PURIFICATION AND CHARACTERISATION OF $\beta$-AMYLASE FROM *BACILLUS SUBTILIS* ISOLATED FROM FERMENTED AFRICAN LOCUST BEAN (*PARKIA BIGLOBOSA*) SEEDS

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$\beta$-amylase was obtained from *Bacillus subtilis* isolated from fermented *Parkia biglobosa* seeds, purified and characterized. Purification was achieved using ion exchange DEAE column and gel filtration (Sephadex G-200) chromatography. Effects of temperature, pH and production time on $\beta$-amylase production were investigated. Also, physicochemical characteristics of the purified enzyme were investigated. The optimum production of $\beta$-amylase was at temperature, pH and time of 37°C, 7.0 and 24 h, respectively. The results showed that purified $\beta$-amylase had more enzymatic activity than crude samples from *Bacillus subtilis* whereby the activity of crude enzyme was 3.21 mM/min/mL while the purified enzyme had an improved activity of 21.46 mM/min/mL. Optimum temperature and pH values of the purified amylase were found to be 50°C and 5.0, respectively. pH stability of the enzyme ranged from 4.0- 9.0. At pH 5.0 and 7.0 it retained 70% and 60% of its activity after 5 h of incubation. Temperature stability ranged between 40°C and 70°C but most stable at 50°C retaining 64% of its activity after 1 h of incubation. The enzyme exhibited maximum activity on soluble starch and sucrose, among other carbohydrate substrates. EDTA, Cu$^{2+}$ and Fe$^{2+}$ inhibited its activity while Ca$^{2+}$ and K$^+$ enhanced it up to 30%. The Lineweaver-Burke plot of the purified $\beta$-amylase activity of *B. subtilis* indicates that the $\beta$-amylase enzyme has apparent Km and Vmax values for the hydrolysis of soluble starch of 17.74 mg mL$^{-1}$ and 14.09U, respectively. The enzyme was purified 18.76 -fold and the molecular weight was 42.2 kDa. The study revealed that $\beta$-amylase from *B. subtilis* can be exploited for starch conversion biotechnologies.

**Keywords:** *Parkia biglobosa*, *Bacillus subtilis*, $\beta$-amylase, Purification, Characterization

INTRODUCTION

Amylases are enzymes whose applications in the industry have been increasing due to widespread use such as in foods, brewing, textiles, adhesives, detergents, pharmaceuticals, and sewage treatments (Osaki and Yoshino, 1988). The
enzymes involved are mainly $\alpha$-amylase (1,4$\alpha$-d-glucan glucohydrolase, EC 3.2.1.1), $\beta$-amylase (1,4$\alpha$-d-glucan maltotrihydrolase, EC 3.2.1.2) and glucoamylase (1,4$\alpha$-d-glucan glucohydrolase, EC 3.2.1.3) (Boldon and Effront, 2000). Though amylases originate from different sources such as plants, animals and microorganisms, the microbial amylases are the most produced and used in industry due to their productivity. Amylases are among 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 (Leveque et al., 2000). These enzymes account for about 30% of the world’s enzyme production. Unlike other members of the amylase family, only a few attempts have been made to study $\beta$-amylases particularly of plant origin while there is a dearth of information on $\beta$-amylase from microbial sources. Bacterial strains belonging to the genera *Bacillus*, *Pseudomonas*, *Clostridium* (Rani et al., 2007); and fungal strains belonging to *Rhizopus* (Forgarty and Kelly, 1990) have been reported to synthesize $\beta$-amylase. The properties of the $\beta$-amylase varies from one source to the other. Some of the microorganisms reported to produce $\beta$-amylases have employed starchy wastes such as cassava, rice husk, potato rice and maize as substrates for production (Forgarty and Kelly, 1990). “Iru”, fermented locust beans used as a condiment in cooking, similar to ogiri and douchi made from fermentation of seeds of the African locust bean tree; *Parkia biglobosa* a perennial leguminous tree belonging to the sub-family Mimosoideae and family leguminoseae (now family Fabaceae) Campbell-plat (1980). The seeds are rich in protein and the food condiment *Iru* is used as a flavor enhancer for soups, stews and also adds protein to a poor-protein diet in the developing countries (Dike and Odunfa, 2003). Studies on the fermentation of African locust bean seeds and other Nigerian condiments of protein origin used for soups and sauces found that fermentation was carried out by *Bacillus subtilis* (Enujiugha, 2009). During fermentation, the reducing sugar content increases, and the total free amino acid content initially decreases; in the end, however, there is a large increase in free amino acid content. Processing of locust bean fruits to food condiment, involves different unit operations after harvesting, such unit operations include de-podding, removal of the yellowish pulp to produce locust bean seeds (Akande et al., 2010). Other processing operations are cleaning, boiling, de-hulling, washing, re-cooking, and then fermentation to produce the food condiment which is used as soup seasoning/spices (flavoring agent) (Enujiugha, 2009). Due to the increasing demand for these enzymes in various industries, there is enormous interest in developing enzymes with better properties; such as raw starch degrading amylases suitable for industrial applications and their cost effective production techniques. The objectives of this project are to: isolate *Bacillus subtilis* microorganism from IRU, a local, cheap and ready legume, purify the $\beta$-amylase enzyme extracted from the isolated *Bacillus subtilis* and determine the effects of some metal ions, pH, and temperature on activity and stability of the pure $\beta$-amylase enzyme.

