangia, type of hyphae, presence or absence of septa on hyphae were examined under the high power objective of a compound binocular microscope.

Bread as a source of carbon

Freshly baked loaves of bread were bought at the bakery of the Department of Food Science and Technology, Obafemi Awolowo University, Ile-Ife, Nigeria. The bread loaf was soaked in distilled water (1% w/v), mercerated with a homogenizer and autoclaved at 15 psi (121 ^oC) for 15 minutes.

One hundred millilitres of the bread medium in conical flasks (250 ml) was inoculated with 1 ml of aqueous spore suspension containing approximately $5x10^5$ spores per ml of isolate. Incubation was at 25 ^{o}C.

On a daily basis, the contents of each flask were filtered through glass fibre filter paper (Whatman GF/A). The protein content of the filtrates was determined as described. The filtrates were assayed for α-amylase activity using the method of Pfueller and Elliott (1969).

Enzyme and protein assays

α-Amylase

α-Amylase activity was determined using the method of Pfueller and Elliott (1969). The reaction mixtures consisted of 2 ml of 0.2% (w/v) starch in 0.2 M citrate phosphate buffer, pH 6.0 as substrate and 0.5 ml of enzyme. Controls consisted of only 2 ml of the prepared substrate. The contents of both experimental and control tubes were incubated at 35 ^{o}C for 20 min. The reaction in each tube was terminated with 3 ml of 1 N HCl. Enzyme (0.5 ml) was then added to the control tube. Two millilitre of the mixture from each of the sets of experimentals and controls was transferred into new sets of clean test tubes. Three millilitre of 0.1 N HCl was added into the contents of each test tube after which 0.1 ml of iodine solution was added. Optical density readings were taken at 670 nm. One unit of enzyme activity was defined as the amount of enzyme which produced 0.1 percent reduction in the intensity of the blue colour of starch-iodine complex under conditions of the assay. Specific activity was calculated as enzyme units per milligram protein.

Protein concentration determination

Protein concentration was routinely determined by the method of Lowry et al. (1951). The Lowry assay, a colometric protein assay, is based on the reaction of the protein with copper in alkali and the reduction of the phosphomolybdic-phosphotungstic reagent (Folin's reagent) by the copper treated protein. Exactly 5 ml of reagent C was added to 1 ml of the test sample. This was thoroughly mixed and left at room temperature for 10 min. Thereafter, 0.5 ml of reagent D was added and allowed to remain at room temperature for 30 min. Absorbance was read at 600 nm. Serial dilutions of Bovine serum albumin were treated likewise and used to plot a standard graph. The unknown protein value in each test sample was intrapolated from the standard calibration graph.

Ammonium sulphate fractionation

The crude enzymes, on the days of optimum activity during daily basis samplings, were treated with ammonium sulphate (analytical grade) at 90% saturation (662 g/L). Precipitation was allowed to continue at 4 ^{o}C for 24 h. The mixtures were centrifuged at 4,000 rpm for 30 minutes at 4 ^{o}C using a high speed cold centrifuge (Optima LE-80K Ultracentrifuge, Beckman, USA) at the Central Laboratory, Obafemi Awolowo University, Ile-Ife, Nigeria. The supernatant was discarded. The precipitate was redissolved in 0.2 M citrate phosphate buffer, pH 6.0. The protein contents were determined using the Lowry et al. (1951) method while α-amylase activity was determined using the method of Pfueller and Elliott (1969).

Dialysis

Using acetylated dialysis tubings (Visking dialysis tubings, Sigma) (Whitaker et al. 1963) and a multiple dialyser, the enzyme preparations were dialysed under several changes of 0.2 M citrate phosphate buffer, pH 6.0 at 4 ^{o}C for 24 h. The protein contents of the dialysed enzymes were determined using the Lowry et al. (1951) method while α-amylase activity was determined using the method of Pfueller and Elliott (1969).

Fractionation of enzyme using Sephadex G-100 column

Dialysed α-amylase from *P. citrinum* was subjected to further purification using Sephadex G-100.

(a)Preparation and packing of Sephadex G-100 column

The Sephadex G-100 resin was suspended in buffer (0.2 M citrate phosphate, pH 6.0 containing 5 mM sodium azide) and allowed to swell for seventy two hours. Fine particles on the suspension were decanted. The suspension was deaerated under vacuum pressure until a slurry of air-free gel particles was obtained. The buffer was also deaerated. The column was half filled with the buffer and the gel slurry added until the column was almost filled. The gel was left to settle in the column. The top of the column was connected to a reservoir and more gel added until the column was filled. The buffer was allowed to flow continuously through the gel throughout the process. When the column had been well equilibrated with the same buffer and resin and a constant bed height obtained, the enzymes were applied. A sample applicator (2.3 cm x 5.0 cm) was placed on top of the gel to prevent distortion when applying sample. Fractions of 5 ml were collected at a flow rate of 10 ml per hour.

