ACTA SCIENTIARUM POLONORUM
Acta Sci. Pol. Technol. Aliment. 14(1) 2015, 71–75
www.food.actapol.net pISSN 1644-0730 eISSN 1889-9594 DOI: 10.17306/J.AFS.2015.1.8

STUDIES ON THE PRODUCTION OF ALKALINE α-AMYLASE FROM *BACILLUS SUBTILIS* CB-18

Ogbonnaya Nwokoro✉, Odiase Anthonia

Industrial Microbiology and Biotechnology Laboratory, Department of Microbiology, University of Nigeria, Nsukka, **Nigeria**

ABSTRACT

Background. Amylases are among the main enzymes used in food and other industries. They hydrolyse starch molecules into polymers composing glucose units. Amylases have potential applications in a number of industrial processes including foods and pharmaceutical industries. Alkaline α-amylase has the potential of hydrolysing starch under alkaline pH and is useful in the starch and textile industries and as an ingredient of detergents. Amylases are produced from plants, however, microbial production processes have dominated applications in the industries. Optimization of microbial production processes can result in improved enzyme yields.

Material and methods. Amylase activity was assayed by incubating the enzyme solution (0.5 ml) with 1% soluble starch (0.5 ml) in 0.1 M Tris/HCl buffer (pH 8.5). After 30 minutes, the reaction was stopped by the addition of 4 mL of 3,5-dinitrosalicylic acid (DNS) reagent then heated for 10 min in boiling water bath and cooled in a refrigerator. Absorbance readings were used to estimate the units of enzyme activity from glucose standard curve. Hydrolysed native starches from cassava, rice, corn, coco yam, maize and potato and soluble starch were adjusted to pH 8.5 prior to incubation with crude enzyme solution. Reducing sugars produced were therefore determined. The effect of pH on enzyme activity of the alkaline α-amylase was determined by using buffer solutions of different pH (potassium phosphate buffer, 6.0–7.0; Tris-HCl buffer 7.5 to 9.0 and carbonate/bicarbonate buffer, pH 9.5–11) for enzyme assay. The pH stability profile of the enzyme was determined by incubating 0.5 ml of α-amylase enzyme in 0.1 M Tris/HCl buffer (pH 8.5) and 0.5 ml of 1% (w/v) soluble starch (Merck) in 0.1 M Tris/HCl buffer (pH 8.5) for 3 h in various buffers. The effect of temperature on enzyme activity was studied by incubating 0.5 mL of the enzyme solution contained in the test tube and 0.5 mL of 1% soluble starch (Merck) solution prepared in 0.1 M Tris/HCl buffer (pH 8.5) for 3 h at various temperatures (25, 30, 35, 40, 45, 50, 55 and 60°C) in a thermo static water bath. The reactions were stopped by adding DNS reagent. The enzyme activity was therefore determined. Thermal stability was studied by incubating 0.5 ml of enzyme solution in 0.1 M Tris/HCl buffer (pH 8.5) and 0.5 ml of 1% (w/v) soluble starch (Merck) in 0.1 M Tris/HCl buffer (pH 8.5) for 3 h at various temperatures (20, 30, 40, 50, 60 and 70°C) for 60 min.

Results. The enzyme displayed optimal activity at pH 8.0 at which it produced maximum specific activity of 34.3 units/mg protein. Maximum stability was at pH 8.0 to 9.0. Maximum activity was observed at temperature of 50°C while thermo stability of the enzyme was observed at 40–50°C. The enzyme displayed a wide range of activities on starch and caused the release of 5.86, 4.75, 5.98, 3.44, 3.96, 8.84 mg/mL reducing sugar from cassava, potato, cocoyam, corn, rice and soluble starch respectively.

Conclusion. This investigation reports some biochemical characterization of alkaline α-amylase from *Bacillus subtilis* CB-18. The substrate specificities of this enzyme on various starches suggested that the alkaline α-amylase enzyme had combined activities on raw and soluble starch.

Key words: alkaline α-amylase, starch hydrolysis, enzyme activity

✉ogb883@yahoo.com, phone +23 48034402414

INTRODUCTION

Starch is the major carbohydrate reserve polymer in maize, wheat, oat, rice, potato, cassava etc. and a potential substrate for the production of sugars and chemicals (Sun et al., 2010). Amylases are a class of industrial enzymes having approximately 30% of the world's enzyme production (Van dar Maarel et al., 2002). Alpha amylases are enzymes that breakdown starch by cleaving internal α-1,4-glucosidic linkages to produce different sizes of oligosacharides. This enzyme has a variety of applications in the food, textile, paper, pharmaceutical, sugar and detergent industries (Gupta et al., 2003). Most amylases of microbial origin have pH optima in the acidic or neutral range which is not good because of the alkalinity of detergents.

