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Purification and Characterization of Beta-Amylase of *Bacillus subtilis* Isolated from Kolanut Weevil

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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 kolanut starch as sole carbon source. The enzyme was partially purified 1.28-fold by acid treatment with ice cold 1.0N HCl and 6.4-fold by gel filtration with Sephadex G-150. The beta amylase had a molecular weight of 39.4 kDa .The enzyme had its optimal activity at pH 5.0 and exhibited maximal activity at temperature of 50^{o}C. The activity of the enzyme was enhanced by Na^{+}, Ca^{2+} and ethylene diaminetetraacetic acid (EDTA), while Hg^{2+} ,Mg^{2+} and Fe^{2+} acted as inhibitors of its activity. The beta amylase had an apparent Michaelis constant K_m of 5.0 mg/ml and maximum velocity (V_{max}) of 50 U/mg proteins.

Keywords: Beta-amylase, *Bacillus*, Kola nut, Starch, Inhibitors, Molecular weight.

1. Introduction

Amylases constitute a class of industrial enzymes which alone form 25% of the enzymes market covering industrial processes such as brewing sugar, textile, paper, distilling industries and pharmaceuticals (Mamo et al., 1999; Pandey et al., 2000; Oudjeriouat et al., 2003). Amylases are employed in the conversion of starch into different sugar solutions (Pandey et al.,

2000; Ray, 2000; Obineme et al., 2003).The enzymes involved are mainly α-amylase (1,4 α-glucan glucaohydrolase, EC3.2.1.1), β – amylase (1,4 α -glucan maltohydrolase, EC 3.2.1.2) and glucoamylase (1,4 α-glucan glucohydrolase, EC 3.2.1.3) (Boldon and Effront, 2000). Though amylases originates from different sources such as plants, animals and microorganisms, the microbial amylases are the mostly produced and used in industry due to their productivity and thermo stability (Reddy et al., 2003). Unlike other members of the amylase family, only a few attempts have been made to study β – amylases particularly of plant origin while there is a dearth on information on β – amylase from microbial sources. Bacterial strains belonging to the genera *Bacillus, Pseudomonas, Clostridium* (Ray, 2000; Rani et al., 2007); and fungal strains belonging to *Rhizopus* (Forgarty and Kelly, 1990) and *Volvariella volvacea* (Olaniyi et al., 2010) have been reported to synthesize β – amylase. In plant, β – amylase is distributed in higher plants such as soybean, sweet potato, barley (Chang et al., 1996; Oudjeriouat et al., 2003). The properties of the β - amylase varies from one source to the other. Some of the microorganisms reported to produce β – amylase has employed starchy wastes such as cassava, rice husk, potato rice and maize as substrates for production. This present study describe the properties of the β – amylase produced by a *Bacillus subtilis* strain isolated from the gut of kola nut weevil *Balanogastris kolae* (Desbr.) when grown in a basal medium containing kola nut starch as carbon source.

2. Materials and Methods

2.1 Collection of Sample

Kola nut sample (*Cola acuminata*) was purchased from the King's market square, Iragbiji, Osun- State, Nigeria.

2.2 Proximate Analysis of Kola Nut

Various parameters such as moisture content, ash content, crude fibre, crude protein, crude fat and carbohydrate contents were determined by the standard methods of the Association of Official Analytical Chemists (AOAC, 1984). The kola nuts were cured by traditional method of wrapping in fresh banana leaves to reduce moisture loss. The moisture content was determined by heating 2.0g of each triplicate sample to a constant weight in a crucible placed in an oven maintained at 105^{o}C. The dry matter was used in the determination of the other parameters. Crude protein was determined by the Kjeldahl method, using 2.0 g samples. Crude fat was obtained by exhaustively extracting 5.0 g of each sample in a Soxhlet apparatus using petroleum ether (boiling point range 40 – 60^{o}C) as the extractant.