(b)Calibration of Sephadex G-100 column

The column was calibrated with proteins of known molecular weight (Andrews, 1964; Olutiola and Cole, 1976). Five milligram per ml each of the standard proteins: catalase (mol. wt., 240, 000), glucose oxidase (mol. wt., 150, 000), human haemoglobin (mol. wt., 68, 000), egg albumin (mol. wt., 45, 000), horse myoglobin (mol. wt., 17, 000) and cytochrome C (mol. wt., 12, 400) (section 3.1) was dissolved in 0.2 M citrate phosphate buffer, pH 6.0 containing 5 mM sodium azide. The total sample volume of each standard protein was 5 ml. The solution was applied to the column. Fractions (5 ml/tube) were collected in tubes using an automated LKB fraction collector (700A Ultro Rac). Protein content of eluted fractions was monitored at 280 nm. The elution volume of each of the standard protein was plotted against the logarithm of its molecular weight to obtain a standard calibration curve (Andrews, 1964).

(c)Application of enzyme to Sephadex G-100 column

Ten milliliter of the dialysed enzyme was applied to the column and eluted with 0.2 M citrate phosphate buffer, pH 6.0 containing 5 mM of sodium azide. Fractions (5 ml/tube) were collected. Protein was monitored at 280 nm. α-Amylase activity was determined as described. Fractions with α-amylase activity with peak ranges were pooled. Also, the molecular weight of the unknown enzyme was extrapolated from the standard curve.

Further fractionation using ion-exchange chromatography

CM-Sephadex C-25 and CM-Sephadex C-50 columns were prepared as described in Pharmacia manual. The resin were swollen in distilled water and equilibrated in the elution buffer. A column (2.5 x 40 cm) was used. Ten milliliter of pooled fractions from the Sephadex G-100 column which exhibited activity were applied to the prepared columns of CM-Sephadex C-50 and CM-Sephadex C-25. The columns were first washed with 0.2 M citrate phosphate buffer pH 6.0 containing 5 mM sodium azide to remove unbound proteins followed by elution with 0.2 M citrate phosphate buffer, pH 6.0, with linear gradients of 0.1 – 0.5 M NaCl. Fractions (5 ml/tube) collected were monitored spectrophotometrically at 280 nm. Amylase activity was determined using the method of Pfueller and Elliott (1969).

Properties of the Partially Purified Enzymes

The effects of temperature, pH, salts and some chemicals on the activities of the purified α-amylases from *P. citrinum* were investigated after the fractionation.

Effect of temperature

The substrate used was 0.2% (w/v) starch in 0.2 M citrate phosphate buffer, pH 6.0. The reaction mixture was 2 ml of substrate and 0.5 ml of enzyme. Incubation was at a range of 4-50 ^{o}C for 1 hr. α-Amylase activity was there after determined.

Stability test at 80 ^{o}C

The effect of heat (80 ^{o}C) on the stability of the partially purified enzymes at different periods, 0, 2, 10, 15 and 20 minutes was examined. The activities of the heated enzymes were determined by incubating 0.5 ml of each enzyme with 2 ml of the citrate phosphate buffered 0.2% starch substrate (pH 6.0) at 35 ^{o}C for 1 hr. α-Amylase activity was there after determined.

Effect of pH

The substrate used was 0.2% (w/v) starch in 0.2 M citrate phosphate buffer at different pH values ranging from pH 3.0 – 8.0. The reaction mixture was 2 ml of substrate and 0.5 ml of enzyme. Incubation was at 35 ^{o}C for 1 hr. α-Amylase activity was then determined.

Effect of substrate concentrations

Different concentrations, 0.08 – 0.4% (w/v) of starch (Sigma) in 0.2 M citrate phosphate buffer, pH 6.0 were used as substrates. The reaction mixture was 2 ml of substrate and 0.5 ml of enzyme, incubated at 35 ^{o}C for 1 hr. α-Amylase activity was afterwards determined.

Effect of salts and some chemicals

The effect of NaCl, KCl, $CaCl_2$ and $MgCl_2$ at varying concentrations (0, 2, 10, 15, and 20 mM) on the activities of the purified α-amylases were examined. Each salt was prepared in 0.2% starch in citrate phosphate buffer pH 6.0. The reaction mixture, 2 ml of substrate and 0.5 ml of enzyme, was incubated at 35 ^{o}C for 1 hr. α-Amylase activity was then determined.

Different concentrations (0, 2, 4, 6 and 8 mM) each of mercuric chloride, 2,4-dinitrophenol and EDTA were prepared in 0.2% starch in citrate phosphate buffer, pH 6.0. These were used as substrates. α-Amylase activity was determined.