Alkaliphiles are a group of extremophilic organisms that thrive at alkaline pH and many of their products, particularly enzymes, have found widespread applications in the industries particularly in detergent and laundry industries. Enzymes from alkaliphiles are stable in detergents due to their inherent tolerance to high pH (Hagihara et al., 2001).

Several species of microorganisms including *Bacillus* species (Saxena et al*.,* 2007; Yang et al., 2011) have been isolated from various alkaline environments and these microorganisms produced large amounts of extracellular α-amylases.

Alkaline α-amylase has high catalytic efficiency and stability at alkaline pH ranging from 9 to 11 (Burhan et al., 2003) and have potential applications to hydrolyse starch under high pH conditions in the starch and textile industries and as ingredients in detergents (Murakami et al., 2007). This present investigation deals with the isolation of *Bacillus subtilis* CB-18 and some properties of its α-amylase.

MATERIAL AND METHODS

Starches. Native starches from cassava, rice, corn, coco yam, maize and potato were prepared according to the method of Obi and Odibo (1984). Briefly, cassava, yam and potato tubers were peeled and washed. These samples, together with rice, corn and maize were separately soaked in water and later reduced to pulp using a hand grater. The pulps were separately homogenised in a blender. From each homogenate, contained in a bag of fine white cloth, starch was leached into a glass vat by churning with excess water. The crude starch suspension was allowed to settle overnight in the vat after which the sediment was separated from the supernatant by decantation and dried at 50°C for 48 h. The resultant flakes were ground to a fine powder and used as native starches.

Medium. The medium for microbial cultivation designated Medium A contained the following: 2% soluble starch (Merck, Darmstadt); 0.5% peptone (Oxoid), 0.2% Na_2HPO_4 and 0.1% KH_2PO_4. The final pH was adjusted to 8.5 using 0.2 M NaOH. The medium was sterilised at 121°C for 15 minutes. The inoculum was grown for 24 h in a Gallenkamp orbital incubator at 100 × g at 30°C. The cells were collected by centrifugation using Gallenkamp Junior centrifuge at 2515 × g for 15 minutes, washed twice with 0.1 M Tris/HCl buffer (pH 8.5).

Microorganism. *Bacillus subtilis* CB-18 was isolated and selected as previously described by Nwokoro and Odiase (2012).

Hydrolysis of starch. Starch was adjusted to pH 8.5 with 0.2 M NaOH and incubated in a gyrator shaker (Fisher Rotor Rack Model 343) with enzyme solution in 0.1 M Tris/HCl buffer (pH 8.5). Samples were withdrawn from the reaction mixture after 24 h and centrifuged at 2515 × g using a Gallenkamp Junior centrifuge for 15 min. The reducing sugars in the supernatant were assayed by the 3,5-dinitrosalicilic acid (DNS) method of Miller (1959).

Enzyme assay. Amylase activity was assayed by incubating the enzyme solution (0.5 ml) with 1% soluble starch (0.5 ml) in 0.1 M Tris/HCl buffer (pH 8.5). After 30 minutes, the reaction was stopped by the addition of 4 ml DNS reagent then heated for 10 min in boiling water bath and cooled in a refrigerator. Absorbance readings were used to estimate the units of enzyme activity from glucose standard curve. One unit of activity was defined as the amount of enzyme that released 1 µg of glucose from starch per minute under the assay condition.

Partial purification of enzyme. Enzyme supernatant fluid was brought to 45% saturation with $(NH_4)_2SO_4$ and was recovered by centrifugation at 2515 × g for 15 min. The supernatant was precipitated with cold acetone (30%) followed by centrifugation at 2515 × g for 10 minutes. Ammonium sulphate was further added to 65% saturation followed by centrifugation. The enzyme solution was dialysed against 0.1 M Tris//HCl buffer (pH 8.5).

The influence of pH on enzyme activity and stability

The effect of pH on activity of the alkaline α-amylase was determined by using buffer solutions of different pH (potassium phosphate buffer, 6.0–7.0; Tris-HCl buffer 7.5 to 9.0 and carbonate/bicarbonate buffer, pH 9.5–11) for enzyme assay. The buffers were used at a concentration of 0.1 Mol/L. The pH activity profile of the enzyme was determined by incubating 0.5 mL of the enzyme contained in test tubes with 0.5 mL of 1% (w/v) soluble starch (Merck) prepared in buffers of different pH values (6.0–11) at 50°C for 2 h. The reaction was terminated by adding DNS reagent and the enzyme activities were determined.

