**CED TECH International Journal of Environmental Science & Biotechnology**
**Volume 2, Number 3, September 2021**
**http: / /www.cedtechjournals.org**
**ISSN: 2756-4541**

# PRODUCTION OF CRUDE AMYLASE FROM *BACILLUS SUBTILIS* ISOLATED FROM WARM WATER

**Timothy Chinedu Nwaulu,[2]Fatimah Buba[1&*2], Ali Ali Abdulrahman[2], Mohammed Adamu Milala[2]**

[1]School of Pharmacy, Faculty of Science and Engineering, University of Wolverhampton, Wolverhampton, WV1-1LY, U.K.
[2]Department of Biochemistry, Faculty of Science, University of Maiduguri, Bama Road, Maiduguri, Nigeria
Email: fatimahbuba@gmail.com

## ABSTRACT

This work investigated the feasibility of generating crude thermostable amylase from *Bacillus subtilis* isolated from warm water, the entire work focused on it activity of the enzyme in terms of temperature, pH and substrate concentration effect. The aim of the findings was to isolate and determine the effect of temperature, pH and substrate concentration on the activity of crude amylase from *Bacillus subtilis*. Glucose oxidase method was used to determine the activity of the amylase. At the production time course, amylase was observed to have a maximum yield of (8.4 μmol/min) at 96 hours of incubation. Maximum crude amylase was observed at pH 7.0 and temperature 60°C (2.47μmol/min). The crude amylase production amylase in this current research might be suitable for applications in industries that require high temperature and neutral pH.

**Keywords**: *Crude Amylase, Bacillus Subtilis, Warm Water and Thermostable*

## INTRODUCTION

Amylases are ubiquitous and distributed throughout plants, animals and microbes with vital role in carbohydrate metabolism. However, microorganisms producing amylases have successfully replaced chemical processing techniques in different industries due to cost effectiveness and technical advantages (Pandey *et al.,* 2000; Vengadaramana, 2013). Due to the expanding application in research areas primarily, focused on the development of new amylase with high pH and temperature stability to achieve maximum rate of catalysis, improved gelatinization of starch, decreased media viscosity and diminished possibility of microbial contamination (Li *et al.,* 2012). The wider occurrence, easy production

and its wider range usage makes alpha-amylase a commercially valuable biocatalyst (Sundarram and Murthy, 2014). Starch is the substrate for amylase production and the mechanism of action of the enzyme on its substrate is referred to as "lock-and-key model" (Prescott et al., 2005). Amylases are starch hydrolyzing enzymes (Reddy et al., 2003). Each of the three types of amylases has a unique way of acting on starch. For instance, alpha amylase (being an endoamylase) cleaves or breaks the $\alpha$-1-4 glucosidic linkages in starch internally to give glucose, maltose, or dextrins (Rao *et al.,* 2007). Beta amylase, as an exoamylase, cleaves the glycolytic bonds removing two glucose units at a time thus producing maltose (*Gupta et al.,* 2003). Glucoamylase, on the other hand, cleaves both the $\alpha$-1, 4 and $\alpha$-1-6 glucosidic linkages to yield glucose, maltose, and limit dextrins (Rao et al., 2007). Due to the significant application of amylases in both food and detergent industries, there is need to alternate source of amylase with high thermostability.

## MATERIALS AND METHODS

### Source of Bacteria and Fermentation Media

The sample of bacteria was isolated from warm water source in Maiduguri metropolis and the bacteria were isolated, identified in Microbiology laboratory, Faculty of Veterinary Medicine, University of Maiduguri. The bacteria sample (*Bacillus subtilis*) was inoculated into 200ml fermentation media (Luria-Bertani culture medium) 2g tryptone, 1g yeast and 0.2g NaCl (Guennadi*et al.,* 2007); crude enzyme was harvested at the interval of 24 hours.

## CHARACTERIZATION OF AMYLASE

### Pre-Determination of Crude Thermostable Amylase Activity

Time course was determined crude amylaseat its maximum activity; a reaction mixture was prepared by mixing 2ml of the crude enzyme, 2ml of NaCl and 2ml of 1% starch. At time 0, a portion of the reaction mixture (0.5ml) was taken and added into a test tube containing 2ml of 1% iodine. The disappearance of blue black colour indicates the absence of starch) which entails hydrolysis of starch. 2ml of this mixture was taken and put in another test tube containing glucose reagent to test for the amount of glucose liberated by the action of crude enzyme. This series of reaction was repeated after 24 hours. It was observed that the enzyme had its maximum activity at 96 hours.