RESULTS

α-Amylase activity of isolate on bread medium

At incubation of 25 ^{o}C, *P. citrinum* grew and exhibited α-amylase activity in the bread medium used in this research.

Fractionation of enzyme on Sephadex G-100

Gel filtration of dialysed enzyme on Sephadex G-100 gave five peaks of protein designated A, B, C, D, E. Only the components of peaks B, C and D possessed α-amylase activity. The molecular weights of components of peaks B, C and D estimated from their elution volumes were approximately 56,234 Daltons, 53,089 Daltons and 11,885 Daltons respectively.

Fractionation of enzyme on CM-Sephadex C-50 and CM-Sephadex C-25

Elution of components of peak B of *P. citrinum* on CM Sephadex C-50 produced three peaks of protein designated Ba, Bb and Bc. Only the components of peak Ba exhibited α-amylase activity. When the components of peak C were separated on CM-Sephadex C-50, two peaks of protein designated Ca and Cb were obtained. Only the components of peak Ca possessed α-amylase activity. When the components of peak D were separated on CM-Sephadex C-25, three peaks of absorption designated Da, Db and Dc were obtained. Only the components of peak Db exhibited α-amylase activity.

Purification folds of approximately 24, 26 and 30 were obtained for peaks Ba, Ca and Db respectively (Table 1).

Properties of the partially purified enzymes

Investigations were carried out to determine the influence of some factors on the activities of the partially purified enzymes (CM-Sephadex C-25 and CM-Sephadex C-50 fractions). The fractions employed were components of Ba, Ca and Db.

Kinetic parameters - Effect of substrate (starch) and Lineweaver-Burk plots

The α-amylase activities of the components of peaks Ba, Ca and Db obtained from *P. citrinum* increased gradually as concentrations of starch increased (Figure 1). The Lineweaver-Burk plots indicated that the apparent K_m for the enzymic hydrolysis of starch were approximately 8.3 mg/ml, 10 mg/ml and 7.14 mg/ml for fractions Ba, Ca and Db respectively.

Effect of pH

The pH of the reaction mixtures affected the activities of the enzymes produced by the fungus. The activities of the enzymes increased with increase in pH and generally decreased after attaining an optimum value. Optimum α-amylase activities of fractions Ba, Ca and Db were at pH 5.5, 6.0 and 6.0 respectively (Figure 2).

Effect of temperature

The temperature of incubation had a remarkable effect on the activities of α-amylases produced by the fungus. The activities of the enzymes increased with increase in temperature of incubation until an optimum was reached after which there was gradual decline. Optimum α-amylase activities of fractions Ba, Ca and Db (Figure 3) for *P. citrinum* were at 35 °C, 30 °C and 35 °C respectively.

Effect of heat

Subjecting the α-amylases to heat at 80 ^{O}C for a period of twenty minutes had substantial effects on the activities of the enzymes. The activity of each enzyme decreased with increase in time of heating. When the α-amylase fractions Ba, Ca and Db of *P. citrinum* were subjected to heat for 2 minutes, activities of approximately 79%, 55% and 83% were lost. Complete inactivations were observed within 10 minutes (Figure 4).

Effect of salts

The salts employed in this investigation stimulated α-amylase activities synthesized by *P. citrinum.* There was a general increase in activity of enzyme with increase in concentration of each cation.

For α-amylase fractions Ba, Ca and Db (Figures 5-7), the highest stimulations were with $MgCl_2$ (for fraction Ba and Db) and $CaCl_2$ (for fraction Ca) at 20 mM concentrations. The least stimulations were with KCl (for fractions Ba and Db) and NaCl (for fraction Ca) at 20 mM concentrations.

Effect of some chemicals

Mercuric chloride ($HgCl_2$), ethylene diamine tetraacetic acid (EDTA) and 2,4-dinitrophenol (DNP) inhibited α-amylase activities of *P. citrinum* used in this research to different extents at different concentrations of the chemicals. As the concentrations of the chemicals increased, α-amylase activities gradually decreased. Inhibition of approximately 91%, 90% and 88% (for fraction Ba); 100%, 90% and 89% (for fraction Ca); 100%, 90% and 95% (for fraction Db) were observed at 8mM concentrations of $HgCl_2$, DNP and EDTA respectively (Figures 8-10).

Table 1: Partial purification of α-amylase obtained from bread deteriorated by *Penicillium citrinum*.