Ash content was determined by the incineration of 10.0 g samples placed in a muffle furnace maintained at 550^{o}C for 5 h. Crude fibre was obtained by digesting 2.0 g of sample with H_2SO_4 and NaOH and incinerating the residue in a muffle furnace maintained at 550^{o}C for 5h. Total carbohydrate was obtained by difference.

2.3 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 1.25 $\times 10^5$ 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.

2.4 Amylase Assay

Amylase activity was estimated by the 3, 5 Dinitrosalicyclic acid (DNSA) method of Bernfield (1955). 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.

2.5 Protein Determination

Protein was determined by the Biuret method of Lowry *et al.* (1951) with bovine serum albumin (BSA) as the standard. The concentration of protein during purification studies was measured at an absorbance of 280nm.

2.6 Purification and Characterization of Beta Amylase

2.6.1 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°C.

2.6.2 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. Forty millilitres of the acid treated fraction was applied to a Sephadex G-150 (Pharmacia) column (1.5×75cm) which had been previously equilibrated with 0.015M $NaHPO_4$ 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^{o}C.

2.6.3 Determination of Molecular Weight

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

2.6.4 Effect of Temperature on Beta-amylase Activity and Stability

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

2.6.5 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.

2.6.6 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.

2.6.7 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.

2.6.8 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.

3. Analysis of Results

The result of the proximate analysis of kola nut is shown on Table 1. The carbohydrate content was 67.49%. The percentage of other parameters were moisture content, 11.48%; crude protein, 9.98%; fat, 5.88% and crude fibre, 1.89%.

Table 1. Proximate composition of *Cola acuminata*

Samples	Composition (%)
Moisture content	11.48
Ash	3.28
Crude fibre	1.89
Crude protein	9.98
Fat	5.88
Carbohydrate content	67.49

The partial purification profile of the extracellular beta amylase produced by *Bacillus* subtilis is summarized in Table 2. The two step purification process revealed a specific activity of 3.01units/mg, yield of 42.16% and fold equals 6.40. The elution profile of the enzyme on Sephadex G- 150 gel filtration column showed a single peak of amylase activity (Fig. 1). The molecular weight of the β – amylase was estimated to be 39.4 kDa. The purified enzyme exhibited maximum activity at 50^{o}C (Fig. 2) and pH 5.0 (Fig. 3). The enzyme was partially stable at 70^{o}C as it retained about 50% of its activity after when heated for 30 minutes (Fig.4). At this temperature, the enzyme was gradually denatured, losing its approximately 80% of its activity after 120 minutes. 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 5.0 mg ml^{-1} and 50.0U respectively. The activity of beta amylase was stimulated by Na^{+}, Ca^{2+} and EDTA, while its activity was mildly inhibited by Mg^{2+}. However Fe^{2+} strongly inhibited the activity of the β – amylase (Table 3).

Table 2. Purification of extracellular β- 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	1001	479	0.47	100	1.00
Acid treatment	50	598	879	1.47	59.74	1.28
Gel Filtration	40	422	1270	3.01	42.16	6.40