**MATERIALS AND METHODS**

**Isolation and Production of $\beta$-amylase**

*Bacillus subtilis* was isolated from fermented African locust bean (*Parkia biglobosa*), samples “iru” obtained from a local market in Akure, Ondo State using the method reported by Yandri et al. (2010). The colonies were picked up and streaked
on nutrient agar plates to get pure culture and to confirm zone formation. The culture was characterised by Gram-staining and other biochemical tests before storing on nutrient agar slants for use. The inoculum was prepared by the addition of sterile distilled water to the freshly grown nutrient agar slants, from this 0.5 mL of cell suspension was inoculated into 100 mL of sterilized fermentation medium and incubated at 35°C for 10 h. The composition of the fermentation medium was [g/L] 6.0 g Bacteriological peptone; 0.5 g MgSO\textsubscript{4} \cdot 7H\textsubscript{2}O; 0.5 g NaCl; 1.0 g Starch; 1.0 g Yeast extract; 0.1% K\textsubscript{2}HPO\textsubscript{4} and 0.02% MgSO\textsubscript{4} \cdot 7H\textsubscript{2}O at pH 7 (Rehman et al., 2011).

Inoculated flasks were maintained in water bath shaker at 150 g for 48 h. Growth and enzyme activity were determined from the aliquots (5 mL) collected at every 6 h. Growth was estimated turbidimetrically and the optical density of the culture broth was measured at 660 nm in spectrophotometer (Spectrophotometer Jenway, 6305).

To study the effect of temperature on amylase production the submerged fermentation was carried out at different temperatures (25°C, 30°C, 35°C and 40°C). About 100 mL of growth medium was taken in one set consisting of 3 flasks, autoclaved and then inoculated with 100 µL of the freshly prepared inocula. The cultures were incubated at the above temperature range in an incubator shaker at 150 rpm. An aliquot of culture was taken at regular intervals (0, 4, 8, 12, 16, 20, 24 and 28 h) to measure absorbance at 600 nm.

**Effect of Starch Concentration on Bacterial Growth**

Growth curves of *B. subtilis* were determined in 1% soluble starch fermentation broth medium. For bacterial isolate 100 mL medium was taken in one set consisting of 3 flasks, autoclaved and then inoculated with 100 µL of the freshly prepared inocula. The cultures were incubated at 45°C in an incubator shaker at 150 rpm. An aliquot of culture was taken at regular intervals (0, 4, 8, 12, 16, 20, 24 and 28 h) to measure absorbance at 600 nm.

