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Characterization of Alpha Amylase from *Bacillus subtilis* BS5 Isolated from *Amitermes evuncifer* Silvestri

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ABSTRACT

Amylase producer *Bacillus subtilis* BS5 was obtained from the hindgut of wood eating termites *Amitermis evuncifer* Silvestri. Amylase enzyme produced by this strain when grown on basal liquid medium with cocoyam starch as substrate was characterized. The enzyme was purified through ion-exchange chromatography and gel-filtration. The purified enzyme had a molecular weight of 63 kDa and was active optimally at pH 6.0 and 50°C. The apparent K_m value and V_{max} of the enzyme during hydrolysis of soluble starch were 16.67 mg mL^{-1} and 3.8 mg/min/mL^{-1}, respectively. The activities of the amylase were stimulated by Ca^{2+}, Na^+ and Mg^{2+} but inhibited by EDTA and $HgCl_2$.

Key words: Amylase, *Bacillus,* cocoyam starch, inhibitors, termite

INTRODUCTION

Starch is an abundant carbon source in nature. α-amylase (1, 4 α D-glucanohydrolase; EC 3.2.1.1) hydrolyses α-1, 4-glucosidic linkage in starch and related molecules. It is one of several enzymes involved in starch degradation. Amylases constitute one of the most important groups of industrial enzymes being extensively used in food, textiles, paper, brewing and distilling industries. Most of the available amylases produced commercially are of microbial origin (Pandey *et al*., 2000). The enzyme is widespread among aerobes and anaerobes. Gram positive bacteria, particularly the genera *Bacillus* and *Clostridia* are prolific producers of amylases (Haddaoui *et al*., 1995; Shih and Labbe, 1995; Obineme *et al*., 2003; Swain and Ray, 2007). Several species of *Bacillus* produces a wide range of extra cellular enzymes of which amylases and proteases are of significant industrial importance. These enzymes are used in the textile and paper industries, in starch liquefaction, as a food additive and in sugar production (Pandey *et al*., 2000). The amylase activity has been shown to be influenced by temperature, pH and presence of some chemicals (Swain and Ray, 2007). A *Bacillus subtilis* strain BS5 was isolated from the hindgut of wood-eating termite, *Amitermes evuncifer* Silvestri (Femi-Ola and Aderibigbe, 2006; Femi-Ola *et al*., 2007). It was selected for further study because of its high amylolytic and cellulolytic activity which was comparable with a reference strain *B. subtilis* NCIB 3610. This study reports a partial purification and characterization of alpha amylase produced by the *B. subtilis* strain BS5.

MATERIALS AND METHODS

Organisms and culture conditions: The *B. subtilis* strain BS5 used in this study was isolated from the hindgut of a wood-eating termite, *Amitermes evuncifer* (Femi-Ola *et al*., 2007). This

experiment was conducted between March 2008 and June 2009. The organism was grown in a basal medium containing (g L^{-1}): K_2HPO_4, 1.5; KH_2PO_4, 0.5; $MgSO_4$, 0.05; NaCl, 1.5; $(NH_4)_2SO_4$, 1.0; $CaCl_2.2H_2O$, 0.02; $FeSO_4.7H_2O$ 0.02; yeast extract, 0.5 and 1% cocoyam starch. The inocula for the experiments were prepared by growing the organism in nutrient broth (NB, Oxoid) at 35°C for 18 h on a rotary shaker (Gallenkamp) and shaked at 120 rpm. Sterilized medium (500 mL) in 1000 mL conical flasks was inoculated with 10 mL of inocula (8.6×10^6 cells mL^{-1}). The flask was incubated at 35°C on a rotary shaker (120 rpm) for 48 h and then centrifuged at 5000 rpm for 20 min in cold to remove bacterial cells. The supernatant obtained was used as the crude extract for further studies.

Amylase assay: Enzyme assay was estimated by the dinitrosalicyclic acid (DNSA) method of Miller (1972). The reaction mixtures consist of 0.5 mL of substrate solution (1% soluble starch in 0.05 M phosphate buffer, pH 6.9) and 0.5 mL of the cell free extract. The reaction mixture was incubated for 3 min at 30°C. The reaction was terminated by the addition of 1 mL of dinitrosalicyclic (DNSA) reagent. The mixture was heated at 100°C for 5 min and cooled. The optical density was read at 540 nm in a spectrophotometer (Jenway, 6305). One unit of alpha amylase activity (U) was defined as the amount of enzyme that liberated reducing sugar equivalent to one micromole of D-glucose from starch under the assay condition.

Purification and characterization of alpha amylase: All chromatography procedures were carried out at 4°C except where stated otherwise.