Fraction	Total Activity (Units)	Total Protein (mg)	Specific Activity (Units/mg protein)	Yield (%)	Purification fold
Crude extract	3110	66.0	47.1	100	1
90% $(NH_4)_2SO_4$ Precipitation	2228	23.8	93.6	71.6	1.9
Sephadex G-100 Gel filtration Chromatography					
Peak B	1149	7.4	155.3	36.9	3.2
C	923	6.3	146.5	29.7	3.1
D	1975	8.2	240.9	63.5	5.1
CM-Sephadex C-50 Ion-Exchange Chromatography					
Peak Ba	905	0.8	1131.3	29.1	24.02
Ca	485	0.4	1212.5	15.6	25.7
CM-Sephadex C-25 Ion-Exchange Chromatography					
Peak Db	859	0.6	1426.7	27.5	30.3

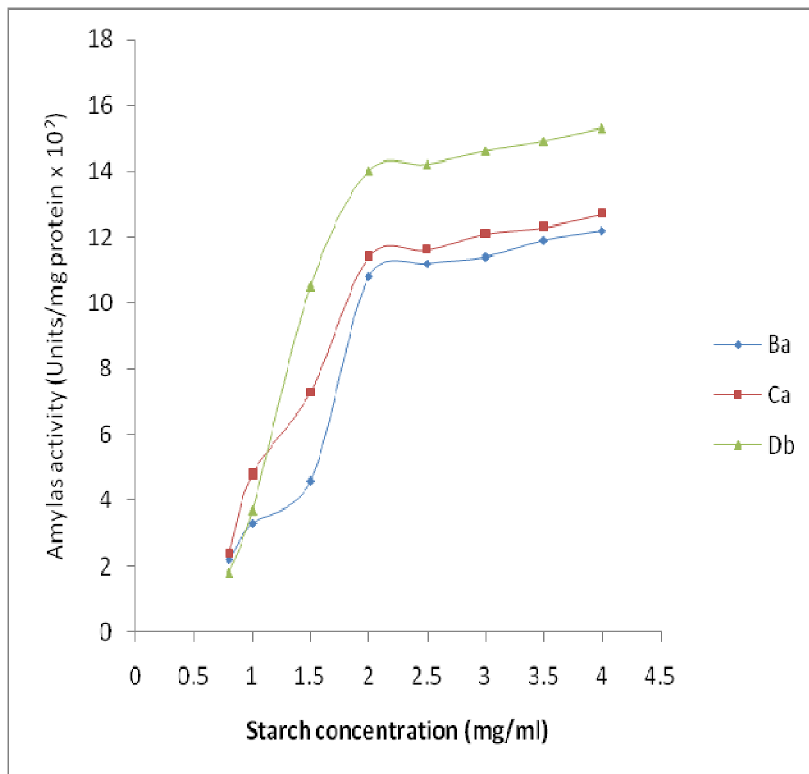

Figure 1: Effect of concentration of starch on the activity of partially purified α-amylases from *Penicillium citrinum*.

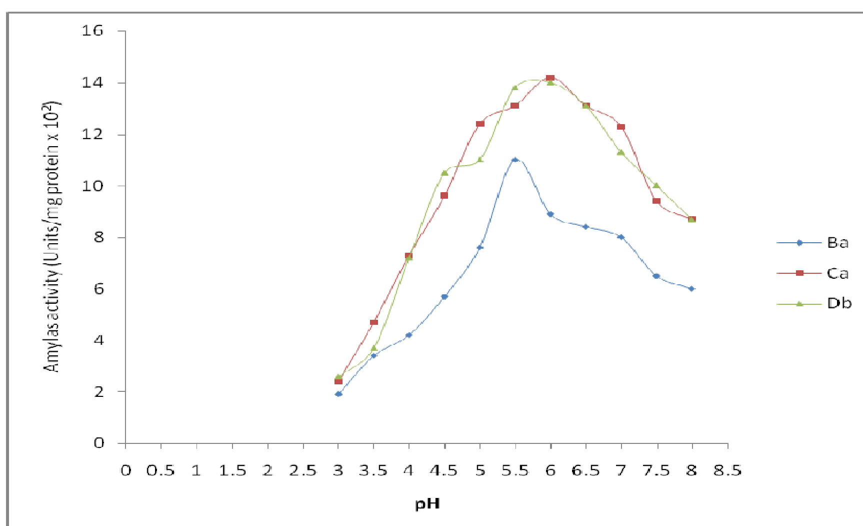

Figure 2: Effect of pH on the activity of partially purified α-amylases from *Penicillium citrinum*.