**PURIFICATION OF β-AMYLASE AMMONIUM SULPHATE FRACTIONATION**

The supernatant was gradually brought to 80% saturation with ammonium sulphate with constant gentle stirring for 1 h after centrifugation (Rehman, 2011). The precipitate was earlier removed after centrifugation at 3,219 g for 30 min. Both enzyme activity (Bernfield, 1955) and protein content (Lowry, 1951) were determined for the separate fractions. The obtained ammonium sulphate precipitate (in solution) was introduced into dialysis bag (Spectra/por standard grade regenerated cellulose dialysis membrane) followed by dialysis against 0.1 M phosphate buffer at pH 6.2 for 48 h while replacing the buffer thrice (Takasaki, 1976). The obtained amylase enzyme preparation was concentrated against crystals of sucrose to remove the remaining salt and kept in the refrigerator at 5°C for further purification.
Ion Exchange Chromatography

Further purification of amylase enzyme was carried out using DEAE (Di-ethyl amino ethyl) cellulose anion exchange chromatography. The dialyzed sample was applied to a DEAE column. The column was washed with 50 mM, Tris buffer pH 8.0, and eluted with serially increasing concentration of NaCl (0.1 M, 0.2 M). The eluted fractions were monitored by UV absorption spectrophotometer at 280 nm.

Gel Filtration Chromatography (Using Sephadex G-200)

Preparation of the gel column and the fractionation procedures was determined as mentioned by Ammar (1975). Sephadex G-200 (Pharmacia, Upsalla, Sweden) was used, 0.1 M Tris-HCl buffer of pH 8.0 was added and the slurry was allowed to swell for 3 days at room temperature. Sodium azide (0.02%) was added to prevent any microbial growth. The enzyme solution was collected and dissolved in Tris-HCl buffer 0.1 M; pH 8.0 and fractionated through the Sephadex G-200 column (2.6 x 7.0 cm) previously equilibrated with the same buffer. Seven (7) mL of the enzyme preparation sample was applied carefully to the top of the gel and allowed to pass into the gel by running the column. Buffer was added without disturbing the gel surface and to the reservoir. Elution was carried out with the respective buffer at a flow rate of 20 mL/h. Fifty fractions (5 mL each) were collected and absorbance read at 280 nm using spectrophotometer (Jenway, 6305). Amylolytic activity and protein content were carried out for each individual fraction. The eluted enzymatically active fractions were pooled and used as the purified enzyme.

AMYLASE ACTIVITY

Beta-amylase Activity was estimated by the 3, 5 Dinitrosalicylic acid (DNSA) method of Bernfield (1955). Appropriately diluted 0.5 mL of enzyme was added to 0.5 mL of 1% (w/v) soluble starch which was dissolved in appropriate buffer solution (sodium acetate buffer, pH4.7). The reaction mixture was prepared in triplicates. The reaction tubes were incubated at room temperature for 3 min. Then 2 mL of color reagent (DNSA) was added to the reaction mixture and placed in boiling water bath (Gallenkamp) for 5 min. The tubes were allowed to cool at room temperature. Then 10 mL of distilled water was further added to the cooled tubes and absorbance at 540 nm was measured using spectrophotometer (Jenway, 6305).

Control tube consisted of 0.5 mL buffer solution plus 0.5 mL soluble starch solution. The assay was also carried out as above. All assays were carried out in triplicate. The amount of maltose liberated was extrapolated from the maltose standard curve. One unit of beta amylase activity was taken 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 released one micromole of maltose from the starch in 5 min.