Ion exchange chromatography: DEAE-Sephadex A-50 (Pharmacia, Uppsala,Sweden) was packed into a column (2.5×40 cm) and equilibrated with 0.015 M Na_2HPO_4 buffer (pH 6.9). Twenty milliliters of the crude enzyme was applied to the cation exchanger column at a flow rate of 20 mL h^{-1}. The fractions containing alpha amylase activity unbound to the gel were pooled and bound proteins were eluted with by a 0-1 M NaCl gradient in the same buffer at a flow rate of 20 mL h^{-1}. The pooled fractions were concentrated by dialysis against glycerol solution at 4°C overnight.

Gel filtration chromatography (using Sephadex G-150): Active fractions from DEAE Sephadex A-50 (Pharmacia) column was applied to a Sephadex G-150 (Pharmacia) column (1.5×75) which had been previously equilibrated with 0.015 M Na_2HPO_4 buffer, pH 4.7. The column was eluted with the same buffer at a flow rate of 25 mL h^{-1}. A fraction of 5.0 mL were collected at interval of 30 min and the absorbance at 280 nm was read using spectrophotometer (Jenway, 6305). For determination of molecular weight by gel filtration the standards used were: lysozyme 14 kDa; lactaglobulin 20 kDa; bovine trypsinogen, 24 kDa; pepsin, 34 kDa; egg albumin, 45 kDa and bovine serum albumin, 66 kDa (Sigma, UK).

Effect of temperature on alpha amylase activity and stability: Alpha amylase activity was assayed by incubating the enzyme reaction mixture at different temperatures ranged from 20°C to 80°C for 3 min. The thermal stability at 70 and 80°C was also determined. Samples were taken at 5 min intervals and analyzed for amylolytic activity.

Effect of pH on amylase activity: Substrates (1% soluble starch) having pH ranging from 3.0 to 8.0 were prepared using 0.05 M of different buffer system (Glycine-HCl, pH 3; acetate buffer, pH 4 and 5; phosphate buffer pH 6 and 7; Tris- HCl, pH 8). Enzyme activity was determined at 35°C.

Effect of substrate concentration on alpha amylase activity: The effect of substrate concentration [S] on the rate of enzyme action was studied using [S] concentration ranged from 2.0 to 10.0 mg mL^{-1}. The Lineweaver-Burke plot was made. Both the V_{max} and K_m of the enzyme were calculated.

Effect of heavy metals on enzyme activity: A stock solution of 0.01 M of $HgCl_2$ and EDTA were prepared. The substrate/chemical mixture was incubated at room temperature for 5 min before it was used in enzyme reaction assay.

Effect of cations: A stock solution of 0.01 M of each salt was prepared. The effects of some salts/cations (NaCl, $CaCl_2$, $CuSO_4$ and MgCl) on enzyme activity was also determined. The substrate/salts mixture was also incubated before it was used for enzyme assay.

RESULTS

Fractionation of the enzyme concentrate on DEAE Sephadex A-50 produced a double peak (Fig. 1). From the elution profile on Sephadex G-150, it was observed that the amylase was eluted as a well resolved single peak of amylase activity (Fig. 2). These purification procedures yielded a pure amylase with specific activity of 0.99 U mg^{-1} proteins and a purification of approximately four fold with 74.62% yield of proteins (Table 1). The molecular weight of the alpha amylase produced was estimated to be 63 kDa. The effect of temperature on the activity of the purified amylase is shown in Fig. 3. Maximum activity was observed at 50°C. The pH value for maximal activity was 6.0 (Fig. 4). The enzyme was partially stable at 70°C as it retained 56.6% of its activity after when heated for 10 min (Fig. 5). At temperature above 70°C, the enzyme was quickly denatured,

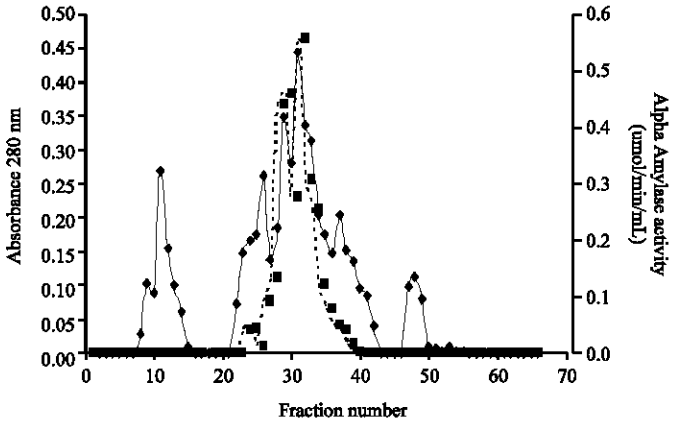

Fig. 1: Elution profile of α-amylase produced by *Bacillus subtilis* from ion exchange column (2.5×40 cm)