The pH stability profile of the enzyme was determined by incubating 0.5 ml of enzyme solution in 0.1 M Tris/HCl buffer (pH 8.5) and 0.5 ml of 1% (w/v) soluble starch (Merck) in 0.1 M Tris/HCl buffer (pH 8.5) for 3 h in various buffers. The reaction was terminated by the addition of DNS reagent and reducing sugar concentration was assayed at 30°C.

Influence of temperature on enzyme activity and stability

The influence of temperature on enzyme activity was studied by incubating 0.5 ml of the enzyme solution contained in test tube and 0.5 mL of 1% soluble starch (Merck) solution prepared in 0.1 M Tris/HCl buffer (pH 8.5) for 3 h at various temperatures (25, 30, 35, 40, 45, 50, 55 and 60°C) in a thermo static water bath (Kottermann, Bremen, Germany). The reactions were stopped by adding DNS reagent. The enzyme activity was therefore determined.

Thermal stability was studied by incubating 0.5 ml of enzyme solution in 0.1 M Tris/HCl buffer (pH 8.5) and 0.5 ml of 1% (w/v) soluble starch (Merck) in 0.1 M Tris/HCl buffer (pH 8.5) at various temperatures (20, 30, 40, 50, 60 and 70°C) for 60 min. The reaction was terminated by the addition of DNS reagent and reducing sugar concentration was therefore determined at 30°C.

Assay procedures

The pH was determined using a glass electrode pH meter (PYE Unicam, England). Protein content was estimated by the method of Lowry et al. (1951) using bovine serum albumin (Sigma-Aldrich) as a standard. Reducing sugar was determined by the dinitrosalicylic acid (DNS) method of Miller (1959) using 50–200 µg glucose as standard.

RESULTS AND DISCUSSION

The result of the hydrolysis of various starches by the alkaline α-amylase enzyme is shown in Table 1. The reaction mixture consisted of crude enzyme solution in 0.1 M Tris/HCl buffer (pH 8.5) and starchy substrates. Hydrolysis was carried out for 24 h at 30 ±2°C. The enzyme produced 8.84 mg/ml reducing sugar from soluble starch after 24 h. Release of reducing sugar by the enzyme was lowest with corn starch (3.44 mg/ml).

Specific enzyme activity gradually increased from pH 6.0 to 8.0 and highest activity of 34.3 units/mg protein was observed at pH 8.0 (Fig. 1). Above pH 8.5, there was a decrease in enzyme activity while at pH 11 only 27% of the maximum activity was detected. The alkaline α-amylase from *Bacillus subtilis* CB-18 retained about 100% of its original activity at pH 8.0–9.0 and about 88% of its original activity

Table 1. Release of reducing sugars from starchy substrates by alkaline α-amylase from *Bacillus subtilis* CB-18

Starch	Reducing sugar, mg/mL
Cassava	5.86
Potato	4.75
Cocoyam	5.98
Corn	3.44
Rice	3.96
Soluble starch	8.84

