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A Thermostable Extracellular α-amylase from *Bacillus licheniformis* Isolated from Cassava Steep Water

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Abstract: The isolation and taxonomic characterization of *Bacillus licheniformis* isolated from cassava steep water and the purification and characterization of its extracellular amylase (α-1, 4-glucano-4-glucanohydrolase, EC 3. 2. 1. 1) were carried out in this study for the potential use of the enzyme for cassava starch hydrolysis for industrial purposes. The enzyme was purified by ion-exchange chromatography on DEAE-Cellulose and gel filtration on Bio-Gel P100 column. The specific activity of the purified enzyme was approximately 855 units per mg of protein (U mg^{-1}). The enzyme is a large protein with apparent molecular weight determined by gel filtration on Bio-Gel P100 of greater than 100, 000 Daltons. The enzyme obeys sigmoidal kinetics with a kinetic constant (K^1) for soluble starch of 1.097±0.027% starch and a V_{max} of 44.54±1.79 U min^{-1}. The optimum pH and temperature for enzyme activity were 7.5 and 90°C, respectively. The enzyme was stable for 45 min at 90°C. The enzyme was activated by Cd^{2+}, Co^{2+}, Mg^{2+} and Ni^{2+} while Fe^{3+} and Mn^{2+} moderately activated the enzyme and Zn^{2+}, Ba^{2+}, EDTA and acetamide were inhibitory. This amylase could be useful for the hydrolysis of soluble starch for the production of maltose.

Key words: α-amylase, cassava, starch, *Bacillus licheniformis*

INTRODUCTION

Amylases are enzymes that catalyze the hydrolysis of starch or other carbohydrates to produce sugar, syrups and dextrin (Sadhukham *et al*., 1992). The hydrolysates thus produced are used as carbon and energy sources in fermentation and in a range of manufactured food and pharmaceutical products (Forgaty and Kelly, 1980). A number of amylases have been considered important because of their different catalytic pattern and yield of products of different configuration (Whitaker, 1972). α-amylase (α-1, 4-glucan-4-glucanohydrolase, EC 3.2.1.1) that hydrolyzes internal α-1, 4-linkages to yield maltose, maltotriose and α-dextrin at the initial step in starch breakdown. β-amylase (1, 4-α-D-glucanmaltohydrolase, EC 3.2.1.2) hydrolyzes α-1, 4-glucosidic linkages of starch in an exo-fashion from the non-reducing end of the starch polymer producing maltose in the β-anomeric configuration and β-limit dextrin (Forgaty and Kelly, 1979). Amyloglucosidase or Glucoamylase (Glucan-1, 4-α-glucosidase) is an exohydrolase that stepwisely hydrolyzes terminal glucose residues from the non-reducing terminal of the substrate molecule. It catalyzes the hydrolysis of α-1, 4 linkages in starch in an exo-fashion hydrolyzing α-1, 6 bonds but at a much slower rate than α-1, 4 bonds. The other types of amylases include the isoamylases and the pullulanases (α-dextrin endo-1, 6-α-glucosidase, EC 3.2. 1.41), which are termed debranching enzymes'. Isoamylase (EC 3.2.1.68) which catalyzes the hydrolysis of the α-(1→6)-glycosidic bonds of amylopectin, glycogen and oligosaccharides. The minimum structural requirements for the substrates are separate maltose and maltotriose units linked by a α-(1→6)-glycosidic bond (Robyt, 1984). Pullulanase catalyses the hydrolysis of the α-(1→6)-glycosidic bonds of the amylopectin and limit dexrins that possess at least a maltose unit in the shortened side chain. The hydrolysis occurs in a random manner to liberate maltotriose (Robyt, 1984). A combination of these enzymes can break down the entire starch polymer.

Amylases are widely distributed in plants, animals and microorganisms and show varying patterns of action depending on the source (Hagenimana *et al*., 1992). The most commonly studied microbes for the production of amylases are fungi and bacteria (Campbell, 1955; Manning *et al*., 1961; Yamamoto *et al*., 1972; Saito,

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1973; Boyer *et al*., 1979; Medda and Chandra, 1980; Forgaty and Kelly, 1980; Nanmori *et al*., 1983; Takasaki, 1985; Srivastava and Baruah, 1986; Sen and Chakrabarty, 1987; Brumm *et al*., 1988) The occurrence of microbial α-amylases with varied catalytic properties has been extensively reviewed by Forgaty and Kelly (1980).

Many of the enzymes used in the industries are extracellular enzymes derived from microorganisms. High value is placed on extreme thermostability and thermoactivity of amylases for use in the bioprocessing of starch. The increased use of bacterial amylases has also created the need to isolate strains producing amylases with characteristics that are more amenable to industrial and other useful applications. The objectives of this study are to isolate and carry out the taxonomic characterization of a bacterium from cassava steep water that is a good amylase producer and to purify and characterize the produced amylase for the purposes of exploiting its potential industrial applications.

