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Kinetic Properties of Beta-Amylase from *Bacillus subtilis*

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Abstract: *An extracellular beta amylase was induced in cultures of Bacillus subtilis isolated from the kolanut weevil, Balanogastris kolae grown in liquid medium that contained cocoyam starch as sole carbon source. The enzyme was partially purified by acid treatment with ice cold 1.0N HCl and gel filtration with Sephadex G 150. The enzyme had its optimal activity at pH 6.0 and exhibited maximal activity at temperature of 50°C. The activity of the enzyme was enhanced by Na^+, while ethylene diaminetetraacetic acid (EDTA), Hg^{2+} and Ca^{2+} acted as mild inhibitors and Fe^{2+} was a strong inhibitor of its activity. The beta amylase had a molecular weight of 27.6 kDa and an apparent Michaelis constant K_m of 4.6 mg/ml and maximum velocity (V_{max}) of 47.62 U/mg proteins.*

Keywords: *cocoyam, starch, inhibitors, molecular weight, kolanut weevil*

I. Introduction

Amylases are one of the most widely distributed enzyme complexes encountered in animal, lower and higher plants and microbes [1].They are starch degrading enzymes and degrade related polymers to yield product characteristic of individual amylolytic enzymes [2]. The enzymes from microbial sources generally meet industrial demands [3]. Three major classes of amylase have been identified. Alpha – amylase and glucoamylase mainly of microbial origin and beta amylases which is of plant origin has been reported from few microbial sources. Sweet potatoes, malted barley, barley, maize, soybean have been the most studied plant sources of β- amylase [4]. *Actinomycetes, Pseudomonads, Clostridium* and *Bacillus* have been reported to produce β – amylase (5, 6). *Bacillus* spp are the most active producer of β – amylase with high saccharifying activity [7, 8].

Microbial amylases have successfully replaced chemical hydrolysis of starch in starch processing industries [9]. Microbial amylases have a wide scale of application ranging from textile to effluent treatment [2]. In recent years the spectrum of amylase application had widened in many fields such as clinical, pharmaceutical, chemical, medical, textile, food and brewing industries with the advent of new frontiers in biotechnology [3, 10]. This paper examines the ability of a strain of *Bacillus subtilis* isolated from the gut of *Balanogastris kolae* to produce β – amylase from cassava starch. Therefore this paper reports the partial purification and characterization of β – amylase from this organism.

II. Materials and methods

1.1. Organism and culture conditions

The isolate of *Bacillus subtilis* used for this research was from kolanut weevil *Balanogastris kolae* Desbr. The organism was grown in a basal medium containing (g/l): K_2HPO_4, 2.5; KH_2PO_4, 3.75; $MgSO_4$, 0.125; NaCl, 3.75; $(NH_4)_2SO_4$, 2.5; $CaCl_2.2H_2O$, 0.05; $FeSO_4.7H_2O$ 0.05; yeast extract, 1.25 and cocoyam starch, 2.5. The inocula for the experiments were prepared by growing the organism in nutrient broth (NB, Oxoid) at 35°C for 18hrs on a rotary shaker (Gallenkamp). Sterilized medium (500ml) in 1000ml conical flasks was inoculated with 5ml of inocula containing 7.05×10^3 cells/ml). The flask was incubated at 35°C on a rotary shaker (120 r. p. m) for 48hrs and then centrifuged at 5000 r. p. m for 20mins in cold to remove bacterial cells. The supernatant obtained was used as the crude extract for further studies.