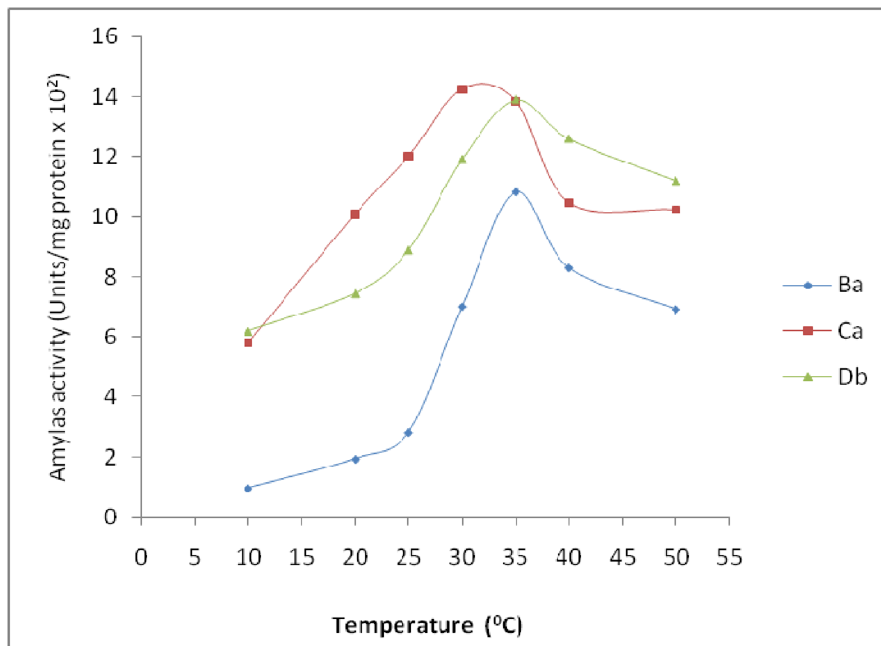

Figure 3: Effect of temperature on the activity of partially purified α-amylases from *Penicillium citrinum*.

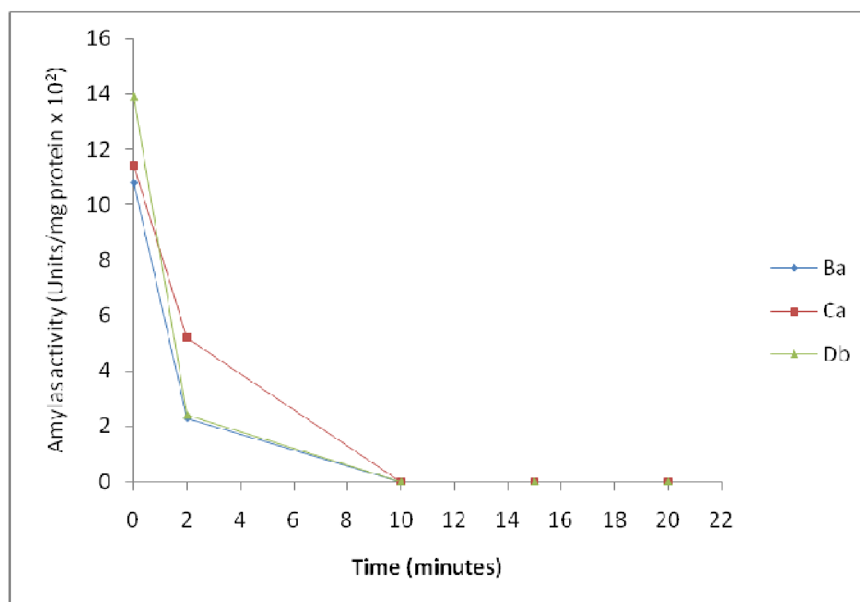

Figure 4: Heat stability test at 80 °C of partially purified α-amylases from *Penicillium citrinum*.

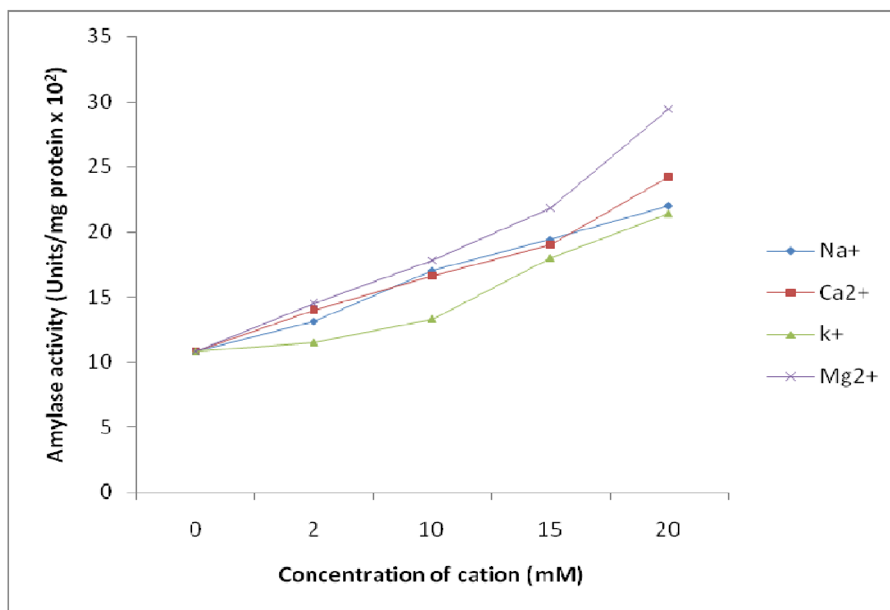

Figure 5: Effect of salts on the activity of the partially purified α-amylase (fraction Ba) obtained from *Penicillium citrinum*.