MATERIALS AND METHODS

This research was conducted between October 2003 and September 2005 at the Obafemi Awolowo University, Ile-Ife, Nigeria and at the Olabisi Onabanjo University, Remo Campus, Ikenne, Nigeria. Twelve to fifteen month-old cassava tuber of the local white variety was harvested from a farm in Ikenne, Ogun State, Nigeria. All media were purchased from Lab M, Lancashire, UK. DEAE-Cellulose and Sephadex G-200 were obtained from Pharmacia AB, Uppsala, Sweden. The High Molecular Weight (HMW) calibration kit for gel filtration was obtained from Sigma Chemicals Company Ltd., St. Louis, MO., USA. Percoated TLC plate (Silica Gel F, 20×20 cm) was purchased from Analtech, Newark, USA. Other reagents were of analytical grade. Glass distilled water was used for all solutions.

Fermentation of cassava tissue: Freshly harvested cassava tuber was peeled, cut into thin cylindrical slices and washed in tap water. Twenty gram of the cassava slices were soaked in 100 mL of tap water in 250 mL capacity conical flask in triplicate. The soaked cassava were allowed to ferment at 30°C for up to 48 h.

Isolation and identification of amylase producing bacteria from cassava steep water: The bacteria were isolated from the cassava steep water at the 24 and 48th h of fermentation. Steep water (10 mL) was taken aseptically from the fermenting cassava tissue and diluted serially in sterile 0.1% peptone water diluent. Aliquots (1.0 mL) of appropriate dilutions were plated in starch agar (Nutrient agar (Oxoid CM3) and 1% soluble starch (Analar)) using the pour plate method. Inoculated plates were incubated at 30°C for 48 h. Representatives of predominant colonies from the plates were purified by repeated streaking on starch agar and pure isolates were preserved on nutrient agar slant in the refrigerator.

The amylolytic potential of the pure bacteria isolates was determined by testing their ability to hydrolyse starch. This was done following standard starch agar plate method as described by Harrigan and McCance (1976). Bacterial isolate with large clearing of the medium after flooding with Gram's iodine solution was selected for this study and was further characterized and identified by standard methods (Harrigan and McCance, 1976; Buchanan and Gibbons, 1974; Collins *et al*., 2004).

Enzyme and protein assays: The amylase activity was measured by incubating 0.2 mL of the appropriately diluted enzyme solution at 37°C for 10 min with 0.8 mL of 1.2% soluble starch in 0.1M citrate phosphate buffer, pH 5.5 containing 80 mM $CaCl_2$. After the incubation, the amount of reducing sugar liberated by the enzyme was measured by the Nelson-Somogyi method (Nelson, 1944; Somogyi, 1945) using maltose as standard. One unit of enzyme activity was defined as the amount of enzyme that released one micromole of reducing sugar as maltose per min under the conditions described above.

The protein concentration was determined by the method of Bradford (1976) using bovine serum albumin as standard.

Enzyme extraction: The pure bacteria culture (18 h old) was used to inoculate sterile starch broth (Nutrient broth (LabM)+1.0% soluble starch) and incubated on an orbital water bath incubator at 37°C for 48 h. The contents of the flasks were then pooled and centrifuged at 7,000 rpm for 20 min. The supernatant was used as the crude enzyme.

Enzyme purification: The *B. licheniformis* α-amylase was purified by a procedure similar to that employed by Giraud *et al*. (1993) with an addition gel filtration step on Bio-Gel P100.

Ammonium sulphate fractionation: The crude enzyme was brought to 70% ammonium sulphate saturation. The precipitated protein after standing in an ice-bath for at least 2 h was recovered by centrifugation at 7,000 rpm at 4°C for 20 min. The precipitate was stored in a minimal volume of 50 mM sodium-phosphate buffer, pH 6.8.

Ion-exchange chromatography on DEAE-Cellulose: DEAE-Cellulose resin was pretreated according to Whatman Product Instruction Manual and equilibrated in

50 mM sodium-phosphate buffer, pH 6.8. The slurry was then packed into a column (2.5×40 cm) and equilibrated further with 50 mM sodium-phosphate buffer, pH 6.8. The precipitate collected from the ammonium sulphate fractionation step above was dialyzed in 50 mM sodium phosphate buffer, pH 6.8. The dialysate was layered on the column and eluted into 3 mL fractions at a flow rate of 30 mL h^{-1} with a 250 mL linear salt gradient (0-0.1M NaCl) in the same buffer. The active fractions were pooled and brought to 70% ammonium sulphate.

Gel filtration on Bio-Gel P100: Bio-Gel P100 resin (12 g) was swollen in distilled water for 72 h, then packed into a column (1.5×40 cm) and equilibrated with 50 mM sodium phosphate buffer, pH 6.8. The precipitated protein from the previous step was dialyzed and gel filtered on the Bio-Gel P100 column eluting with the same buffer.