1.2. Amylase assay

Dinitrosalicyclic acid (DNSA) method of [11] was used in estimating amylase activity. It measures the increase in the reducing power of the digests in the reaction between starch and the enzyme. Appropriately diluted 0.5ml of enzyme was added to 0.5ml of 1% (w/v) soluble starch which was dissolved in appropriate buffer solution (phosphate buffer, 6.9). The above reaction mixture was made in three test tubes. The reaction tubes were incubated at room temperature for 3 minutes. Then one ml of colour reagent (DNSA) was added to the reaction mixture and place in boiling water bath (Gallenkamp) for 5 mins. The tubes were allowed to cool at room temperature. After which 10ml of distilled water was further added to the cooled tubes and absorbance at 540nm was measured using spectrophotometer (Jenway, 6305). Control tube consisted of 0.5ml buffer solution plus 0.5ml soluble starch solution. The assay was also carried out as explained above. All assays were done in

triplicates. The amount of maltose liberated was extrapolated from the maltose standard curve. One unit of beta amylase activity was defined as the amount of enzyme required to produce one micromole of maltose from starch under the assay condition. That is, amount of the enzyme which releases one micromole of maltose from the starch in 3mins.

1.3. Protein determination

Protein was determined by the Biuret method of Lowry *et al.* [12] with bovine serum albumin (BSA) as the standard. The concentration of protein during purification studies was measured at an absorbance of 280nm.

1.4. Purification and characterization of alpha amylase

1.4.1. Acid treatment

Fifty millilitres (50 ml) of crude enzyme at a pH of 4.7 was adjusted to pH 3.6 with ice cold 1.0N HCl. This was allowed to stand for 10 minutes. The acidified enzyme was readjusted to pH 4.7 with 3 % NH_4OH solution. The acid treated sample was concentrated overnight in cold 4.0M sucrose solution at 4^oC.

1.4.2. Gel filtration chromatography (using Sephadex G-150)

Sephadex G-150 was swollen in 0.015M acetate buffer at pH 4.7 for 3 days before it was packed into column. Twenty millilitres of the acid treated fraction was applied to a Sephadex G-150 (Pharmacia) column (1.5×75) which had been previously equilibrated with 0.015M acetate buffer, pH 4.7. The column was eluted with the same buffer at a flow rate of 20ml/hr. A fraction of 5.0ml were collected at interval of 30mins and the absorbance at 280nm was read using spectrophotometer (Jenway, 6305). Fractions with amylase activity were pooled and concentrated in glycerol solution at 30^oC.

1.4.3. Determination of molecular weight

The apparent molecular weight of the beta amylase was estimated from the gel filtration column using alpha chymotrypsinogen (25.7kDa), ovalbumin (45 kDa), bovine serum albumin (68 kDa), creatine phosphokinase (81 kDa) and Gamma Globulin (150 kDa) (Sigma, UK) as reference proteins.

1.4.4.Effect of temperature on beta-amylase activity and stability

Beta amylase activity was assayed by incubating the enzyme reaction mixture at different temperatures, 20^oC to 80^oC for 3mins. The thermal stability at 70^oC and 80^oC was also determined. Samples were taken at 5mins intervals and assayed for amylolytic activity.

1.4.5.Effect of pH on beta-amylase activity

Buffer (0.05M) of different pH ranging from 3.0 to 8.0 were prepared using different buffer system, Glycine-HCl, pH 3.0; acetate buffer, pH 4.0 and 5.0; phosphate buffer pH 6.0 and 7.0; Tris- HCl, pH 8.0. Each of this buffer solution was used to prepare 1% soluble starch solution used as substrate in assaying the enzyme. The assay was carried out according to standard assay procedure.

1.4.6.Effect of substrate concentration and determination of kinetic parameters

The effect of substrate concentration [S] on the rate of enzyme action was studied using [S] values of 2.0mg/ml to10.0mg/ml. The Lineweaver-Burke plot was made. Both the maximum velocity (V_{max}) and Michaelis – Menten constant (K_m) of the enzyme were calculated.

1.4.7.Effect of heavy metals on enzyme activity

A stock solution of 0.01M of $HgCl_2$ and EDTA were prepared. Two milliliter of each salt solution was mixed with 2ml of enzyme solution. The mixture was incubated for 5mins at room temperature. A 0.5ml of the mixture was withdrawn and assay according to standard assay procedure.

1.4.8. Effect of cations

A stock solution of 0.01M of each salt was prepared. The salts used were NaCl, $CaCl_2$, $FeCl_2$ and $MgCl_2$. Two milliliter of salts solution was mixed with 2ml of enzyme solution. And the same procedure for heavy metals was followed.