## GLUCOSE ASSAY

Glucose oxidase kit was used to determine the glucose released from the hydrolysis of starch. The experiment was carried out using glucose oxidase method as modified by Keston in the early 1950's. The principle behind this assay is to oxidized glucose by glucose oxidase to yield hydrogen peroxide and gluconic acid. The hydrogen peroxide then oxidatively couple with 4-aminophenazone and phenol substitute (p-HBS), in the presence of peroxidase to yield a red-violet quinoneimine dye as indicator. The amount of coloured complex formed is proportional to glucose concentration and can be measured photometrically (Keston, 1956).

$$\text{Glucose} + H_2O_2 \xrightarrow{\text{glucose oxidase}} H_2O_2 + \text{Gluconic acid}$$

$$H_2O_2 + \text{4-aminophenazone} + \text{Phenol} \xrightarrow{\text{peroxidise}} \text{Quinoneimine} + 4H_2O$$

The reaction mixture was prepared and the mixture was incubated at 37°C water bath and the intensity of resulting colour was measured photometrically at 500nm against a blank containing distilled water and reagent 1 (buffer). The absorbances obtained in triplicate were used in calculating the activity. The result was calculated as follows: Glucose concentration = (Absorbance $_{test}$/ Absorbance $_{standard}$) x concentration standard

## EFFECT OF TEMPERATURE ON CRUDE THEMOSTABLE AMYLASE ACTIVITY

Crude amylase (0.5ml) was added in a test tube containing 1ml of NaCl and Starch (2ml) to produce a reaction mixture which was put into a water bath to attain temperature of 30°C. Aliquot of 0.5ml was taken from the reaction mixture and added into a test tube containing 2ml of 1% iodine solution to test for the presence of starch. A faint blue black colour was obtained showing that the enzyme has hydrolyzed some of the starch. This mixture (0.5ml) was added into a test tube containing 0.5ml of glucose oxidase. The mixture was allowed to stand for 20 minutes for colour to develop. A pink colour was obtained indicating the presence of glucose. The concentration of the colour obtained was directly proportional to the amount of glucose produced. The absorbance of the solution was taken using a spectrophotometer at 500nm. The steps above were repeated at temperature 30°C, 40°C, 50°C, 60°C, 70°C and 80°C respectively.

## EFFECT OF pH ON CRUDE THERMOSTABLE AMYLASE ACTIVITY

The effects of pH on crude thermostable amylase activity were determined by varying the pH values 4.0, 5.0, 6.0, 7.0 and 8.0.

## EFFECT OF SUBSTRATE CONCENTRATION ON CRUDE THERMOSTABLE AMYLASE ACTIVITY

Amylase activity with different concentrations of the substrate was determined by varying the substrate into 0.5 mg/ml, 1.0 mg/ml, 1.5 mg/ml, 2.0 mg/ml, and 2.5 mg/ml.

## DETERMINATION OF $V_{Max}$ AND $K_M$

The $V_{Max}$ and $K_M$ was determined by Lineweaver-Burk plot 1934.

## RESULTS AND DISCUSSION

Time course for production of amylase enzyme by *Bacillus subtilis* was investigated and fermentation was carried out for 144 hours as depicted in (Figure 1).The result of the time course production of amylase by *Bacillus subtilis* at incubation period of 96 hours shows optimum level of amylase (8.4μmol/min). The time of fermentation has an important impact on the product formation; enzyme production is related to time of incubation. Incubation time depends on the characteristics of the culture, growth rate (rate of substrate utilization) and enzyme production (Baysal*et al.,* 2003). A gradual increase was seen in enzyme activity from 24 hours to 96 hours after which a gradual decrease was observed. Incubation period of 96 hours was optimum for amylase enzyme production by the organism (*Bacillus subtilis*). This finding relate with the results of (Ozdemir*et al.,* 2009) who reported incubation time of 72 hours as optimum incubation time for the production of amylase, though the current finding has exceeded 72 hours but was within the range. The decrease in enzyme yield after 96 hours of incubation as shown in (Figure 1) may be as a result of denaturation or decomposition of amylase due to interaction with other components in the medium, as is reported by (Ramesh and Losanne, 1987). Moreover, the reaction for maximum enzyme production at 96 hours could be due to the fact that the microorganism was in its exponential phase. At the later stage, when nutrients are depleted, it has reached its stationary phase and may start producing secondary metabolites, thus resulting in a lower yield of enzyme (Ramachandran *et al.,* 2004).