Determination of the native molecular weight: Bio-Gel P100 resin was prepared as described above and packed into a 2.5×70 cm column. The molecular weight markers were cytochrome c (M_r 12,400; 4 mg mL^{-1}), carbonic anhydrase (M_r 29,000; 3 mg mL^{-1}), albumin (M_r 66,000; 10 mg mL^{-1}) and hexokinase (M_r 96,000; 2 mg mL^{-1}). The proteins were eluted with 50 mM sodium/potassium phosphate buffer, pH 6.8 at a flow rate of 20 mL h^{-1}. The void volume of the column was determined with blue dextran (2 mg mL^{-1}). The calibration and estimation were done according to Andrews (1964 and 1965).

Determination of the kinetic parameters: The kinetic constants were determined using soluble starch as substrate. The enzyme was assayed routinely at varying substrate concentration from 0.10-1.20%. The kinetic constant (K^1) and V_{max} were estimated from the plots of velocity (v) against [S] and the Hill plot of log v/V_{max}-v versus log [S] according to Segel (1975).

Effect of pH and temperature: The optimum pH of the enzyme was determined by assaying the enzyme at various pH values using 0.2 M of the following buffers-sodium acetate (pH 3.5-4.5), citrate-phosphate (pH 5.5), phosphate (pH 6.5-7.5) and tris (pH 9.20). The optimum temperature of the enzyme was determined by incubating the enzyme at different temperatures between 40 and 100°C and assaying its residual activity. The thermal stability of the enzyme at 90°C was also carried out by incubating for 45 min while aliquots were withdrawn at 5 min intervals for enzyme assay.

Effect of salts and reagents: The effects of some salts (Ni^{2+}, Ba^{2+}, Cd^{2+}, Co^{2+}, Mg^{2+}, Zn^{2+}, Cu^{2+}, Mn^{2+}, $Sn^{2+}$and Fe^{3+}), EDTA and acetamide on the enzyme activity were investigated by assaying the enzyme in the presence of 1 and 5 mM of the chemicals.

Analysis of the hydrolysate by thin-layer Chromatography: The hydrolysate obtained after the incubation of 2 mL of the enzyme with 2 mL of 1% analyzed soluble starch for 2 h at room temperature were identified using the thin-layer chromatography method of Ivor and Feinberg (1965). The standard sugars were D (+) glucose (G), D (+) maltose (M), D (+) sucrose (S) and D (+) fructose (F).

RESULTS

Characterization and identification of the isolated amylolytic bacterial strain: The morphological and biochemical characteristic of the isolated amylase producing bacteria is shown in Table 1. The bacteria isolate was identified as *B. licheniformis*.

Enzyme purification: The elution profiles on DEAE-Cellulose and Bio-Gel P100 are shown in Fig. 1 and 2, respectively. There were more than one peak of amylase activity but the one with highest activity was purified further. The results of the purification procedures were summarized in Table 2. The procedure yielded a α-amylase with a specific activity of 854.8 U mg^{-1} of protein with a yield of 43.17%.

Table 1: Morphological and biochemical characteristics of the isolated amylolytic bacterial strain

Characterization tests	Bacterial strain
Gram reaction/cell shape	Positive/Rod
Catalase	Positive
Spore	Present
Spore position	Central (MG1)
Starch hydrolysis	Positive
Nitrate reduction	Positive
Gelatin liquefaction	Positive
VP (acetoin)	Positive
Citrate utilization	Positive
Oxidative-Fermentative test	Oxidative
Oxygen relationship	FA
Indole production	Negative
Anaerobic growth	Positive
Acid from sugar fermentation	
Glucose	Positive
Sucrose	Positive
Mannitol	Positive
Xylose	Positive
Maltose	Positive
Growth at:	
45°C	Positive
50°C	Negative
Probable identity	*Bacillus licheniformis*

FA: Facultative anaerobe; VP: Voges Proskauer, MG1: Morphological group 1 (ellipsoidal and cylindrical spore that do not exceed the diameter of the sporangium)

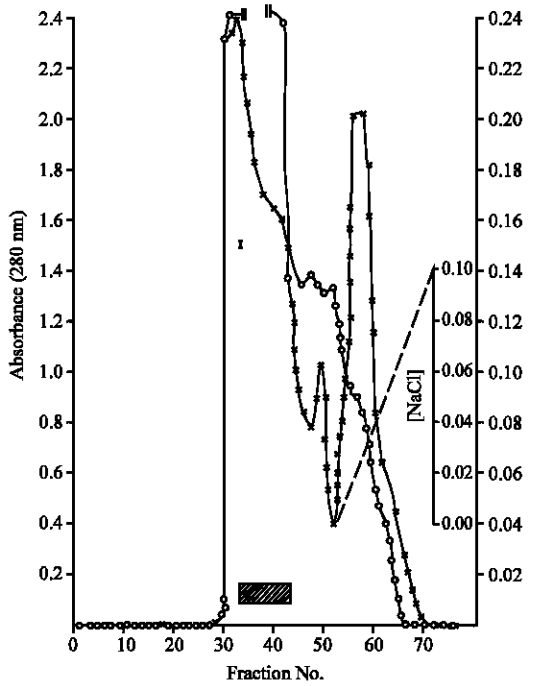

Fig. 1: Elution profile of the *Bacillus licheniformis* amylase from cassava steep water from DEAE-Cellulose ion-exchange chromatography. The column was equilibrated with 50 mM sodium/potassium phosphate buffer, pH 6.8. The enzyme was eluted with a 250 mL linear salt gradient (0-0.1 m NaCl) as described in the text. o-o-o (protein), x-x-x (enzyme activity), --- (salt gradient) and – (pooled fractions)