AMYLASE ASSAY USING DIFFERENT SUBSTRATES

The amylase activity was also assayed by measuring the reducing sugar released during the reaction, using complex polysaccharide substrates (soluble starch, carboxymethylcellulose (CMC), corn starch, cassava starch, rice bran, lactose and sucrose). The reaction mixture contained 0.5 mL of 1% solution of the
substrate separately prepared in 2 mM sodium acetate buffer of pH 4.7 and 0.5 mL of enzyme solution.

Effect of pH on Purified β-amylase Activity
The effect of pH on activity of β-amylase was determined by assaying for enzyme activity at different pH values ranging from 3.0-9.0. Buffer (0.05 M) of different pH ranging from 3.0 to 9.0 were prepared using different buffer systems, 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 and 9.0. Each of these buffer solutions 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.

Effect of pH on Stability of the Purified Enzyme
The stability of purified enzyme was determined by measuring the residual activity of the enzyme being incubated for a specific period at different pH values at room temperature based on the method applied by Yang et al. (1996) and Rehman et al. (2011). This was determined by mixing aliquots of 1 mL enzyme with 2 mL buffer solution earlier described. The mixture was incubated at room temperature for 6 h. At one hour intervals, an aliquot of 0.5 mL from the mixture was assayed for residual activity under standard assay condition except that each buffer solution was used to prepare 1% soluble starch used as substrate in assaying the enzyme activity.

Effect of Temperature on Purified β-Amylase Activity
To find the optimum temperature, the variations of temperature used were 30°C to 80°C. Beta amylase activity was assayed by incubating the enzyme reaction mixture of 0.5 mL enzyme and 1% soluble starch in sodium acetate buffer pH 5.0 at different temperatures (30°C to 80°C) for 10 min. After treatment the residual enzyme activity was assayed and absorbance was determined at 540 nm.

Effect of Temperature on β-amylase Stability
The thermal stability of the enzyme was determined by incubating about 4 mL of the pooled enzyme fractions at temperatures ranging from 30°C to 80°C without the substrate (soluble starch) for 1 h. At intervals of 10 min, aliquot of 0.5 mL of the incubated enzyme was assayed for residual activity.

Determination of Kinetics Data of Purified Enzyme
The Michaelis constant (Kₘ) and the maximum reaction velocity (Vₘₐₓ) of the amylase for starch was determined at different substrate concentrations. They were evaluated by plotting the data on a Lineweaver-Burk double-reciprocal graph (1/vi) versus (1/[S]) (Lineweaver and Burk, 1934).

Polyacrylamide Gel Electrophoresis
Sodium dodecyl sulphate-Polyacrylamide gel electrophoresis (SDS-PAGE) at 12% was carried out to determine the purity and molecular weight of the enzyme, as described by Laemmli (1970). The molecular weight of the enzyme was estimated using a low molecular weight calibration kit with markers consisting of (I) Phosphorylase b, 103.14 kDa; (II) Bovine serum albumin, 81.35 kDa; (III) Ovalbumin, 47.05 kDa; (IV) Carbonic anhydrase, 34.17 kDa; (V) Soybean trypsin inhibitor, 27.26 kDa and (VI) Lysozyme, 17.67 kDa. The molecular weight was taken as a measure of its purity.
**Effect of Heavy Metals and Cations on Enzyme Activity**

A stock solution of 0.01 M of HgCl$_2$ and Ethylenediaminetetraacetic acid (EDTA) were prepared. Two milliliter of each salt solution was mixed with 2 mL of enzyme solution. The mixture was incubated for 5 min at room temperature. 0.5 mL of the mixture was withdrawn and assayed according to standard assay procedure. Also, a stock solution of 0.01 M of each salt was prepared. The salts used were NaCl, CaCl$_2$, FeCl$_2$, and MgCl$_2$. Two milliliters of salts solution was mixed with 2 mL of enzyme solution, and the same procedure for heavy metals was followed.