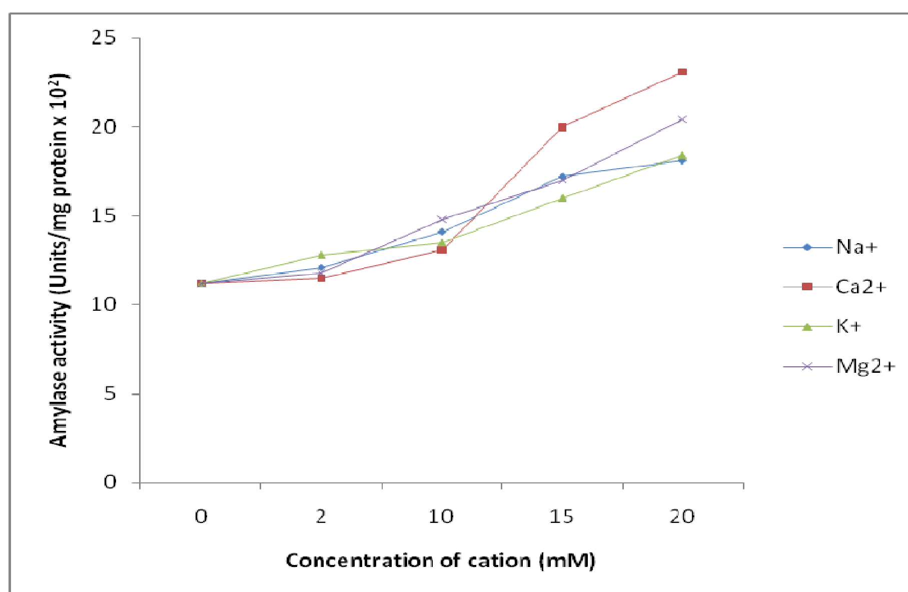

Figure 6: Effect of salts on the activity of the partially purified α-amylase (fraction Ca) obtained from *Penicillium citrinum*.

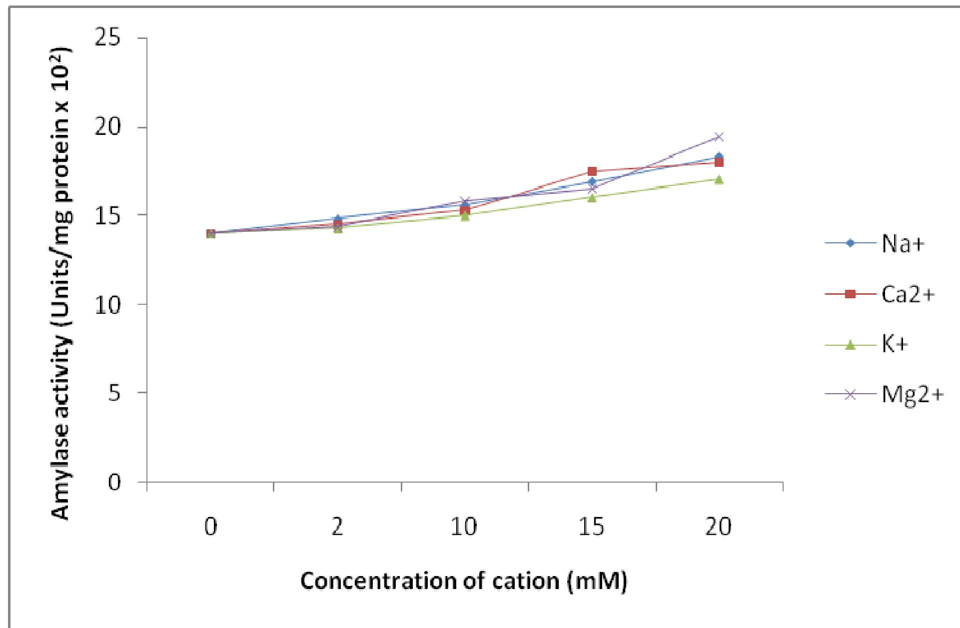

Figure 7: Effect of salts on the activity of the partially purified α-amylase (fraction Db) obtained from *Penicillium citrinum*.