III. Results and Discussion

The crude culture supernatant was purified to apparent homogeneity by gel filtration on DEAE Sephadex G-150 column. The elution profile from the gel filtration showed a single peak of amylase activity (Fig. 1). The summary of purification procedure is shown in Table 1. The two step purification process revealed a specific activity of 3.57units/mg, yield of 51.1% and fold equals 5.35. The molecular weight of the β –

amylase was estimated to be 27.6 kDa.This is much lower than the molecular weight reported in a wide variety of β – amylase isolated from other sources. For example, 209 kDa and 105 kDa were reported for β – amylase from *Bacillus megaterium* [6], 69kDa was reported by Olaniyi *et al.* [9] for β – amylase from *Volvariella volvacea.* Buonocore *et al.* [13] has suggested that the discrepancy observed between the apparent molecular mass determined by gel filtration may be due to the interaction of the enzyme with the gel, resulting in retardation of its mobility and thus an underestimation of its molecular weight.

The purified enzyme exhibited maximum activity at 50^{o}C (Fig. 2) and pH 6.0 (Fig.3). The enzyme was partially stable at 80^{o}C as it retained 50% of its activity after when heated for 20 minutes (Fig.4). At this temperature, the enzyme was gradually denatured, completely losing its activity after 120 minutes. The optimal temperature (50^{o}C) of the β – amylase in this investigation is similar to the β – amylase isolated from *Bacillus polymyxa* 26-1[14] and *Clostridium perfringes* which showed maximum activity at 50^{o}C in a starch based medium [15]. It has been reported that enzyme structure are disrupted and always a loss of activity at high temperature values above the optimum [16]. The optimum pH 6.0 observed for the β – amylase activity in this study correlates with the pH values that had been reported for most bacterial amylases and yeast amylases [17]. Changes in pH greatly influence enzymatic activities. Enzyme denaturation of essential cofactor may be due to decrease in reaction rates or enzyme activities at high or low pH values [18]. Enzyme being proteins, like other proteins consist of long chains of amino acid held together by peptide bonds [19]. Extreme pH can initiate chemical reactions that can destroy amino acid residues of the protein molecules of the enzyme, thus resulting in irreversible reaction [20]. A Lineweaver-Burke plot of the purified β – amylase activity of *B. subtilis* (Fig.5) indicates that this enzyme has apparent K_m and V_{max} values for the hydrolysis of soluble starch of 4.6 mg ml^{-1} and 47.62U respectively.

In this study, it was observed that NaCl enhanced the activity of the β – amylase, while its activity was slightly inhibited by salts such as EDTA, $CaCl_2$ and $MgCl_2$ (Table 2). The inhibition of the β – amylase activity by EDTA may suggest that the enzyme may contain inorganic groups, which form inactive complexes with EDTA. Fogarty and Kelly [21] 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 the enzyme's activity. Increase in heat treatment of *Bacillus subtilis* β – amylase at 80^{o}C resulted into complete loss of its activity after 120 minutes. Denaturation of enzyme protein at temperature higher than 70^{o}C has been reported by Gupta *et al.* [22]. Inactivation due to heat has been associated with a two-step process [20]. The reversible thermal unfolding of an enzyme as a result of increase in vibration and rotational motion of reacting molecules, which may also lead to dissociation in case of multi-subunit enzyme [22].

IV. Conclusion

The results obtained in this report indicate that the β – amylase produced by *Bacillus subtilis* possess properties of an industrial enzyme. The stability of the enzyme to heat may be an indication of its possible application in industrial processes.