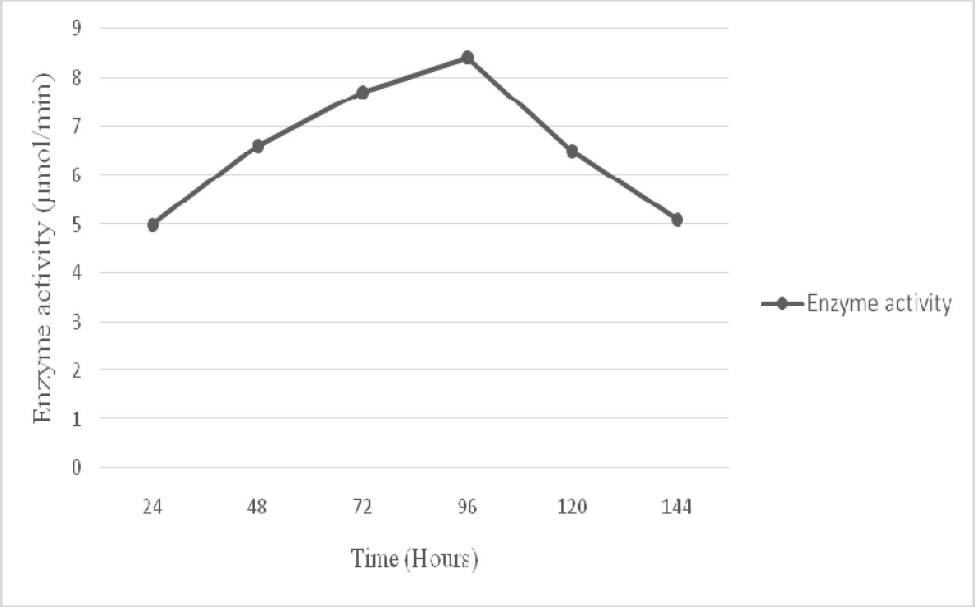


Fig. 1: Time course production of amylase by *Bacillus subtilis.*

However, temperature is a critical factor that influences enzyme activity and has to be regulated in any bioprocess to maintain optimum activity. In this study, the activity of amylase increased gradually as the temperature increases from 30°C – 50°C and reached a peak (2.47 µmol/min) when the temperature was at 60°C (Figure 2). This relates to the findings of Goyal *et al.,* (2005) who reported 70°C as optimum temperature for activity of amylase isolated from *Bacillus specie.* Though, the current findings were lower than the reported temperature by Goyal *et al.,* (2005). Although a further increase in temperature in this study resulted to a decrease in amylase activity. At 70°C there was slight decrease in activity (1.9µmol/min) and a significant decrease in activity (0.9µmol/min) at 80°C was observed. Thus, the activity was swiftly declined. This trend can be attributed to denaturation, which is the process in which proteins lose their native structure by application of external stress such as high temperature. Enzymes are prone to denaturation, high temperature tends to disrupt inter and intra molecular forces holding the enzyme molecule together and this results to change in enzyme structure which in turn affects its activity ( Bandos, 2014).