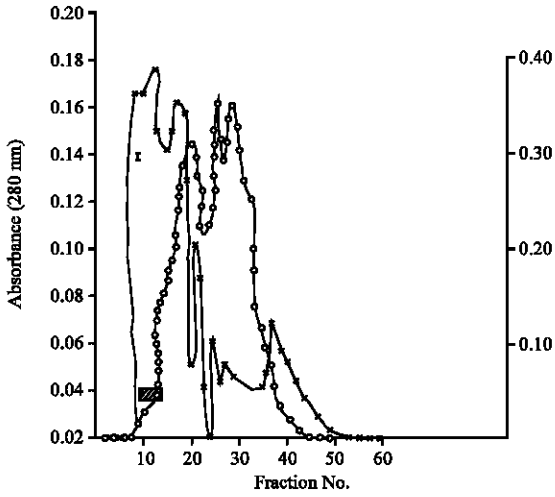

Fig. 2: Elution profile of the post ion exchange fraction on Bio-Gel P100 gel filtration column. The eluant was 50 mM sodium/potassium phosphate buffer, pH 6.8. Details are as described in the text. o-o-o (protein), x-x-x (enzyme activity) and – (pooled fractions)

Properties of α-amylase of *B. licheniformis* from Cassava Steep water: The molecular weight obtained by gel filtration on Bio-Gel P100 was greater than 100,000 Daltons. The V_{max} was estimated as 44.54±1.79 U min^{-1} (Fig. 3). The kinetic constant (K^1) and the number of binding site estimated from the Hill plot were 1.097±0.027% starch and 2, respectively. The pH and temperature profiles of the purified α-amylase are shown in Fig. 4 and 5, respectively. The optimum pH was 7.5.

Table 2: Summary of the purification procedure

Fraction	Volume (mL)	Activity (U)	Total protein (mg)	Specific activity (U mg^{-1})	Yield (%)	Purification fold
Crude 70%	1338	1782.22	1070.40	1.67	100.00	1.00
$(NH_4)_2SO_4$	160	974.03	43.75	22.26	54.65	13.33
DEAE-Cellulose (Fraction I)	55	650.08	24.89	26.12	36.48	15.64
Bio-Gel P100 (Fraction I)	18	420.98	1.24	339.50	23.62	203.29

Table 3: Effect of cations and other reagents

	Activity (%)	
Cations/Reagents	1 mM	5 mM
$CaCl_2$	100.00	100.00
$NiCl_2$	84.72	77.92
$MgCl_2$	85.71	85.71
$CoCl_2$	85.71	82.79
$CdSO_4$	87.66	87.79
$FeCl_3$	77.92	78.90
MnO_2	68.18	61.37
$SnCl_2$	43.83	59.42
$BaCl_2$	36.99	22.88
$ZnSO_4$	24.35	22.40
$CuSO_4$	15.58	11.69
Acetamide	56.40	64.28
EDTA	35.06	18.51

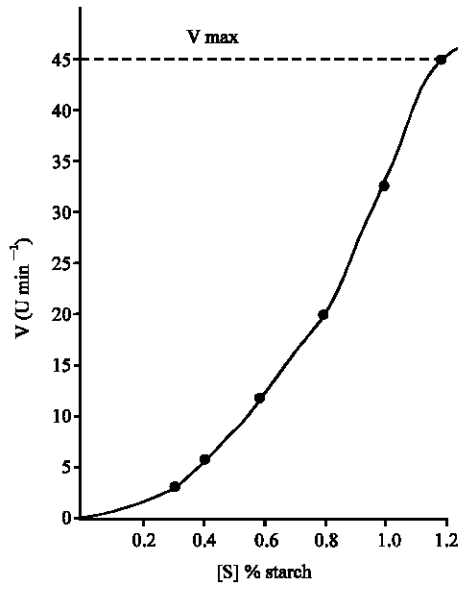

Fig. 3: Plot of v (U min^{-1}) versus [S] % starch for the determination of V_{max}