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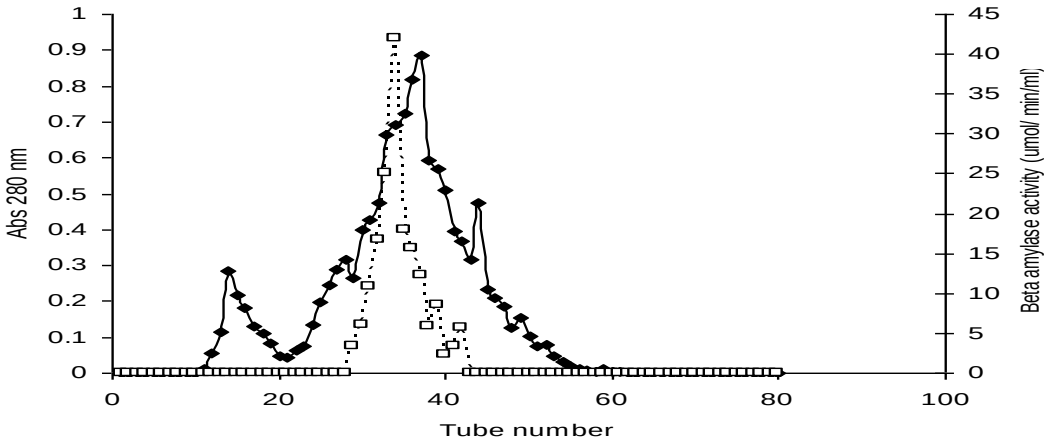

Fig 1: Elution profile of β-amylase produced by *Bacillus subtilis* grown in production mediausing Sephadex G – 150 (1.5 x 75) column equilibrated with 0.015M acetate buffer, pH 4.7.
Abs 280 nm (♦♦♦♦♦♦♦♦♦♦), β-amylase activity (units/ml) □□□□□□□□□□

Table 1: Purification of extracellular □- amylase of *Bacillus subtilis*

Fraction	Vol. (ml)	Protein Content (mg/ml)	β-amylase activity (U)	Specific activity (U/ mg of protein)	Yield (%)	Purification fold
Crude enzyme	50	1841	1230	0.66	100	1.00
Acid treatment	50	1607.5	1730	1.07	87.3	1.61
Gel Filtration	60	942	3372	3.57	51.1	5.35

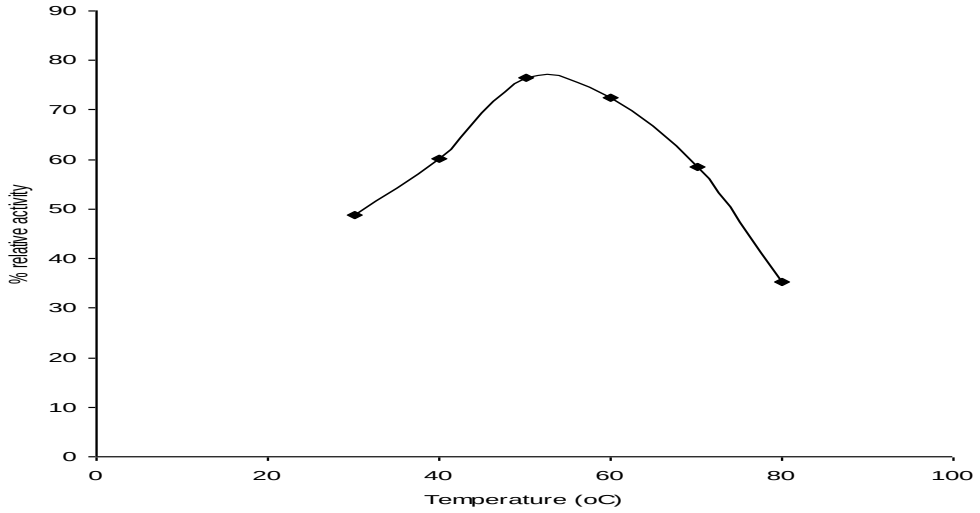

Fig 2: Effect of temperature on □-amylase activity of *Bacillus subtilis*.