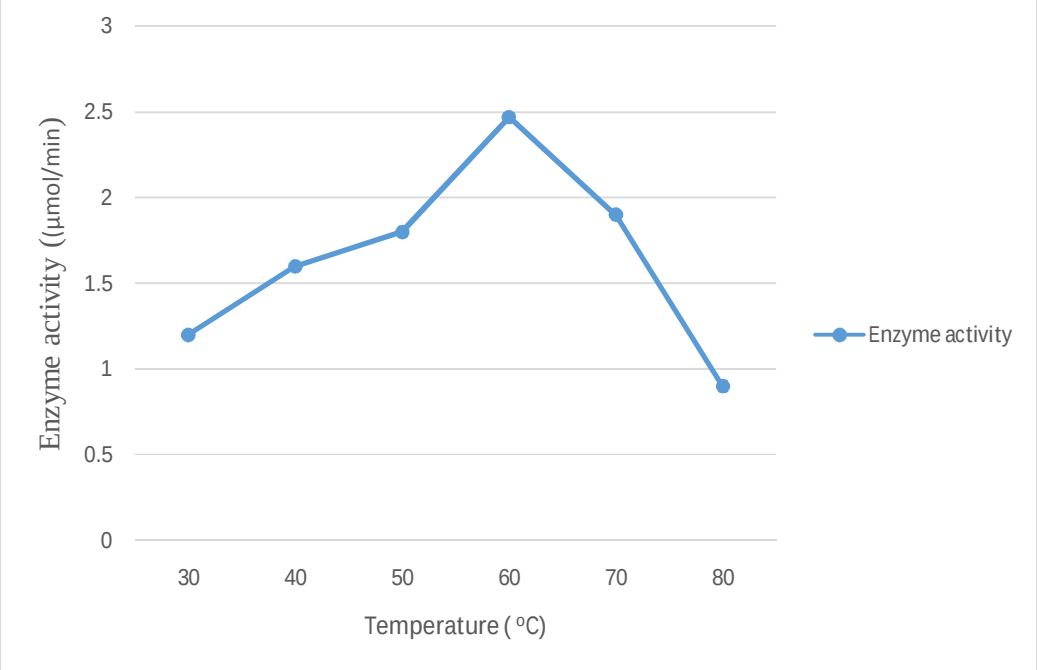


Fig. 2: Effect of temperature on crude amylase activity.

The maximum crude enzyme activity was observed to be 8.0µmol/min at pH 7.0. Conversely, the minimum enzyme activity of the crude amylase was observed to be 1.4µmol/min at pH 4.0. Therefore, pH is also an important parameter that influences Enzyme activity. Enzymes activity is controlled by the pH of the medium; this dictates its stability and activity for long duration (Pal and Khanum, 2010). Excess of either $H^+$ or $OH^-$ ions affect the secondary, tertiary and quaternary structures of protein (enzyme) by disrupting H-bond and van der Waals interactions. When the crude amylase was treated at different pH, maximum activity was obtained at pH 7.0 retaining 8µmol/min activity (Figure 3). Crude amylase activity declined when pH changed from 7.0 to 8.0 (retaining 5.4 µmol/min activities). The possible reason may be the inhibition of enzyme active site by alterations in the concentrations of hydrogen ions and also amylases was proven to be inactive acid medium but active in neutral or alkaline medium. This finding agrees with the report of (Goyal *et al.,* 2005), who reported pH 7.0 as the optimum pH for amylase isolated from *Bacillus specie.*

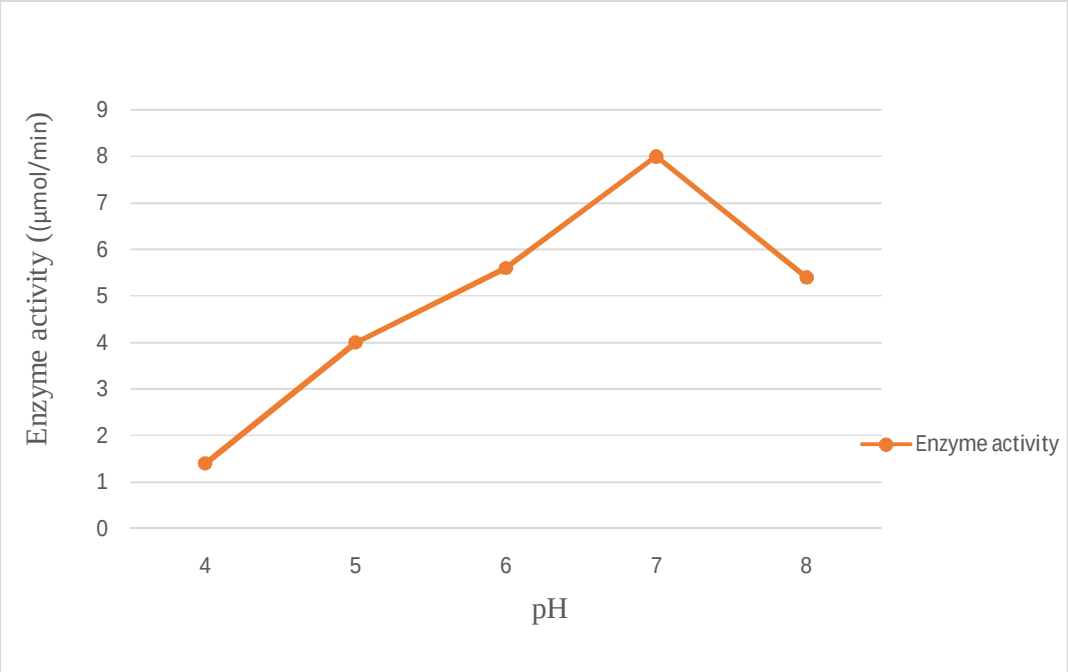


Fig. 3: Effect of pH on crude amylase activity.

