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Production of Alpha-Amylase from Mixed Actinomyces Spp Cultured at Room Temperature Using Nelson's Colorimetric Method

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ABSTRACT

The present study aimed at highlighting the biochemical properties of pH, temperature and inhibitors of α-amylases from mixed Actinomyces species cultured at room temperature in a view to assess its industrial application. The enzyme activity was assayed using Nelson's colorimetric method. The crude enzyme extract showed optimum activity at pH 7 and at 30°C, but retains 60% of its activity at temperature range 40°C to 60°C. It was also found stable from the pH range of 4-6. Both EDTA and PMSF were strongly inhibitory to enzyme activity with that of PMSF being stronger. The inhibitory effect of EDTA and PMSF suggests that the enzyme is a serine protease and requires Ca^{2+} as cofactor for activity.

Key words: α-amylase, mixed actinomyces Spp, Nelson's colorimetric method, optimum activity, pH 7, 30°C, EDTA, PMSF

INTRODUCTION

The amylases is a family of enzymes that catalyse the hydrolysis of starch into sugars. The members of this family include α-amylase, β-amylase and γ-amylase. Alpha-amylase (EC 3.2.1.1) is alternatively called 1,4-α-D-glucan glucanohydrolase belong to a group of enzyme called transferases. It is a calcium metalloenzyme, that catalyses the transfer of carbohydrate radical to water thereby producing the hydrolysis of their substrate. They hydrolyse α, 1-4 glycosidic linkages found in polysaccharide such as amylose; producing maltotose and maltose and amylopectin; producing maltose, glucose and limited dextrin (Richard, 2002; Talammond *et al*., 2006). Its optimum pH is 6.7 to 7.0. β-amylase (EC 3.2.1.2) also known as 1,4-α-D-maltohydrolase, saccharogen amylase or glycogenase., acting from none reducing end, it cleaved off maltose from polysaccharide. β-amylase has its optimum pH between 4.0-5.0 (Rejzek *et al*., 2011). The γ-amylase (EC 3.2.1.3) is also known as Glucan 1,4-α-glucosidase, amyloglucosidase. It cleaves α (1,6) glycosidic bonds and last α (1,4) glycosidic bonds at the non-reducing end of amylose and amylopectin yielding glucose. It has a pH optimum of 3.0.

Many bacteria do secrete these amylases extracellular during and after fermentation of starch. Although there is some information on the thermostability of amylases especially α-amylase; a survey of the literature indicates that the actinomyces enzymes have not been adequately investigated. Kuo and Hartman (1966) reported the first isolation of thermostable α-amylase from *Thermoactinomyces vulgaris*, while Shimzu *et al*. (1978) reported 70°C as the temperature activity optimum for the enzyme of their strain of *Thermoactinomyces vulgaris*. Hidaka *et al*. (1974) found in *Streptomyces hygroscopicus* α-amylase capable of retaining 75% activity at 80°C.

Thermostable,α-amylase enzyme have wide applications in industrial processes such as brewing, baking, textiles, pharmaceuticals, starch processing and detergents (Rejzek *et al*., 2011). The process of enzymatic conversions of starch to either fructose in syrup manufacture or maltose in wort production involves action of α amylase on starch at temperature of 70°C and above of the amylases of the various strains; those of bacillus are highly thermostable and will be useful for possible industrial appreciation (Pandey *et al*., 2000; Obi and Odibo, 1984). Amylases are some of the most commonly used enzymes in the industrial enzyme sector and account for nearly 25% of the enzyme market (Sidhu *et al*., 1997; Blain, 1974).

The aim of the study therefore is to highlight the Biochemical properties of α-amylases from mixed actinomyces species cultured at room temperature in a view to assess its industrial application.

MATERIALS AND METHODS

Preparation of cassava starch: Some cassava were first peeled and reduced to pulp using hand grater subsequently the pulps as well as the grain (after soaking for 1 h in water) were separately homogenized in a warring blender (10 burst of 305 each) from each homogenate. Contained in a bag of fine white cloth, starch was leached into a glass not by chumming with excess water. The crude starch suspension in the aliquot was allowed to settle overnight, after which the sediment was separated from the supernatant by decantation and dried at 50°C the resultant flakes were ground to a fine powder and used as cassava starched.

Preparation and inoculation of media for isolation of actinomyces: Strains of actinomyces were isolated from soil on a medium containing the following otherwise known as Erogorov medium. One gram (gram per liter) of K_2HPO_4, 1 g of NaCl, 1 g of NH_2SO_4 1 g of $MgSO_4$, 10 g of cassava starch and 15 g of Agar-agar all in I litre of distilled water at pH 7.0. The plates were incubated at room temperature for 3 days after which strains considered as probable producers of α-amylase were selected by flooding the plate with I mMol iodine solution (Shinke *et al*., 1974), subsequently subjected to serial dilution of 1×10^5 with phosphate buffer to confirm the pure isolate.