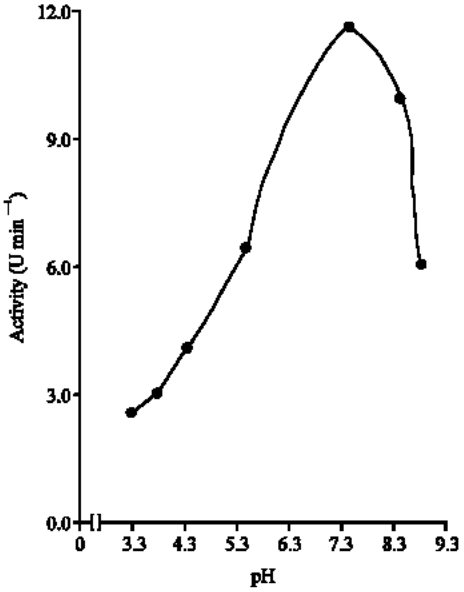

Fig. 4: The effect of pH on enzyme activity

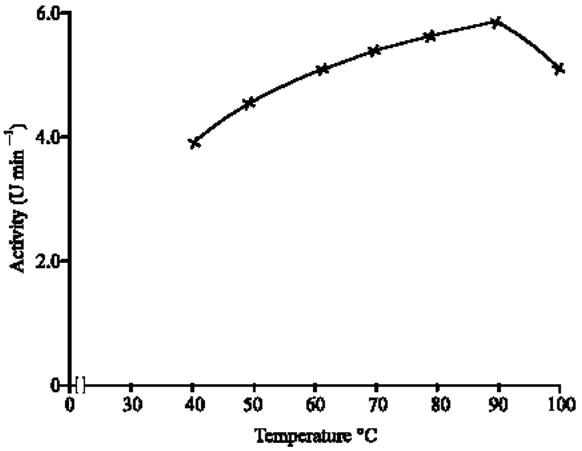

Fig. 5: The effect of temperature on enzyme activity

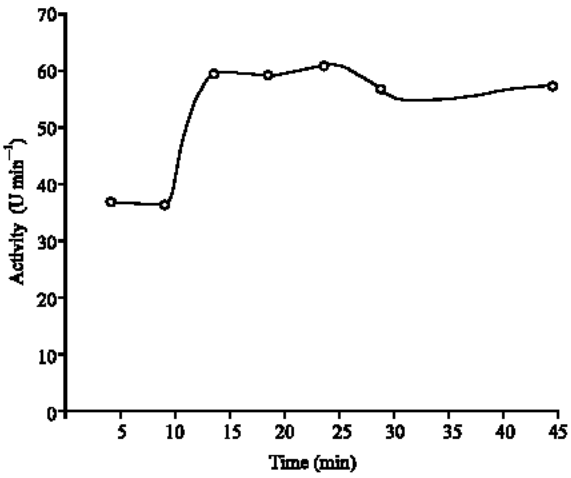

Fig. 6: The thermal stability curves of the enzyme at 90°C

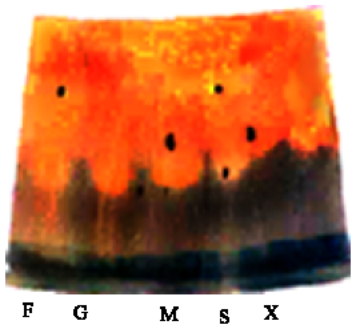

Fig. 7: The Silica gel chromatogram. The photograph of the chromatogram showing the hydrolysate on hydrolyzing soluble starch with the purified enzyme (X).The chemical markers are fructose (F), glucose (G), maltose (M) and sucrose (S)

The enzyme showed maximum activity at 90°C and retained a substantial amount of activity at 100°C. The thermal stability curve at 90°C is shown in Fig. 6. The effect of some cations and other reagents on the activity of *B. licheniformis* α-amylase in the presence of $CaCl_2$ is presented in Table 3. The hydrolysate of the reaction of the enzyme with starch when separated by thin layer chromatography contained mainly maltose (Fig. 7).

DISCUSSION

Many bacteria produce extracellular amylases during the fermentation of starch. α-amylases have been purified and crystallized from different sources including *B. licheniformis* (Robyt, 1984). *B. licheniformis* is a ubiquitous bacterium of importance in the environment as a contributor to nutrient cycling due to the production of amylase and other enzymes.

Varied molecular weights have been reported for α-amylase from various sources. As a result, the choice of a gel filtration medium for the determination of the molecular weight of this α-amylase was rather difficult. An unusually low molecular weight of 15,600 was reported for *Bacillus stearothermophilus* α-amylase (Manning *et al*., 1961). Bernfeld (1951) and Koivula *et al*. (1993) had obtained molecular weights in the range 45,000-90,000 Daltons for most α-amylases. *Bacillus licheniformis* 44MB82-A strain α-amylase had also been reported to have a molecular weight of 58,000 (Ivanova *et al*., 1993). α-amylase has many times been found to occur in aggregates as in the case of homodimer of molecular weight of 100,000 Daltons (Dong *et al*., 1997). The conditions favouring aggregation is however, not known. One can only suggest that it may depend on the duration of storage during the purification procedure.

It is noteworthy to find that the *B. licheniformis* α-amylase obeys sigmoidal kinetics (Fig. 5). It may appear from this that either the enzyme is oligomeric or it forms aggregates. In all, it is possible to assume that there is more than one substrate binding site. Earlier, Seigner *et al.* (1985) have reported that α-amylase from porcine pancreas showed sigmoidal response.