**RESULT: SUMMARY OF PURIFICATION TABLE**

A summary of the results from purification of the $\beta$-amylase obtained from *Bacillus subtilis* isolated from Parkia biglobosa seeds “iru” is presented in Table 1. The table shows that specific activities for crude extract, ammonium sulphate precipitation, ion exchange chromatography, and gel filtration were 0.19, 0.52, 0.79, and 3.56 (U/mg) respectively. Purification (fold) for crude extract, ammonium sulphate precipitation, ion exchange, and gel filtration were 1, 2.75, 4.25, and 18.74, respectively, an indication that purification increased with each purification step while percentage enzyme yield reduced with each purification step.

**SDS PAGE Electrophoresis**

From the electrophoretogram (Figure 7), the protein band occurred as a single band and it was spotted between protein standard iii and IV; which are Ovalbumin and Carbonic anhydrase respectively. The estimated molecular weight for purified $\beta$-amylase was 42.20 kDa.

**Effect of Starch Concentration on Production of *Bacillus subtilis***

The effect of soluble starch concentration on growth of *B. subtilis* from fermented African locust bean seed shows that Log phase was from 0-5 h, lag phase continued from 5 h to 20 h while stationary growth was at 20-25 h and decline in growth started from 25 h. There was increase in growth at 6 h of production steadily increasing after 12 h and 18 h, at 18-24 h, 1% was found to be higher than 0.5% by 19% and 2% by 12%, respectively. Additionally bacterium achieved optimum growth at 24 h incubation period but the growth pattern and the growth curve of the 1% starch was significantly different from those of 0.5% and 2%. There was a steady growth decline after 25 h with a further 24 % drop in growth at 28 h incubation (Figure 1).

<table>
<thead>
<tr>
<th>Purification Step</th>
<th>Total protein (mg)</th>
<th>Total enzyme activity (U)</th>
<th>Specific activity (U/mg)</th>
<th>Purification (fold)</th>
<th>Yield (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Crude enzyme</td>
<td>68800</td>
<td>12840</td>
<td>0.19</td>
<td>1</td>
<td>100</td>
</tr>
<tr>
<td>$\text{NH}_4\text{SO}_4$</td>
<td>24325</td>
<td>12530</td>
<td>0.52</td>
<td>2.75</td>
<td>97.59</td>
</tr>
<tr>
<td>Precipitation DEAE Cellulose</td>
<td>234.4</td>
<td>186.08</td>
<td>0.79</td>
<td>4.25</td>
<td>1.45</td>
</tr>
<tr>
<td>Sephadex G-200</td>
<td>45.23</td>
<td>160.95</td>
<td>3.56</td>
<td>18.74</td>
<td>1.25</td>
</tr>
</tbody>
</table>

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Effect of Temperature on *B. subtilis* Production

The optimum temperature for the growth of *Bacillus subtilis* revealed that the optimum *B. subtilis* production (14.42 µmole/ min/mL) was recorded at 37°C. There was an increase (about 52%) in production from 30 h to 37 h, after which a drop in growth was experienced from 38 h to 40 h. There was a growth decline at temperatures above 40°C and the minimum growth (5.4 µmole/ min/mL) was recorded at 25°C.

Effect of pH on Growth of *Bacillus subtilis*

The effect of various pH on *Bacillus subtilis* growth after 24 h incubation at 37°C shows that optimum *Bacillus subtilis* production (19.78 µmole/min/mL) was observed at pH 7.0. Growth was noticeable from slightly acidic through neutral to the slightly alkaline pH. With increase in pH above 7.0, there was a decrease in yield. Minimum yield of growth (6.0 µmole/min/ml) was recorded at pH 5.0. It was observed that a slight change in pH of medium adversely affected the growth of the bacterium. Growth showed that at peak growth for pH 7.0 was about 59% higher than peak growth for pH 6.0 while decline in peak of 25% for pH 7.5 and 8.0, respectively.