Substrate concentration is another critical factor that influences enzyme activity and has to be controlled in any bioprocess to achieve optimum activity (Vander et al., 2002). Carbon is a major requirement of the cell and the rate at which a carbon source is metabolized can often influence the production of metabolites. The carbon/energy source is necessary for growth and product formation in microbial cultivation (Vengadaramana 2013). The nature and characteristics of these substrates has a predominant role to play in the metabolism of microorganism (Anderson and Jayaraman, 2003). Among different concentration of the substrate (starch) studied, 2.5 mg/ml of the starch showed highest enzyme activity (3.10 µmol/min) and lowest enzyme activity (0.25 µmol/min) was observed at 0.5 mg/ml substrate concentration. As the substrate concentration increases there is an increase in crude amylase activity. This result relates to the work of (Padul*et al.,* 2014) which showed an increase in the enzyme activity as substrate concentration increased. From the Lineweaver-Burk plot (Figure 4), kinetic parameters $V_{Max}$ and $K_M$ were 6.716 µmol/min and 12.73mg/ml respectively. A low Km implies high affinity of the enzyme for substrate while high Km entails low affinity of the enzyme for the substrate (Vasudevan*et al.,* 2013).

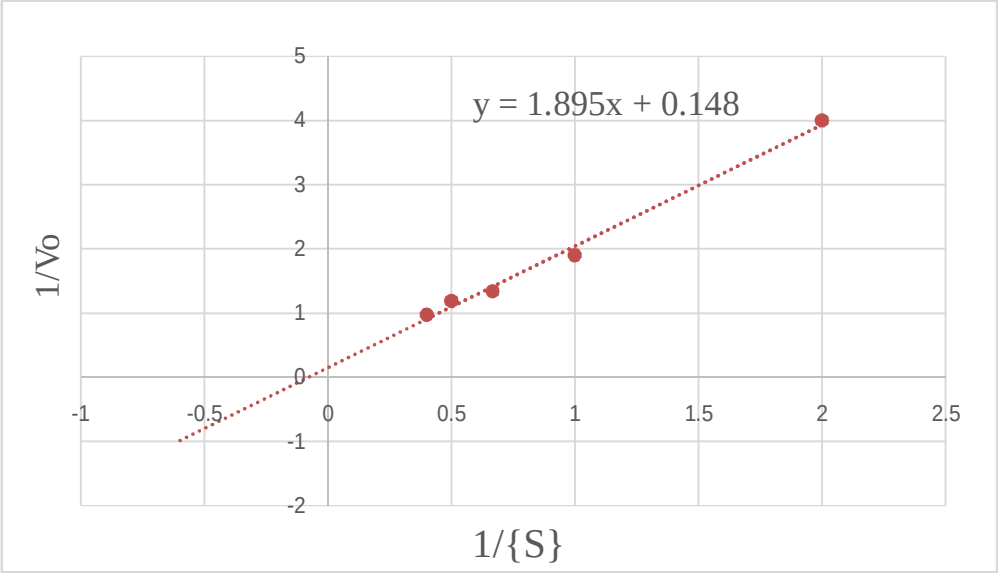


Fig.4: Lineweaver-Burk plot of crude amylase. (Vmax=6.716μmol/min, Km=12.73)

## CONCLUSION

Conclusively, it was an established fact that high temperature in product production could stand as a barrier in achieving a standard finished product that requires the application of this enzyme thus, the crude amylase produced in this current study might be suitable for applications in industries that require high temperature and neutral pH.

## REFERENCES

Anderson, R. K. I. and Jayaraman, K. (2003). Influence of carbon and nitrogen sources on the growth and sporulation of *Bacillus thuringiensis* var Galleriae for Biopesticide production. *Chem. Biochem*. ***17***: 225– 231

Bandos, S. (2014). Protein folding. *Access science,* ***10***:1036-1070.