Amylases give bell-shaped curves when activity is plotted against pH (Robyt and Whelan, 1968). The pH range of stability of α-amylase has been observed to be between 4.0 and 11.0 (Bernfield, 1951; Buonocore *et al.*, 1976). Human saliva and porcine pancreas amylases were also reported to have optimum pH of between 6.0 and 7.0 (Fischer and Stein, 1960). *Bacillus licheniformis* CUMC 305 α-amylase showed maximal activity at pH 9.0 (Krishnan and Chandran, 1989).

α-amylases have been considered as either thermolabile or thermostable. The optimum temperature has been found to range between 27 and 55°C (Khoo *et al.*, 1994; Chang *et al.*, 1995; Egas *et al.*, 1995; Jeang *et al.*, 1995; Dong *et al.*, 1997; Aktypis *et al.*, 1998) while Ivanova *et al.* (1993) reported an optimum temperature of 90°C for *B. licheniformis* 44MB82A strain. *B. licheniformis* CUMC 305 (Krishnan and Chandran, 1989). Thermostable α-amylases have been found to require calcium ion as a protection against high temperature (Violet and Meunier, 1989; Savchenko *et al.*, 2002). Although, the cation is not an integral part of the active site of the enzyme, Ca^{2+} is believed to stabilize the overall conformation of the enzyme (Robyt, 1984).

The study on the effect of some cations and reagents showed inhibition (Table 3) by Cu^{2+}, EDTA, Zn^{2+}, Ba^{2+}, Sn^{2+}, Mn^{2+} and acetamide. This is in agreement with the work of Krishnan and Chandra (1989) and Vihinen and Mantsala (1990), Jeang *et al.* (1995), who reported that Cu^{2+}, EDTA, Zn^{2+} are inhibitors of α-amylases. Recently, Ramachandaran *et al.* (2004) reported a considerable inhibition of a α-amylase by Mn^{2+} from a fungus. Cu^{2+}, Zn^{2+}, Cd^{2+}, Hg^{2+} and Pb^{2+} are inhibitory to the enzyme from *B. stearothermophilus* (Vihinen and Mantsala, 1990). Other inhibitory compounds are dinitrofluorobenzene (DNFB) and EDTA (Jeang *et al.*, 1995). On the other hand, the activity of the enzyme increased significantly with Cd^{2+}, Co^{2+}, Mg^{2+}, $Ni^{2+}$and Fe^{3+}. Saboury (2002) had reported that cobalt activates α-amylases. However, a low inhibition at a high concentration of Co^{2+} had been reported for α-amylase from *Salvadora oleides* fruit (Memon *et al.*, 1987; Witt and Sauter, 1996). Generally, α-amylases have long been observed to be ion dependent. They require calcium ions (Ca^{2+}) or sodium ions (Na^{+}) (Vihinen and Mantsala, 1990; Stefanova and Emanuilova, 1992; Feller *et al.*, 1992; Egas *et al.*, 1998) and rarely magnesium ion (Mg^{2+}) (Adeleye, 1990) for thermostability. The Ca^{2+} is required to maintain structural integrity of α-amylase (Violet and Meunier, 1989). The removal of calcium leads to decreased thermostability and/or decreased enzymatic activity or susceptibility to proteolytic degradation (Machius *et al.*, 1995).

The major hydrolysis product was maltose in accordance with the mechanism outlined by Robyt and French (1970).

In conclusion, the occurrence of this enzyme at high specific activity as well as its apparent thermostalibity makes it a target in our novel approach to exploit the organism further, through mutagenesis, in our quest to develop locally a highly active, thermostable and alcohol tolerant α-amylase for the emerging cassava-ethanol industry in our country. Moreover, the hydrolysis products are also useful as sweeteners in food and pharmaceutical industries.

REFERENCES

Adeleye, A.I., 1990. Production and Control of extracellular α-amylase in *Micrococcus varian*. J. Basic Microbiol., 30: 723-727.

Aktypis, A., G. Kalantzopulous, H.H. Huis-an't-veld and B. Ten-Brink, 1998. Purification and Characterization of thermophilin T, a novel bacterium produced by *Streptococcus thermophilus* ACA-DC 0040. J. Applied Microbiol., 84: 568-576.

Andrews, P., 1964. Estimation of molecular weights of proteins by Sephadex gel filtration. Biochem. J., 91: 222-233.

Andrews, P., 1965. The gel filtration behaviour of proteins related to their molecular weights over a wide range. Biochem. J., 96: 595-606.

Bernfield, P., 1951. Enzymes of starch degradation and synthesis. Adv. Enzymol., XII: 379-428.

Boyer, E.N., M.B. Ingle and G.D. Mercer, 1979. Isolation and Characterization of unusual bacterial amylases. Starke, 31: 166-171.

Bradford, M.M., 1976. A rapid and sensitive method for the quantification of microgram quantities of proteins utilizing the principle of protein-dye binding. Anal. Biochem., 72: 248-254.