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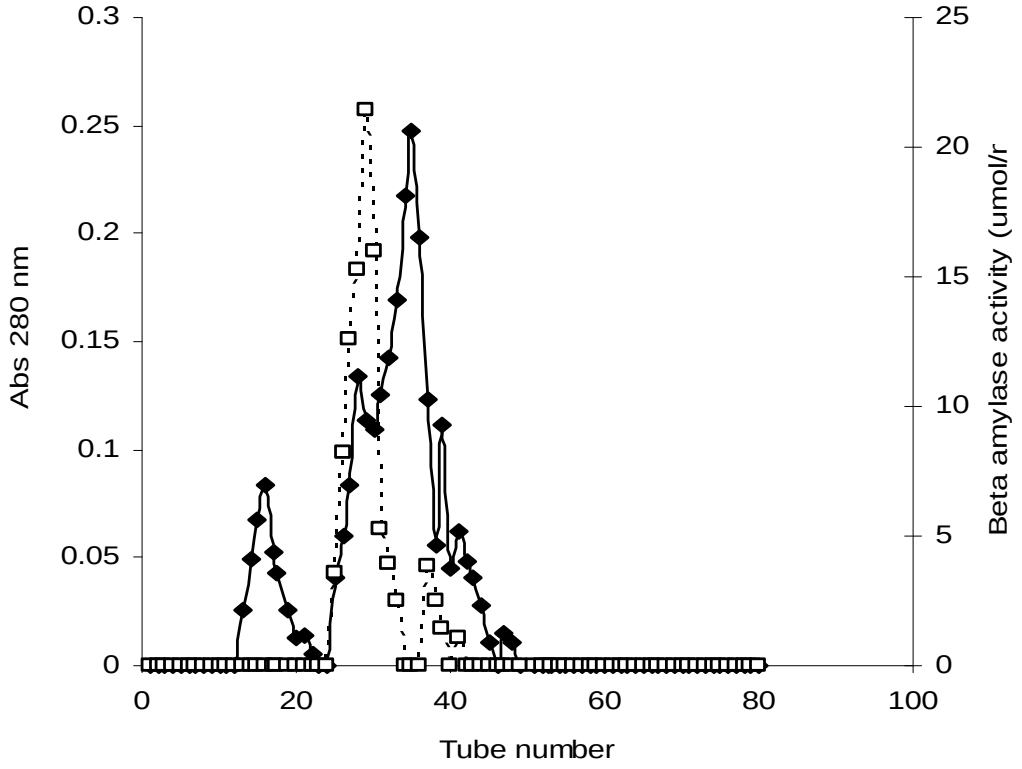

Figure 1. Elution profile of β-amylase produced by *Bacillus* sp. grown in production media using sephadex G – 150 (1.5 x 75) column equilibrated with 0.015M acetate buffer, pH 4.7. Abs 280 nm (♦♦♦♦♦♦♦♦♦♦), β-amylase activity (µmol/min/ml) □□□□□□□□□□

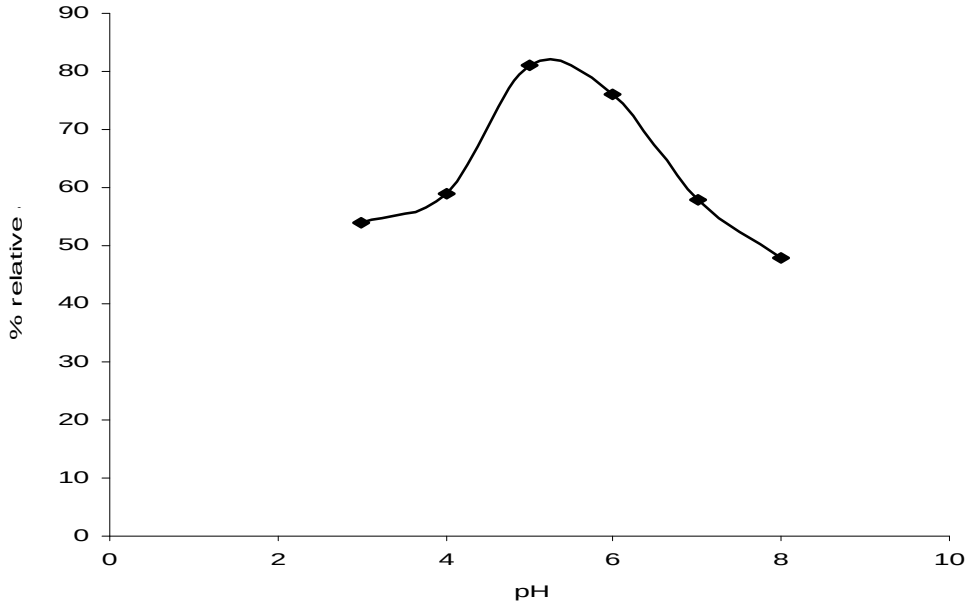

Figure 2. Effect of pH on β-amylase activity of *Bacillus sp* grown in production media.