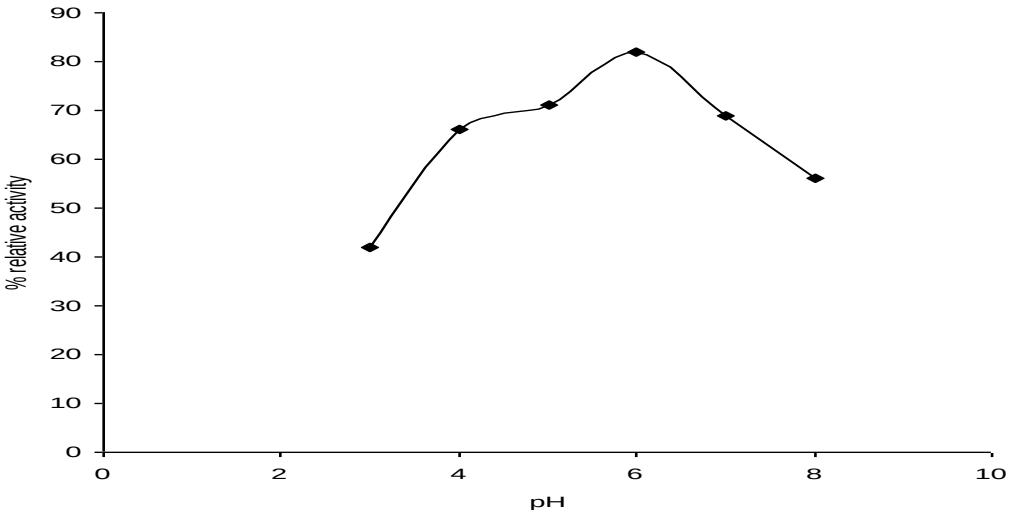

Fig 3: Effect of pH on β-amylase activity of *Bacillus subtilis*.

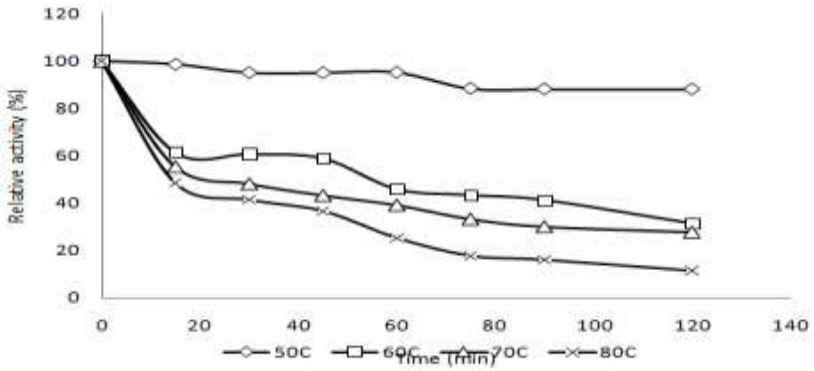

Fig. 4: Thermal stability of □-amylase produced by *Bacillus subtilis*.

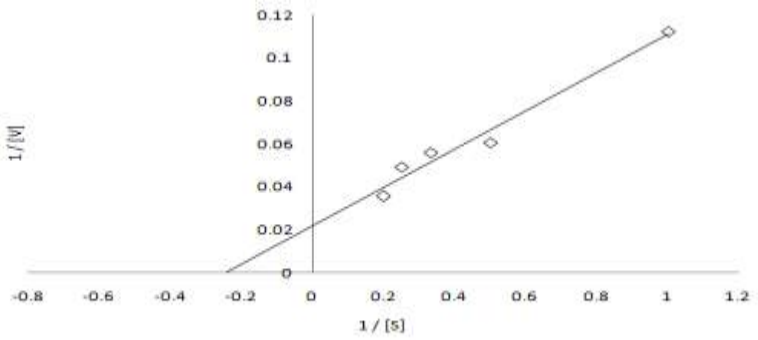

Fig 5: Lineweaver- Burke plot □-amylase produced by *Bacillus subtilis*

Table 2: Effect of Salts on □-amylase activity of *Bacillus subtilis*

Salt	% Relative activity
Control	100
NaCl	104
$CaCl_2$	98
EDTA	93
$MgCl_2$	77
$HgCl_2$	53
$FeCl_2$	17