Brumm, P.J., R.E. Hebeda and W.N. Teaque, 1988. Purification and Properties of a new commercial thermostable *Bacillus stearothermophilus* alpha amylase. Biotechnol. Lett., 11: 541-544.

Buchanan, R.E. and N.E. Gibbons, 1974. Bergey's Manual of Determinative Bacteriology. 8th Edn., Williams and Wilkins.

Buonocore, V.C., M. Caparole, D. Rosa and A. Gambocorta, 1976. Stable inducible thermoacidophilic α-amylase from *B. acidocaldarius*. J. Bacteriol., 128: 515-521.

Campbell, L.L., 1955. Purification and some properties of a α-amylase from a facultative thermophilic bacteria. Arch. Biochem. Biophys., 54: 154-161.

Chang, C.I., M.S. Tang and C.F. Lin, 1995. Purification and Properties of α-amylase from *Aspergillus oryzae* ATCC 76080. Biochem. Mol. Biol. Int., 36: 185-200.

Collins, C.H., P.M. Lyne, J.M. Grange and J.O. Falkinham, 2004. Collins and Lyne`s Microbiological Methods. 8th Edn., Arnold Publishers.

Dong, G., C. Vieille, A. Savchenko and J.G. Zeikus, 1997. Cloning Sequencing and expression of the gene encoding extracellular α-amylase from *Pyrococcus furiosus* and biochemical Characterization of the recombinant enzyme. Applied Environ. Microbiol., 63: 3569-3576.

Egas, M.C., M.S. Da Costa, D.A. Cowan and E.M. Pires, 1998. Extracellular alpha-amylase from *Thermus filiformis* Ork A_2: Purification and biochemical characterization. Extremop., 2: 23-32.

Feller, G., C. Lonhienne, C. Deroanne, J. Libioulle, J. Van Beeumen and C. Gerday, 1992. Purification, characterization and nucleotide sequence of the thermolabile alpha-amylase from the Antarctic psychrotroph *Alteromonas haloplanctis* A23. J. Biol. Chem., 267: 5217-5221.

Fischer, E.H. and E.A. Stein, 1960. α-amylases in The Enzymes, Vol. 4. Boyer, P.D., H. Lardy and K. Myrback (Eds.), Academic Press, London, pp: 313-314.

Forgaty, W.M. and C.T. Kelly, 1979. Starch-degrading enzymes of microbial origin. Prog. Ind. Microbiol., 15: 87-150.

Forgaty, W.M. and C.T. Kelly, 1980. Amylases, Amyloglucosidases and Related Substance. In: Economic Microbiology, Microbial Enzymes and Bioconversion, Vol. 5. Rose, A.H. (Ed.), Academic Press, New York, pp: 115-170.

Giraud, E., L. Gosselin, B. Marin, J.L. Parada and M. Raimbault, 1993. Purification and Characterization of an extracellular amylase from *Lactobacillus plantarum* strain A6. J. Applied Bacteriol., 75: 276-282.

Hagenimana, V., L.P. Vezina and R.E. Simard, 1992. Distribution of amylase within sweet potato (*Ipomea batatas* L.) root tissue. J. Agric. Food Chem., 40: 1777-1783.

Harrigan, W.F. and M.E. McCance, 1976. Laboratory Methods in Food and Dairy Microbiology, Academic Press, London.

Ivanova, V.N., E.P. Dobreva and E.L. Emanuilova, 1993. Purification and characterization of a thermostable alpha-amylase from *Bacillus licheniformis*. J. Biotechnol., 28: 277-289.

Ivor, S. and G. Feinberg, 1965. Paper and Thin Layer Chromatography and Electrophoresis. A Teaching Level Manual. 2nd Edn., Shandon Scientific Co. Ltd., pp: 62-65.

Jeang, C.L., Y.H. Lee and L.W. Chang, 1995. Purification and Characterization of a raw-starch digesting amylase from a soil bacterium *Cytophaga* spp. Biochem. Mol. Biol. Int., 35: 549-551.

Khoo, S.L., A.A. Amirul, M. Kamaruzanaz, N. Nazulun and M.N. Azizan, 1994. Purification and characterization of α-amylase from *Aspergillus flavus*. Folia Microbiol., 39: 392-398.

Koivula, T.T., H. Hemila, R. Pakkanen and M. Sibakov, 1993. Cloning and sequencing of a gene encoding acidophilic amylase from *Bacillus acidocaldarius*. J. Gen. Microbiol., 139: 2399-2407.

Krishnan, T. and A.K. Chandra, 1989. Purification and characterization of α-amylase from *Bacillus licheniformis* CUMC 305. Applied Environ. Microbiol., 46: 430-437.

Machius, M., G. Wiegand and R. Huber, 1995. Crystal structure of calcium-depleted *Bacillus licheniformis* α-amylase 2.2-Å resolution. J. Mol. Biol., 246: 545-559.