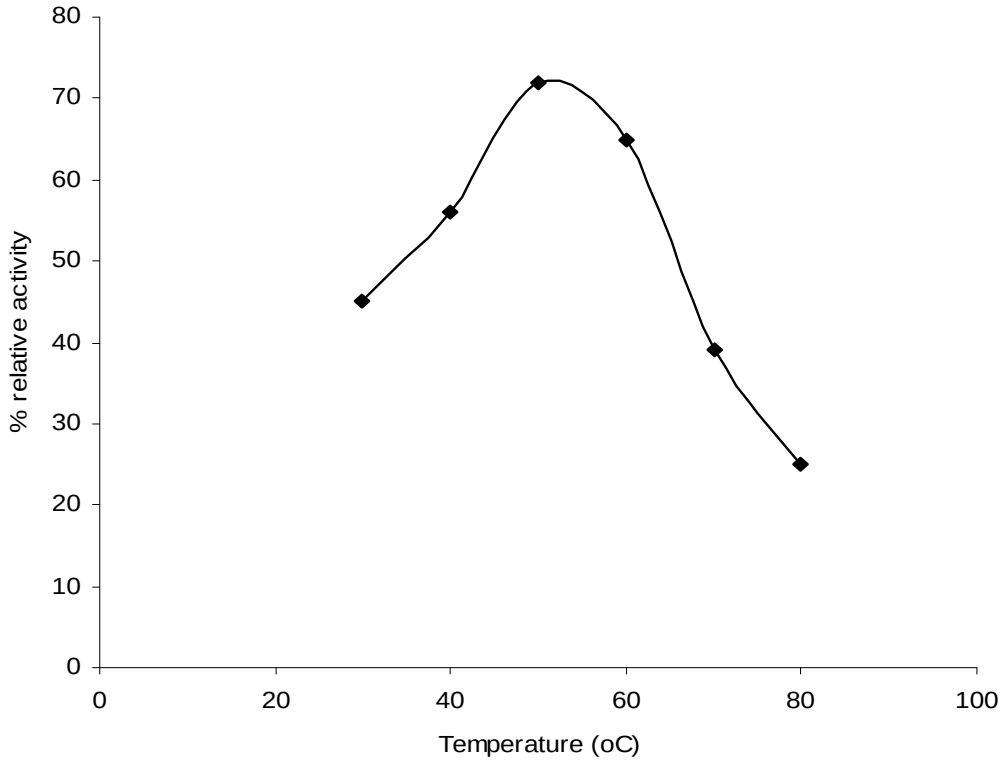

Figure 3. Effect of temperature on β-amylase activity of *Bacillus* sp. grown in production media.