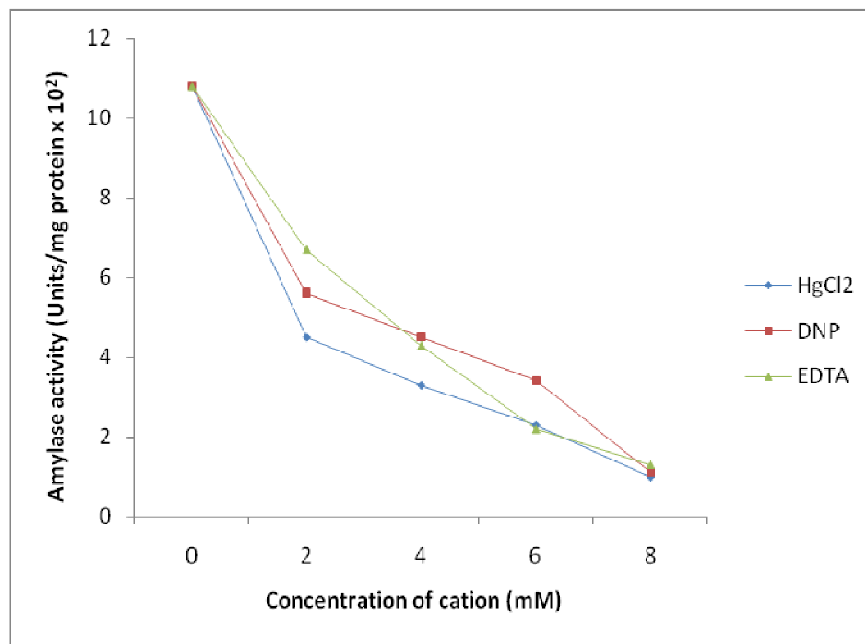

Figure 8: Effect of some chemicals on the activity of partially purified α-amylase (fraction Ba) obtained from *Penicillium citrinum*.

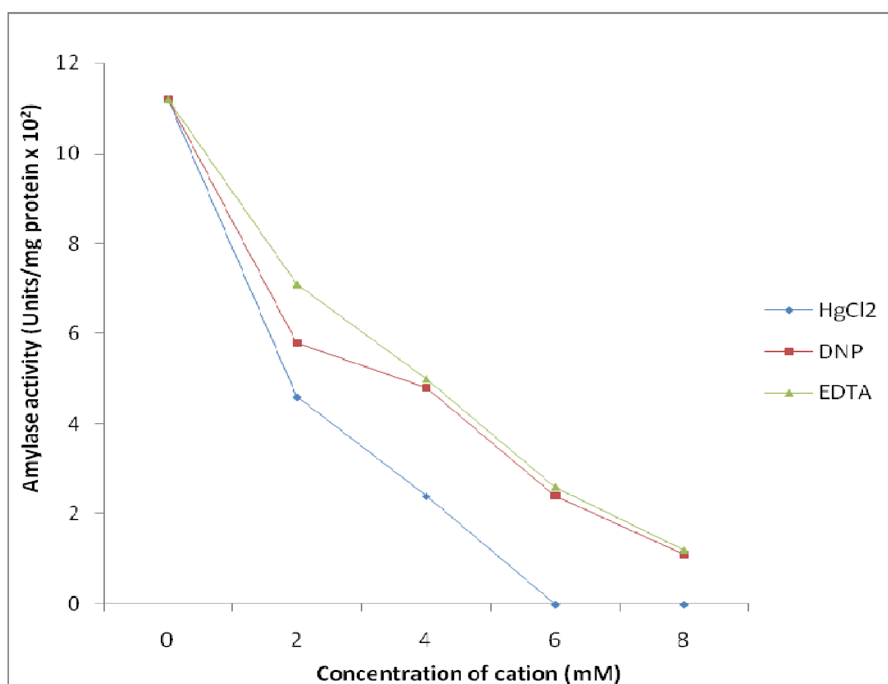

Figure 9: Effect of some chemicals on the activity of partially purified α-amylase (fraction Ca) obtained from *Penicillium citrinum*.