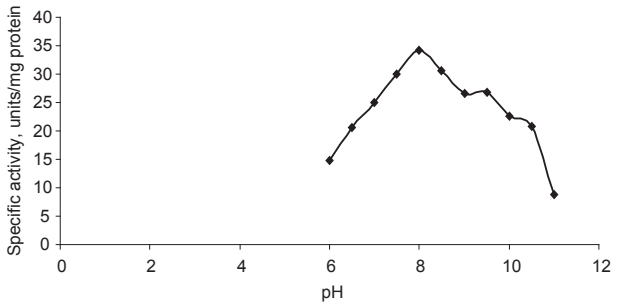

Fig. 1. Effect of pH on enzyme activity

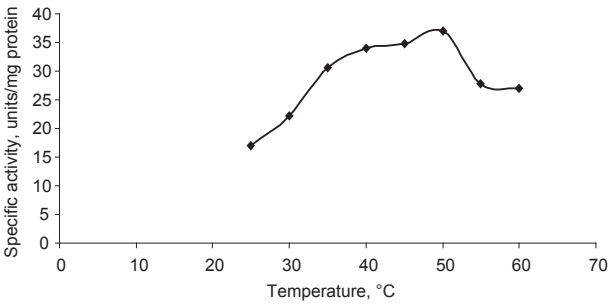

Fig. 3. Effect of temperature on enzyme activity

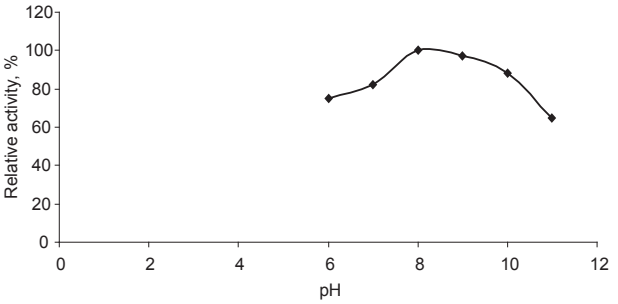

Fig. 2. Effect of pH on enzyme stability

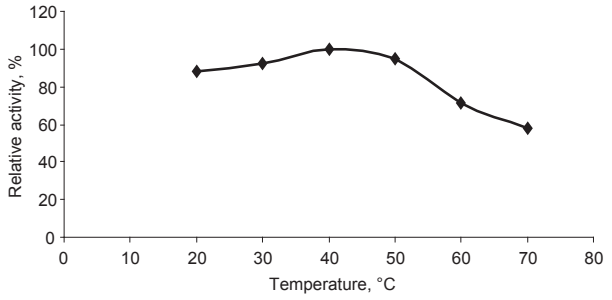

Fig. 4. Effect of temperature on enzyme stability

at pH 10.0. At higher pH values, there was a gradual decrease in enzyme stability (Fig. 2). The pH values of media used for alkaline α-amylase production were 9.0 (Lin et al., 1996; Burhan et al., 2003); 8.0 (Shanmugapriya, 2009); 10.5 (Horikoshi, 1971) and 11.0 (Arikan, 2008). Alkaline α-amylases are stable at pHs 6–13 (Kim et al., 1996); 8–8.5 (Igarashi et al., 1998); 7–11 (Burhan et al., 2003) and 8.5–11 (Dastager et al., 2009).

The optimum temperature for alkaline α-amylase was 50°C at which the enzyme specific activity of 37 units/mg protein was produced. The enzyme showed an increase in activity up to a temperature of 50°C but showed a gradual reduction in its activity as the temperature was increased beyond 50°C (Fig. 3). The specific enzyme activity was reduced to 73% of its maximum at 60°C. Maximum enzyme stability was at 40–50°C but activity was lowest at 70°C at which only 58% of the original activity was observed (Fig. 4). Temperatures used for α-amylase production were 55°C (Horikoshi, 1971; Igarashi et al., 1998); 50°C (Boyer and Ingle, 1972); 60°C (Kim et al., 1996) and 40°C (Zhao et al., 2008). Temperature stabilities for α-amylases were at 55–60°C (Hagihara et al., 2001); 75–80°C (Mamo and Gessesse, 1999) and 50–60°C (Murakami et al., 2007).

CONCLUSION

This work reports some biochemical studies on an alkaline α-amylase from *Bacillus subtilis* CB-18. The enzyme showed its best activity at pH 8.0 and best stability at pH of 8.0–9.0. Best enzyme activity was observed at 50°C and temperature stability in the range of 40–50°C. The enzyme displayed a broad spectrum activity in the hydrolysis of raw and soluble starches and may be useful for some biotechnological applications.

REFERENCES

Arikan, B. (2008). Highly thermostable, thermophilic, alkaline SDS and chelator resistant amylase from a thermophilic *Bacillus* sp. isolate A3-15. Biores. Technol., 99(8), 3071–3076.

Boyer, E. W., Ingle, M. B. (1972). Extracellular alkaline amylase from *Bacillus* species. J. Bacteriol., 110, 992–1000.

Burhan, A., Nisa, U., Khan, G., Gokhan, C., Ashabil, A., Osman, G. (2003). Enzymatic properties of a novel thermostable, thermophilic alkaline and chelator resistant amylase from an alkaliphilic *Bacillus* sp. isolate ANT-6. Process Biochem., 38, 1397–1403.

Dastager, G. S., Agasar, D., Pandey, A. (2009). Production and partial purification of α-amylase from a novel isolate *Streptomyces gulbargensis*. J. Ind. Microbiol. Biotechnol., 36, 189–194.

Gupta, R., Gigras, P., Mohapatra, H., Goswami, V. K., Chauhan, B. (2003). Microbial α-amylases: a biotechnological perspective. Process Biochem., 381, 599–1616.