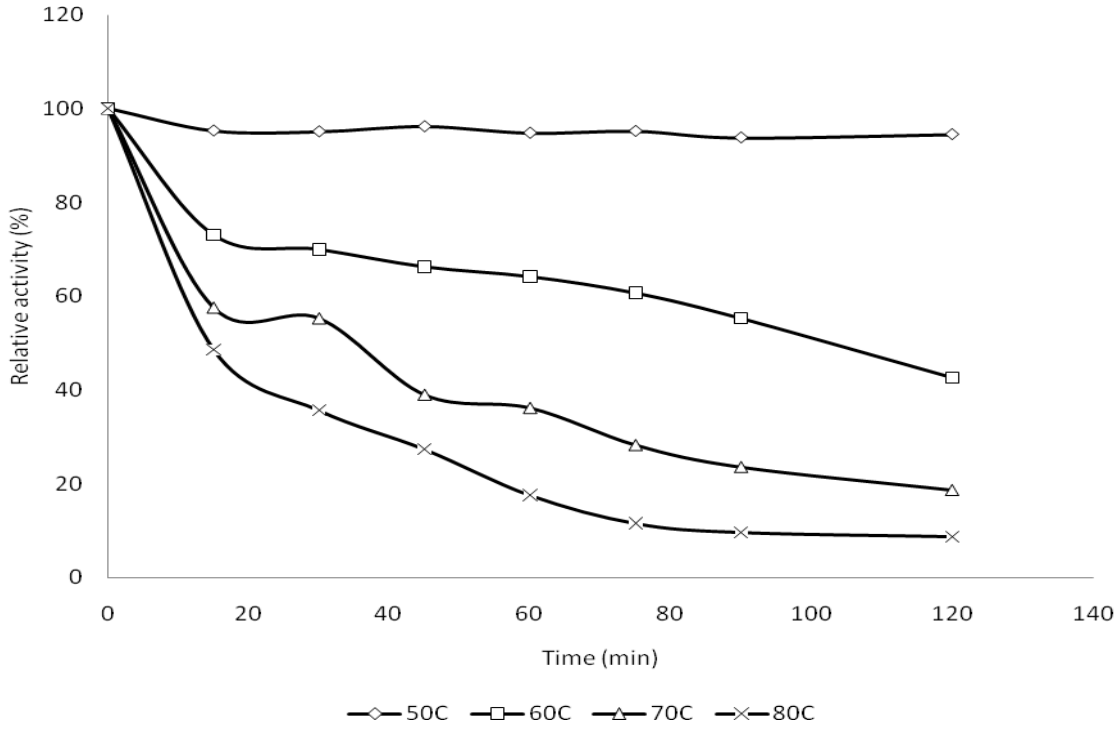

Figure 4. Thermal stability of β-amylase produced by *Bacillus* sp. in a growth medium.

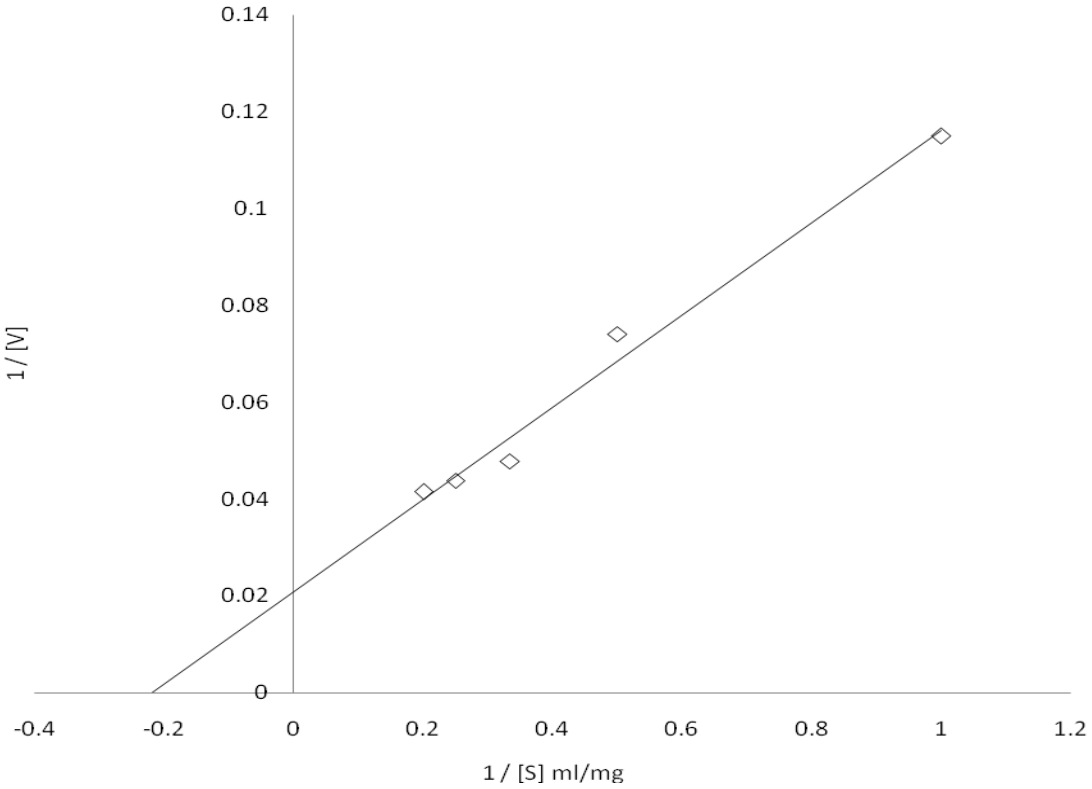

Figure 5. Lineweaver- Burke plot β-amylase produced by *Bacillus* sp.