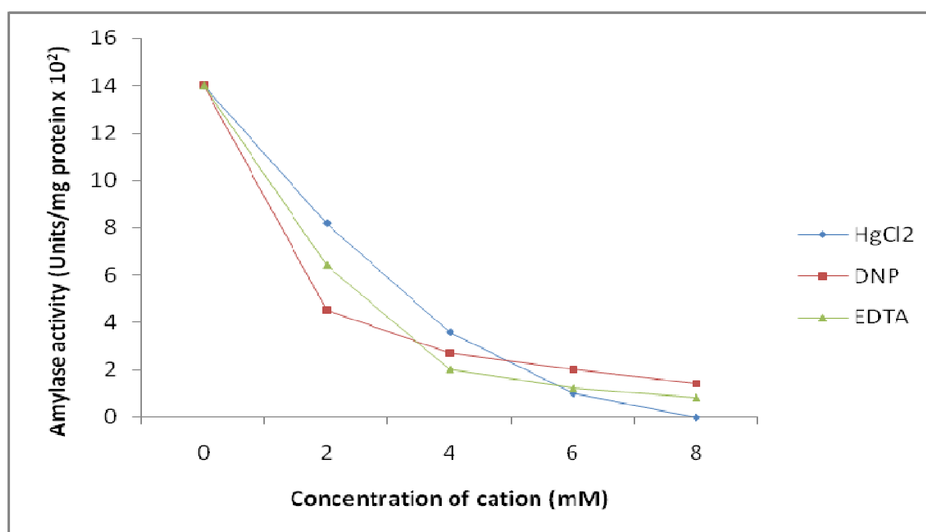

Figure 10: Effect of some chemicals on the activity of partially purified α-amylase (fraction Db) obtained from *Penicillium citrinum*.

DISCUSSION

Several factors influence rate of enzyme reactions (Adejuwon, 2013b; Adejuwon, 2013c). In the present investigation, pH influenced α-amylase activity produced by *P. citrinum.* Activities were optimum at pH 5.5 and 6.0 in reactions.

Starch degradation by *Rhizopus oryzae* was favourable at pH 6.0 (Huang et al., 2005). However, a mutant of *Bacillus amyloliquefaciens* has been reported to synthesize alpha-amylase with optimum activity at pH 7.0 (Bessler et al., 2003). A bacillus sp. isolated from piglet caecum produced an extracellular alpha-amylase optimally active at pH 7.0 (Peng et al., 2005).

Non stability of an enzyme outside its pH of optimum activity in which the enzyme loses its tertiary structure necessary for the conformation of its active site or the ionization of basic and acidic groups at the active centre of an enzyme are the influence of pH on enzyme catalyzed reactions (Murray et al., 2003).

α-Amylase activities produced by *P. citrinum* in reaction procedures of this study were optimum at 30 °C and 35 °C. Ajayi et al. (2014) reported amylase from *Aspergillus chavilieri* with optimum activity at 35 °C. Recently, Adejuwon (2015a) reported an alpha-amylase from *Penicillium rubrum* stable at 25 °C.

In this investigation, activities of α-amylases from *P. citrinum* were completely lost within 10 minutes of heat treatment at 80 °C. There were 78.7%, 54.4% and 82.7% loss of α-amylase activities.

The cations of NaCl, KCl, $CaCl_2$ and $MgCl_2$ in this investigation stimulated α-amylase activities produced by *P. citrinum* and *P. rubrum*. Stimulation was at varying degrees. Recombinant α-amylase from *Pyrococcus woesei* is stable on calcium salt (Synowiecki et al., 2006). Yun et al. (2004) reported a soil microorganism during cloning using recombinant *Escherichia coli* as expressing amylase activity stabilized by Ca^{2+}. Ca^{2+} and Mg^{2+} stimulated cloned alpha amylase from *Lipomyces starkeyi* (Kang et al., 2004). According to Ladokun and Adejuwon (2011), amylase from a strain of *Aspergillus fumigatus* associated with deterioration of rice (*Oryzae sativa*) was stimulated by the cation Al^{3+}.

According to Adejuwon (2013a), enzyme activities are influenced by metal ions. For instance, in metalloenzymes, metal ions function as electrophilic catalysts, stabilizing formed negative charges (Murray et al., 2003).

EDTA, 2,4-dinitrophenol and $HgCl_2$ in this study inhibited α-amylase activities produced by *P. citrinum*. Ajayi et al. (2014) reported the production of alpha amylase from *Aspergillius chevalieri* inhibited by EDTA and $HgCl_2$.

The results of this research show that the rate of enzyme reaction increased steadily with increase in concentration of substrate. As the concentration of starch as substrate gradually increased, α-amylase activities by *P. citrinum* steadily increased.

Conclusion

Bread inoculated with *Penicillium citrinum* is an ideal source of alpha amylase. Currently, amylases comprise a fair percentage of the world's enzyme production. α-Amylases produced by *Penicillium citrinum* in this study can be inhibited at temperatures extreme from 30 °C and 35 °C. Hydrogen ion concentrations (pH) extreme from 6.0 and 5.5 seem not to support alpha amylase activity obtained from the fungus. Also, bread and other grain products can be preserved at temperatures and pH inhibiting fungal growth and α-amylase activity. However, the industrial production of alpha-amylases in the tropics using bread as a cheap source of substrate for fungal growth is encouraged.

Competing interests

The authors declare they have no competing interests.

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