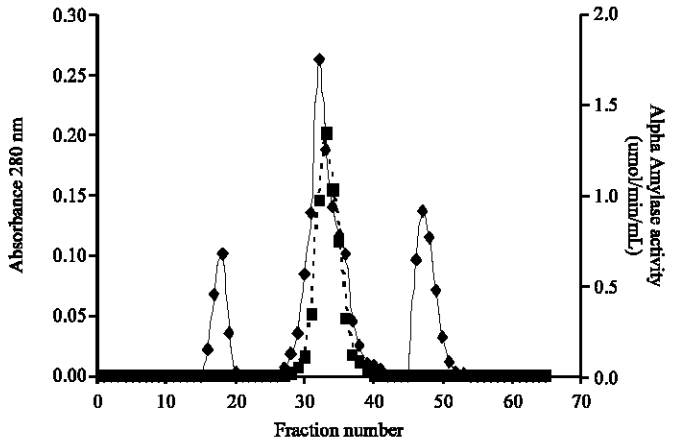

Fig. 2: Elution profile of α-amylase produced by *Bacillus subtilis* from Gel filtration column (1.5×75 cm)

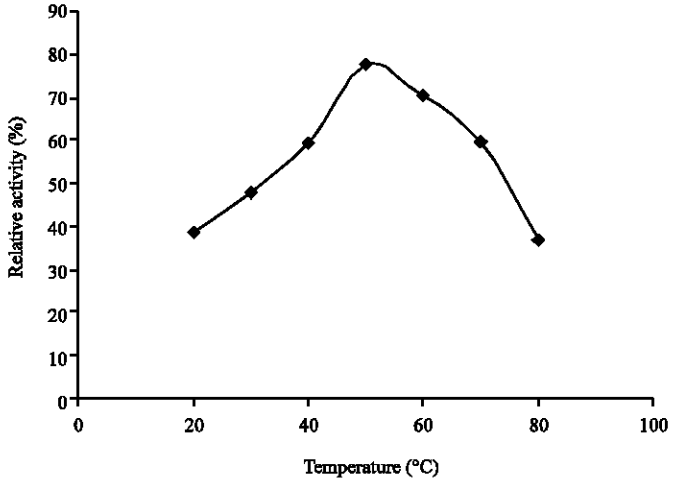

Fig. 3: Effect of temperature on the activity of partially purified alpha amylase of *Bacillus subtilis* BS5

Table 1: Purification of extracellular amylase of *B. subtilis* BS5

Fraction	Volume (mL)	Protein content (mg mL^{-1})	Amylase activity (U)	Specific activity (U mg^{-1} of protein)	Yield (%)	Purification fold
Crude enzyme	20.00	171.60	48.20	0.28	100.00	1.00
Ion exchange	33.50	175.54	94.79	0.54	102.29	1.93
Gel filtration	42.40	128.05	127.20	0.99	74.62	3.50

completely lost its activity after 10 min. A Lineweaver-Burke plot of the purified Alpha amylase activity of *B. subtilis* (Fig. 6) indicates that this enzyme has apparent K_m and V_{max} values for the hydrolysis of soluble starch of 16.67 mg mL^{-1} and 3.82 U, respectively. The activity of alpha amylase was stimulated by Ca^{2+}, Na^{+} and Mg^{2+} while heavy metals inhibited the activity (Table 2).

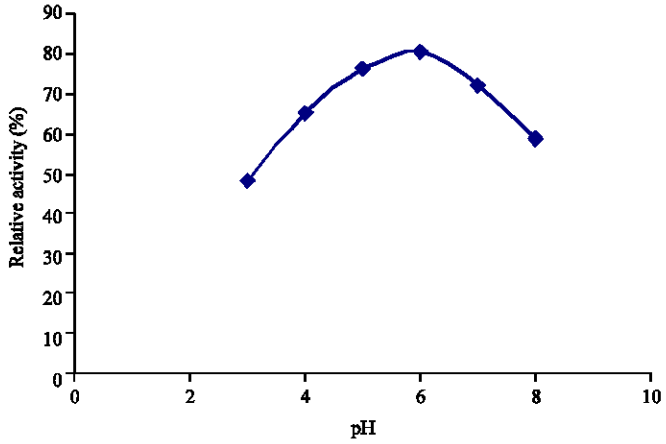

Fig. 4: Effect of pH on alpha amylase production by *B. subtilis* BS5

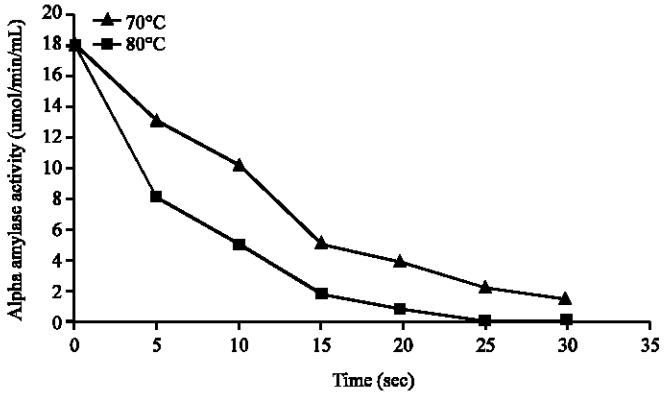

Fig. 5: Effect of heating at 70 and 80°C on alpha amylase activity of *B. subtilis* BS5