Manning, G.B., L.L. Campbell and R.J. Foster, 1961. Thermostable α-amylase from *Bacillus stearothermophilus*. J. Biol. Chem., 236: 2958-2961.

Medda, S. and A.K. Chandra, 1980. New strains of *Bacillus licheniformis* and *Bacillus coagulans* producing thermostable alpha-amylase active at alkaline pH. J. Applied Bacteriol., 48: 47-58.

Memon, A.N., A.R. Memon and M.V. Dahot, 1987. α-amylase activity in *Salvadora oleides* fruit. J. Pure Applied Sci., 6: 5-9.

Nanmori, T., R. Shinke, K. Aoki and H. Nishira, 1983. Purification and Characterization of β-amylase from *Bacillus cereus* BQ10-SI Sp 0II. Agric. Biol. Chem., 47: 941-947.

Nelson, N., 1944. A photometric adaptation of the Somogyi method for the determination of glucose. J. Biol. Chem., 153: 375-380.

Ramachandaran, S., A.K. Patel, K.S. Nampoothiri, S. Chandran, G. Szakas, R.C. Soccol and A. Pandey, 2004. α-amylase from a fungal culture grown on oil cakes and its properties. Brazil. Arch. Biol. Technol., 47: 1-13.

Robyt, J.F., 1984. Enzymes in the Hydrolysis and Synthesis of Starch. In: Starch Chemistry and Technology. 2nd Edn., Vol. 4. Whistler, R.L., J.N. Be Miller and E.F. Paschall (Eds.), pp: 88-90.

Robyt, J.F. and D. French, 1967. Multiple attack hypothesis of α-amylase action: Action of porcine pancreatin, human salivary and *Aspergillus oryzae* α-amylases. Arch. Biochem. Biophys., 122: 8-16.

Robyt, J.F. and W.J. Whelan, 1968. Starch and its Derivatives. 4th Edn., Vol. 13, Radley, J.A. (Ed.), Chapman and Hall, London, pp: 423-477.

Saboury, A.A., 2002. Stability, activity and binding properties study of α-amylase upon interaction with Ca^{2+} and Co^{2+}. Biologia Bratislava, 57 suppl., 11: 221-228.

Sadhukham, R., S.K. Roy, S.S. Raha, S. Manna and S.L. Chakrabarty, 1992. Induction and regulation of α-amylase synthesis in a cellulolytic thermophilic fungus *Myceliophthora thermophilia* D14 (ATCC 48104). India J. Exp. Biol., 30: 482-486.

Saito, N., 1973. A thermophilic extracellular amylase from *Bacillus licheniformis*. Arch. Biochem. Biophys., 155: 296-298.

Savchenko, A., C. Vielle, S. Kang and G. Zeikus, 2002. *Pyrococcus furiosus* α-amylase stabilized by calcium and zinc. Biochemistry, 41: 6193-6201.

Segel, I.H., 1975. Biochemical Calculations: Multisite and Allosteric Enzymes. John Wiley and Sons, New York, pp: 303-311.

Seigner, C., E. Prodanov and G. Machius-Mouren, 1985. In: Porcine pancreatic α-amylase action: Kinetic evidence for the binding of two malto-oligosaccharide molecules. Eur. J. Biochem., 148: 161-162.

Sen, S. and S.L. Chakrabarty, 1987. Amylase from *Lactobacillus cellobiosus* isolated from vegetable wastes. Enz. Microbiol. Technol., 9: 112-115.

Somogyi, M., 1945. A new reagent for the determination of sugars. J. Biol. Chem., 160: 61-68.

Srivastava, R.A.K. and J.N. Baruah, 1986. Culture conditions for production of thermostable amylase by *Bacillus stearothermophilus*. Applied Environ. Microbiol., 52: 179-184.

Stefanova, M. and E. Emanuilova, 1992. Characterization of a thermostable α-amylase from *Bacillus brevis*. Eur. J. Biochem., 207: 345-349.

Takasaki, Y., 1985. An amylase producing maltotriose from *Bacillus subtilis*. Agric. Biol. Chem., 49: 1091-1097.

Tester, R.F., J. Karkalas and X. Qi, 2004. Starch structure and digestibility enzyme-substrate relationship. World's Poult. Sci. J., 60: 190-195.

Vihinen, M. and P. Mantsala, 1990. Characterization of thermostable *Bacillus stearothemophilus* α-amylase. Biotechnol. Applied Biochem., 12: 427-435.

Violet, M. and J.C. Meunier, 1989. Kinetic study of the thermal denaturation of *Bacillus licheniformis* α-amylase. Biochem. J., 263: 665-670.

Whitaker, J.R., 1972. Principles of Enzymology for the Food Sciences. Marcell Dekker. Inc., pp: 442-454.

Witt, W. and J.J. Sauter, 1996. Purification and characterization of α-amylase from poplar leaves. Phytochemistry, 41: 365-372.

Yamamoto, M., Y. Tamaka and K. Kirokoshi, 1972. Alkaline amylase of alkalophilic bacteria. Agric. Biol. Chem., 36: 1819-1823.