Effect of pH on the Activity of β-amylase

The influence of pH on the activity of β-amylase is presented on Figure 2 and the enzyme was found to be active in the acidic pH range of 3.0-5.0 and had optimum relative activity (4.32 mM/ min/mL) at pH 5.0. There was a decline in the activity of enzyme from slightly acidic pH 6.0 but again experiencing slight induction and retained 60% of its activity at pH 7.0 of that at optimal pH. From the plot, it was also found that residual activity at peak pH 5.0 was 9.5%, 12.5%, 59.0%, 34.0%, 45.0%, 36.0% greater than residual activity of the enzyme at pH 3.0, 4.0, 6.0, 7.0 and 9.0, 8.0 and 12.0, 10.0 and 11.0, respectively.

Figure 1: Effect of Starch on *B. subtilis* growth

![Graph showing the effect of starch on *B. subtilis* growth](image)
Effect of Temperature on the Activity of β-amylase

Figure 3 shows the influence of temperature on activity of the enzyme. The activity of β-amylase from *B. subtilis* increased with temperature from 30-50°C. The optimum activity (6.32 mM/min/mL) was at 50°C; afterwards, there was a gradual decline as temperature increases. The enzyme retained 60% of its activity at 80°C but very little activity at 90°C. It was found that peak residual activity at 50°C
was greater than residual activity for 30°C, 40°C, 60°C, 70°C, 80°C and 90°C by 25%, 17%, 18%, 12%, 37%, and 72%.

**Effect of pH on the Stability of β-amylase**

The plot of the percentage residual activity of the enzyme at pH 4.0-10.0 against time of incubation at room temperature is illustrated in Figure 4. At
all the pH examined, the enzyme was relatively stable for 4 h. After 4 h, it was still able to retain almost 70% of its activity except at pH 7.0 where it dropped to 60%.

**Effect of Temperature on Stability of β-amylase**

The thermostability of β-amylase at various temperatures is shown in Figure 5. β-amylase from *B. subtilis* was stable from 30 -60°C, showing relative residual activity of 70, 80 and 60% at 40, 50 and 60°C after an hour of incubation. At higher temperatures, there was a decline in residual activity such that at 70°C, there was 30% residual activity.

**Effect of Different Carbohydrate Substrate on β-amylase**

The result revealed that maximum β-amylase activity was observed in 1% soluble starch followed by sucrose. The least activity was observed in 1% CMC (Carboxymethylcellulose), corn starch and cassava starch. Cassava starch had a 34% residual activity in relation to soluble starch (control), corn starch had 33% residual activity, CMC was less at 27% in relation to soluble starch, lactose 47% while sucrose residual activity in relation to the control was at a high by 92.0% and rice bran residual activity was 49% of the control.

**Effect of Metal Salts**

In enzyme action, metallic cofactors are important because their presence or absence regulates enzyme activity. The presence of specific metallic ions along with food content can inhibit or enhance amylase activity, and therefore

![Figure 6: Effect of Metal Ions on β-amylase Activity](image-url)
the rate of digestion (Mar et al., 2008, Kiran and Chandra, 2008).

The effects of various metals ions at two different concentrations on the activity of β-amylase are reported in Figure 6 where it showed that Fe$^{2+}$, Cu$^{2+}$ as divalent ions almost or completely inactivated the enzyme activity. Of the monovalent ions, Na$^+$ had inhibitory action on the enzyme while K$^+$ exhibited activation of the enzyme at different concentrations. Also Ca$^{2+}$ and Mg$^{2+}$ increased the activity of the enzyme especially at higher concentrations of the metal ions. Presumably because of its chelating properties (Kiran and Chandra, 2008), from the study, EDTA also inhibited the β-amylase enzyme.

**Effect of Substrate Concentration**

The Lineweaver-Burke plot of the purified β-amylase activity of *B. subtilis* revealed that the β-amylase enzyme has apparent $K_m$ and $V_{max}$ values for the hydrolysis of soluble starch of 17.74 mg mL$^{-1}$ and 14.09U respectively.