Baysal, Z., Uyar, F., Aytekin, C. (2003).Solid state fermentation for production of a-amylase by a thermotolerant *Bacillus subtilis* from hot-spring water, *Process Bioch* ***38***:1665-1668.

Goyal, N., Gupta, J.K., Soni, S.K., (2005). A novel raw starch digesting thermostable a- amylase from *Bacillus* specie I-3 and its use in the direct hydrolysis of raw potato starch, *Enzyme Microb. Technology*,***37***:723–734.

Guennadi, S., Daniele, J.P., and Richard, D. (2007).Escherichia coli physiology in Luria- Bertani Broth. *Journal of Bacteriology,* ***189***:8746-8749.

Gupta, R., Gigras, P., Mohapatra, H., Goswami, V. K., Chauhan, B. (2003). Microbial Alpha amylases: A Biotechnological Perspective. *Process Biochemistry,* ***38***: 1599-1616.

Keston, A.S. (1956). Colorimetric, enzymatic reagents for glucose.Abstracts of papers, 129th Meeting ACS, 131C.

Li, S., Yang, X. and Yang, S. (2012). Technology prospecting on enzymes: application, marketing and engineering. *Computed Structural Biotechnology Journal,* ***2***:1–11.

Ozdemir, S., Guven, K., Baysal, Z., and Uyar, F. (2009).Low cost medium for alpha-amylase production by *bacillus subtilis. Food technology and biotechnology,* ***47***:364-369.

Padul, M.V., Chougale, A.D., Danna, L.B., Gadge, P.P., Shaikh, F.K., Chopde, R.M. and Vadan, S.S. (2014). Amylase from sugarcane willy aphid (*CeratovacunaLanigera Zehntner*). *International peer-reviewed journal,* ***1***:50-51.

Pal, A., Khanum, F. (2010). Production and extraction optimization of xylanase from aspergillus niger DFR-5 through solid-state fermentation. *Bioresource Technology,* ***101***: 7563-7569.

Pandey, A.; Nigam, P.; Soccol, C.R.; Soccol, V.T.; Singh, D.; Mohan, R. (2000). Advances in microbial amylases. *Biotechnological Appl. Biochem,* ***31***:35-152.

Prescott, L. M., Harley, J. P., and Klein, D. A. (2005). Metabolism: Energy, Enzymes, and Regulation. *In Microbiology (6th edition)*.Published co. New York: McGraw-Hill. PP 156-159, 171-172.

Ramachandran, S., Patel, A.K., Nampoothiri, K.M., Francis, F., Nagy, V., Szakacs, G., Pandey, A. (2004).Coconut oil cake – A potential raw material for the production of a–amylase, *Bioresource Technology,* ***93***:169–174.

Ramesh, M.V., Lonsane, B.K. (1987). A novel bacterial thermostable alpha-amylase system produced under solid state fermentation, *Biotechnol. Lett*.**9**:501–504.

Rao, D. M., Swamy, A. V. N., and Siva Rhama Krishna, G. (2007). Bioprocess Technology Strategies, Production, and Purification of Amylases: An Overview. *The Internet Journal of Genomics and Proteomics, **2***: 30-34.

Reddy, N. S., Nimmagadda, A., and SambasivaRoa, K. R. S. (2003).An Overview of the Microbial Alpha-amylase Family. *African Journal of Biotechnology, **2***: 645-648.

Sundarram, A. and Murthy, T.P.K. (2014). Amylase Production and Applications: A Review. *Journal of Applied & Environmental Microbiology*, ***2***:166-175.

Vander, M.J.E.C., Maarel, B., VanderVeen, J.C.M., Uitdehaag, H.L.M., Dijkhuizen, L. (2002). Properties and application of starch converting enzymes of the alpha amylase family. *Biotechnology*, ***94***:137-155.

Vasudevan, D.M., Sreekumari, S., Kannan, V. (2013). Text book of Biochemistry for Medical Students (Seventh Edition).Jaypee Brothers Medical Publishers (P) Ltd, Ansari road, Daryaganj, New Delhi, India. PP. 57-58.

Vengadaramana, A. (2013). Industrial Important Microbial alpha-Amylase on Starch- Converting Process. *Journal of Pharmacy, **2***:209-221.

Vengadaramana, A. (2013). Industrial Important Microbial alpha-Amylase on Starch- Converting Process. *Journal of Pharmacy, **2***:209-221.