Table 3. Effect of salts on β-amylase activity of *Bacillus* sp.

Salt	% Relative activity
Control	100
NaCl	101
$CaCl_2$	105
EDTA	103
$MgCl_2$	79
$HgCl_2$	53
$FeCl_2$	36

4. Discussion

The proximate analysis of the carbon source; kola nut (*Cola acuminata*) revealed high percentage of carbohydrate (67.49%) which was comparable with 69% reported by Arogba (1999). However, Jayeola (2001) reported a carbohydrate content of 88.10%. The β – amylase was purified 6.40-fold with 42.16% yield. The low purification factor may be due to the presence of low amounts of other proteins to be separated. However a single major activity peak of the β – amylase was found in the elution profile during gel filtration on Sephadex G-150. The molecular mass of the purified enzyme was estimated as 39.4kDa. Obi and Odibo (1984) reported a lower molecular mass of 31.6kDa for β – amylase of an *Actinomycete* sp. however β – amylase of high molecular weight have been reported in some other sources. Ray (2000) reported 209kDa and 105kDa for β – amylase from *Bacillus megaterium* while Olaniyi et al.(2010) reported a molecular weight of 69kDa for β – amylase of *Volvariella volvacea*. The

β – amylase showed maximum activity at pH 5.0, thus exhibiting similarity to most β – amylase that have acidic pH optimum ranging from 4.5-6.2 (Mikami et al., 1982). Obineme et al. (2003) and Olaniyi et al. (2010) reported similar pH optimum of 5.0 for the β – amylase of *Aspergillus niger* and *Volvariella volvacea* respectively. Sarowar et al. (2009) reported a pH optimum of 6.0 for the β – amylase of *Raphanus sativus* root.

The temperature and thermal stability studies showed that the activity of the enzyme was optimal at 50^{o}C. This is a widely reported attribute of β – amylase. Shinke et al. (1974) reported 50 to 60^{o}C as optimum for the activity β – amylase of *Streptomyces* sp. The enzyme retained 70% of its activity after incubation at 60^{o}C for 60min. β – amylase isolated from *Curculigo pilosa* was also reported by Dicko et al. (1999) to have an optimum temperature 55^{o}C and retained 80% of its activity after incubation at 65^{o}C for 90min. Denaturation of enzyme protein at temperature higher than 70^{o}C has been reported by Gupta et al. (2003). Inactivation due to heat has been associated with a two-step process (Creighton, 1990). 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 (Gupta et al., 2003). According to Daniel et al. (1996), extremely high temperature could also lead to deamination of asparagines and glutamine residues, hydrolysis of the peptide bonds at aspartic acid residues, thiol disulphide interchange and destruction of disulphide bonds and oxidation of amino acid side chains of protein molecule of the enzyme.

The affinity of the enzyme for the substrate was investigated using soluble starch as substrate. The K_m and V_{max} value of the purified *Bacillus subtilis* β – amylase obtained from the Lineweaver-Burk double reciprocal plot was 5mg/ml and 50 U/mg. This is closely related to the K_m value of 5.9mg/ml of Alfalfa (*Medicago sativa*) root β– amylase reported Douglas et al. (1982). The activity of the partially purified β – amylase was enhanced by Na^{+}, Ca^{2+} and ethylene diaminetetra acetic acid (EDTA). This observation is similar to those obtained for β – amylase from other sources (Obi & Odibo, 1984; Sarowar et al., 2009). However, the activity of the enzyme was inhibited by Mg^{2+}, Fe^{2+} and Hg^{2+}. Heavy metals are known to react with protein sulphydryl groups, thus converting them to mercaptides (Dixon & Webb, 1971). Hydrolytic degradation of disulphide bond by mercury has also been reported (Whitaker, 1972).

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