**DISCUSSION**

**The microbial growth:** The features confirmed from the growth was consistent with reported literature Rehman et al. (2011), Ray and Nanda (1996). Absence of lactose shows that the fermentation of “iru” was not lactic acid fermentation and the presence of glucose revealed that the organism broke down the medium into simple sugars. The growth features including the elevation, serrated edges and color was consistent with *B. subtilis* growth reported by Rehman et al. (2011) and Nouadri et al. (2010).

**Optimal Growth Conditions**

The study revealed that growth of *B. subtilis* was at its peak when growth medium contained 1% soluble starch, at pH 7.0, under temperature of 37°C and for 24 h. This was consistent with findings by Rehman et al. (2011), Annamalai (2011), Lin (1998), Sivakumar et al. (2012) and Mazutti et al. (2007).

**Effect of Starch Concentration on Growth**

A growth curve often time consists of the Log phase, Lag phase, Stationery phase and the Logarithmic decline phase Fankhauser (2012). The effect of starch concentration on production of *B. subtilis* followed the production growth curve of most microbial growth. The growth curve pattern was studied by growing the bacterium in 0.5, 1 and 2% soluble starch. The bacterium achieved optimum growth at 24 h incubation period during the lag phase of growth as reported by Rosenberger (2008) and Fankhauser (2012) but the growth pattern of the 1% soluble starch was significantly different from those of 0.5 and 2%.

**Effect of pH on Growth**

The optimum *B. subtilis* production (19.78 μmole/min/mL) was observed at pH 7.0. At increase in pH above 7.0, there was a decrease in *B. subtilis* yield. Minimum yield of *B. subtilis* (6.0 μmole/min/mL) was recorded at pH 5.0. This study has recorded optimization of culture conditions for growth and amylase production at pH, 7.0, 37°C, 24 h and 1% of substrate concentrations consistent with (Annamalai, 2011 and Lin, 1998). Also Vijayalakshmi (2009) reported 35°C optima temperature for amylase production by *Bacillus* species.

The effect of initial pH on SSF of amylase production shows that the pH range of 5.0-7.0 produced more amount of amylase (Sivakumar et al., 2012). Amylase production by microbial strains strongly depends on the extracellular pH,
as culture pH strongly influences many enzymatic reactions and also for the transport of various components across the cell membrane (Nahas and Waldermanin, 2002).

**Effect of Temperature.**
The maximum yield of β-amylase by *B. subtilis* was obtained at 37°C; this was close to the value reported by Sivakumar et al. (2012) using *Bacillus cereus* isolated from a vermicompost site. 35°C was observed as the optima temperature for production of amylase by *Bacillus sp.* (Vidyalakshmi, 2009). Pandey et al. (2001) stated that increase in incubation temperature decreased the production of enzyme. It could be that at high temperature, the growth of the bacteria was greatly inhibited and hence enzyme formation was also prohibited. In contrast, low temperature values may reduce the metabolism of the microorganism (Mazutti et al., 2007) consequently the enzyme synthesis.

**Effect of Temperature on Enzyme Activity**
The optimum activity (6.32 mM/min/mL) was at 50 °C; afterwards, there was a gradual decline as temperature increases. The enzyme retained 60% of its activity at 80°C but very little activity at 90°C. Optimal activity at 50°C is widely reported as attribute of β-amylase. Temperature optimal between 40-50°C was shown by the β-amylase of *B. polymyxa* (Gasparian et al., 1992); *B. subtilis* (Castro et al., 1993) and *B. circulans* (Kwan et al., 1994). Ojo et al. (2007) reported the optimum temperature of 60°C for β-amylase from cassava peel. The thermostability of β-amylase at various temperatures showed that β-amylase from *B. subtilis* was stable from 30-60°C, showing relative residual activity of 70, 80 and 60% at 40, 50 and 60°C after an hour of incubation. At higher temperatures, there was a decline in residual activity such that at 70°C, there was 30% residual activity. Denaturation of enzyme protein at higher temperature has been reported by Gupta et al. (2003) who also surmise that extremely high temperature could 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. Also resistance to thermal denaturation of an enzyme is regarded as one of the most important criteria for industrially appreciable enzymes (Sarowar et al., 2012).