Hagihara, H., Igarashi, K., Hayashi, Y., Endo, K., Ikawa--Kitayama, K., Ozaki, K., Kawai, S., Ito, S. (2001). Novel alpha amylase that is highly resistant to chelating reagents and chemical oxidants from alkaliphilic *Bacillus* isolate KSM-K38. Appl. Environ. Microbiol., 67, 1744–1750.

Horikoshi, K., 1971. Production of alkaline enzymes by alkalophilic microorganisms 11. Alkaline amylase produced by *Bacillus* No A-40-2. Agric. Biol. Chem., 35, 1783–1791.

Igarashi, K., Hatada, Y. Hagihara, H., Saeki, K, Takaiwa, M., Uemura, T. (1998). Enzymatic properties of a novel liquefying α-amylase from an alkaliphilic *Bacillus* isolate and entire nucleotide and amino acid sequence. Appl. Environ. Microbiol., 64, 3282–3289.

Kim, T. U., Gu, B. G., Jeong, J. Y., Byun, S. M., Shin, Y. C. (1996). Purification and characterization of a maltotetrose – forming alkaline α-amylase from an alkalophilic *Bacillus* strain GM8901. Appl. Environ. Microbiol., 61, 3105–3112.

Lin, L. L., Chyau, C. C., Hsu, W. H. (1996). Production and properties of a raw starch – degrading amylase from the thermophilic and alkaliphilic *Bacillus* sp. TS-23. Biotechnol. Appl. Biochem., 28, 61–68.

Lowry, O. H., Rosebrough, N. J., Farr, A. L., Randall, R. J. (1951). Protein measurement with folin-phenol reagent. J. Biochem., 193, 265–275.

Mamo, G., Gessesse, A. (1999). Purification and characterization of two raw – starch digesting thermostable α-amylases from a thermophilic *Bacillus.* Enz. Microbiol. Technol., 25, 433–438.

Miller, G. L. (1959). Use of dinitrosalicic acid reagent for determination of reducing sugar. Anal. Chem., 31, 426–428.

Murakami, S., Nishimato, H., Toyama, Y., Shimamoto, E., Takenaka, S., Kaulpiboon, J., Prousoontorn, M., Limpaseni, T., Pongsawasdi, P., Aoki, K. (2007). Purification and characterization of two alkaline, thermotolerant alphamylases from *Bacillus halodurans* 38C-2-I and expression of the cloned gene in *Escherichia coli*. Biosc. Biotechnol. Biochem., 71 (10), 2393–2401.

Nwokoro, O., Odiase, A. O. (2012). Influence of media composition on the production of alkaline α-amylase from *Bacillus subtilis* CB-18. Acta Sci. Pol. Technol. Aliment., 11(3), 231–238.

Obi, S. K. C., Odibo, J. F. C. (1984). Some properties of a highly thermostable α-amylase from *Thermoactinomyces* sp. Can. J. Microbiol., 30, 780–785.

Saxena, R. K., Dutt, K., Agarwal, L., Nayyar, P. (2007). A highly thermostable and alkaline amylase from *Bacillus* sp. PN5. Biores. Technol., 98, 260–265.

Shanmugapriya, S., Kiran, G. S., Selvin, J., Gandhimathi, R., Baskar, T. B., Manilal, A., Sujith, S. (2009). Optimization, production and partial characterization of an alkalophilic amylase produced by sponge associated marine bacterium *Halotacterium salinarum* MMDO47. Biotechnol. Bioproc. Eng., 14, 67–75.

Van der Maarel, M. J. E. C., Van der Veen, B., Uitdehaag, J. C. M., Leemhuis, H., Dijkhuizen, L. (2002). Properties and applications of starch-converting enzymes of the α-amylase family. J. Bacteriol., 94, 137–155.

Yang, H., Liu, L., Li, J., Du, G., Chen, J. (2011). Heterologous expression, biochemical characterization and overproduction of alkaline α-amylase from *Bacillus alcalophilus* in *Bacillus subtilis*. Microbial. Cell Fact., 10, 77–85.

Zhao, J., Lan, X., Su, J., Sun, L., Rahman, E. (2008). Isolation and identification of an alkaliphilic *Bacillus flexus* XJU-3 and analysis of its alkaline amylase. Wei Sheng Wu Xue Bao, 48(6), 750–756.

Received – Przyjęto: 12.06.2014

Accepted for print – Zaakceptowano do druku: 2.10.2014

For citation – Do cytowania

Nwokoro, O., Anthonia, O. (2015). Studies on the production of alkaline α-amylase from *Bacillus subtilis* CB-18. Acta Sci. Pol. Technol. Aliment., 14(1), 71–75. DOI: 10.17306/J.AFS.2015.1.8