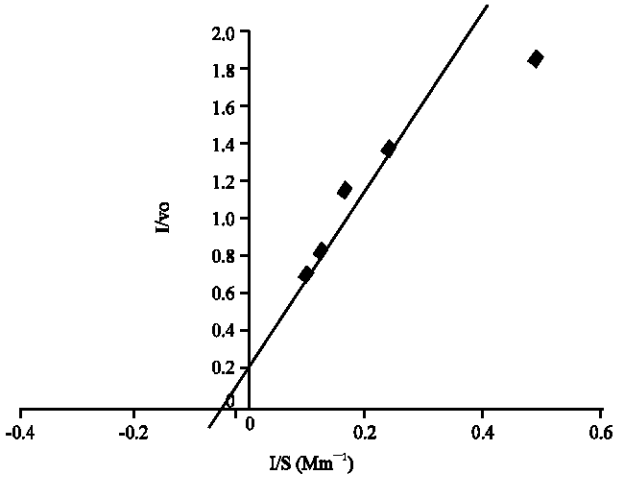

Fig. 6: Lineweaver-Burke plot for the hydrolysis of starch by partially purified α-amylase from *Bacillus subtilis* BS5

Table 2: Effect of salts on the activity of alpha amylase of *B. subtilis*

Salts	Residual activity (%)
$CaCl_2$	118.67±4.70
$CuSO_4$	64.09±8.20
NaCl	130.24±2.29
EDTA	74.46±0.00
$MgCl_2$	83.75±2.30
$HgCl_2$	25.54±2.29
Control	100.00

DISCUSSION

The current result of this investigation showed that temperature considerably affected the activity of the amylase enzyme. The optimum temperature for the α-amylase activity was 50°C. Other investigators working on alpha amylase have reported various temperature optima in this range. Obineme *et al.* (2003) and Shelby (1993) reported an optimal activity of α-amylase at 50°C for *Aspergillus oryzae* and *Streptococcus bovis* JBI respectively. The α-amylase of *B. subtilis* BS5 still retained 56.6 and 30.46% of its activity when heated for 10 min at 70 and 80°C, respectively. Denaturation of enzyme proteins at temperature higher than 70°C has been reported (Aderibigbe, 1998). The optimum pH for the α-amylase activity in this study was 6.0. This optimal α-amylase activity pH value was within the range of values reported for most starch degrading bacterial strain (Gupta *et al.*, 2003). Mishra and Behera (2008) had also reported a pH of 6.0 and 7.0 for normal growth and enzyme activity for alpha amylase of *Bacillus* strains isolated from soil receiving kitchen waste. Similar values for bacterial amylases production by *Streptococcus bovis* JBI, new *Clostridium* isolate and *Clostridium acetobutylicum* SAI were reported (Chojecki and Blascheck, 1986; Madi *et al.*, 1987; Shelby, 1993). It was also noticed that amylolytic activity increased with substrate concentration. Among the physiological parameters, optimum temperature, substrate concentration and the pH of the growth medium plays an important role in production and activity of microbial enzymes.

This study also revealed that amylolytic activity was affected by the concentration of cations at 0.04 M. Ca^{2+} and Na^{+} had stimulatory effect on the activity of *B. subtilis* amylase, while heavy metals, Hg^{2+} and EDTA inhibited the activity. The inactivation of the enzyme by heavy metals poisoning are well documented (Kim *et al.*, 2005; Pandey *et al.*, 2000). In their reports, it was suggested that EDTA acted by chelating Ca^{2+} and once the Ca^{2+} content of the enzyme was completely removed by EDTA, there followed a quick loss in activity of the enzyme. Heavy metals are known to react with protein sulphydryl groups thus converting them to mercaptides. The inhibition of the enzyme activity by EDTA suggests that the enzyme contains metallic ions which are removed by the chelating agent, forming an active complex with EDTA (Dixon and Webb, 1971).

The results presented in this study indicate that the α-amylase produced by *B. subtilis* BS5 possesses properties of an industrial enzyme; productivity and thermo stability.

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