**Effect of pH on Enzyme Activity**
The enzyme was assayed at pH ranges from 3.0-12.0. The optimum activity (4.32 mM/min/mL) was at pH 5.0. There was a slight decline in the
enzyme activity from pH 6.0 but retained 60% of its activity at pH 7.0. The optimum pH observed agrees with those reported from purified β-amylase from different sources. Most of the microbial β-amylases have an optimum pH at 5.0-7.0 as reported from B. megaterium (Ray et al., 1996); B. subtilis (Castro et al., 1992); while Olaniyi et al. (2010) reported similar optimum pH of 5.0 for the β-amylase of Aspergillus niger. The plot of the percentage residual activity of the enzyme at pH 4.0-10.0 against time of incubation at room temperature is illustrated in Figure 7. At all the pH examined, the enzyme was relatively stable for 4 h at almost all the pH. After 4 h, it was still able to retain almost 70% of its activity except at pH 7.0 where it dropped to 60%. The pH stability is also a very important quality for continuous production of enzymes because in large scale fermenters, pH change is a usual and natural occurrence (Sarowar et al., 2012).

**Effect of Metals and Ions**

In enzyme action, metallic cofactors are important because their presence or absence regulates enzyme activity. The presence of specific metallic ions along with food content can inhibit or enhance amylase activity, and therefore the rate of digestion. The effects of various metals ions is reported in Figure 6 where it showed that Fe^{2+}, Cu^{2+} as divalent ions almost or completely inactivated the enzyme activity which is consistent with the findings reported by Sarowar et al. (2012), Ojo and Ajele (2011). Of the divalent ions, Na^+ had inhibitory action on the enzyme while K^+ exhibited activation of the enzyme at different concentrations (Sarowar et al., 2012). Also Ca^{2+} and Mg^{2+} increased the activity of the enzyme especially at higher concentrations of the metal ions. Usually, the role of Ca^{2+} in stability and maintaining the structure of the β-amylase enzyme has been well documented (Parkin, 1993). It is well known that amylases contain Ca^{2+} as an essential component of the enzyme molecule and are often inhibited by the chelating reagent EDTA (Kiran and Chandra, 2008) presumably because of its chelating properties, from the study; EDTA also inhibited the β-amylase enzyme thereby confirming the above assertion. It was observed that NaCl enhanced the activity of the β-amylase, while its activity was slightly inhibited by salts such as CaCl_2 and MgCl_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 (1990) 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.

The β-amylase was purified 18.7-fold. The purification factor is a measure of purity of the β-amylase and the fact that it may be approaching a homogenous state.

**SDS PAGE ELECTROPHORESIS**

The molecular weight of the β-amylase was estimated to be 42.2 kDa migrating as a single protein band in SDS-PAGE 12% gel which indicates its homogeneity. The estimated molecular weight of 42.2 kDa from its mobility relative to those of standard proteins on SDS-PAGE indicated that the purified enzyme is a monomer (Ray and Nanda, 1996).

This is lower than the molecular weight reported in a wide variety of β-amylase isolated from other sources. For example, 105 kDa was
reported for $\beta$-amylase from *Bacillus megaterium* (Ray, 2000), 69 kDa was reported by Olaniyi *et al.* (2010) for $\beta$-amylase from *Volvariella volvacea*. It was higher than 27.6 kDa reported by Femi-Ola *et al.* (2013) for $\beta$-amylase from *Bacillus subtilis*. Buonocore *et al.* (1976) 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.

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