Fermentation preparation of media for amylase production: The production medium otherwise known as mineral salt solution medium contains the following ingredients; 3 g of NaCl, 0.05 g of K_2HPO_4, 1 g of $(NH_4)_2SO_4$, 0.02 g of $MgSO_4.7H_2O$, 0.19 g $CaCl_2.2H_2O$, all in 1 L of distilled water pH7.0 then meat extract 0.2% (i.e. 0.69 g), 3 g of starch were all added into only 300 mL of the medium in a beaker.

The mineral salt solution was dispersed into each of four 25 mL Erlenmeyer flask and plunged with cotton wool. A loopful of the organism (actinomyces) was inoculated into each flask and the flasks incubated at room temperature for 6 days. This is called static culture. Thus, it requires periodical shaking vigorously so that the spores will be evenly distributed while growing.

Confirmation test for actinomycets species: This was done by gram staining and by microscopy.

Substrate

Standard solution: About 200 mg of pure anhydrous glucose is dissolved in water and diluted to 100 mL in volumetric flask (2 mg mL^{-1}) volumes of 0.2, 0.4, 0.6, 0.8 and 1.0 mL were placed in 5 different tubes. Standard solutions should contain 40, 80, 120, 160 and 200 mg mL^{-1}.

Sample solution: A starch solution is prepared by adding cold water slurry of soluble starch to boiling water. This is cooled and diluted to the correct volume to give 20 mg of starch per milliliter. The starch solution is then diluted 1:1 with a 0.2 mL of phosphate buffer at pH 7.0. And the solution heated to dissolve the starch.

Neleson's copper reagent; As reported by Nelson (1944): Read absorbance or O.D using green filter in a colorimeter at 520 nm.

Enzyme assay: Enzyme assay was done using Nelson (1944) while total protein estimation using Lowry *et al.* (1951) The absorbance is measured after 10 min with a green filter with a colorimeter or at 520 nm using spectrophotometer. A blank is prepared by using 1.0 mL of the substrate and 1.0 mL of water.

EFFECT OF SUBSTRATE CONCENTRATION

On enzyme activity: Varying concentrations of substrate concentration were made up to 1.0 mL with distilled water. (0.2, .04, 0.5, 0.8, 1.0) then 1.0 mL of enzyme solution is added and mixture incubated for 10 min at the end of which they were tested for activity using Nelson's colorimetric method Nelson (1944).

Effect of pH: One milliliter of the buffer (phosphate buffer using Na_2PO_4 and $NaHPO_4$) pH 4-8) of the above pH were incubated with One mL of enzyme reducing sugar solution using Nelson method (1 mL of enzyme+1 mL of substrate solution). Activity was determined at the end of the incubation time for preparation of buffer of various pH.

Effect of temperature: Two milliliter of enzyme abstract was mixed with 2 mL of substrate solution and incubated at 40, 50, 60, 70, 80°C. The mixtures were assayed for α-amylase activity using Nelson's calorimetric method.

Effect of Inhibitors:

- EDTA
- PMSF (phenylmelhylsulfonylfluoride)

One milliliter of each inhibitor was added to 1 mL of enzyme reducing sugar using Nelson's colorimetric method activity was tested.

Control is prepared by adding 1 mL of enzyme to 1.0 mL of substrate 1.0 mL of the mixture was used to assay for amylase activity. For blank, 1.0 mL of each inhibitor is added to 1.0 mL of water in separate tubes, respectively. One milliliter of each solution was added to 1.0 mL of substrate and Nelson's method of enzyme activity determination.

RESULTS AND DISCUSSION

Determination of enzyme activity: One unit of enzyme activity was defined as:

1 m mole of reducing sugar (glucose) produced per min
1 mole of glucose = 180 g
1 mole of glucose = 180×1000 mg = 180, 000 mg

1 mm of glucose = 180, 000
1 mm of glucose = 180,000/10^6 = 0.180 mg
One unit of enzyme activity is = 0.18 mg of glucose

DISCUSSION

The preliminary studies showed that the enzyme gave a clear zone of starch hydrolysis on starch agar plate following the iodine test. This is suggestive of the presence of amylases (Shinke *et al*., 1974).

In Fig. 1 , the optimum pH for the enzyme activity was observed around pH 7. However, the enzymes retained at least 61% of activity at pH 4-6. The observed pH profile is in consonance with characteristic single pH peaks shown by most amylases (Robyt and Whelan, 1968).

In contrast, however, with some other reports on the amylase group. Ramachandran *et al*. (1978) and Morgan and Priest (1981) had reported the possession of two pH optima for the thermostable α-amylase of *Aspergillus niger* and *B. licheniformis*, respectively. This phenomenon has been attributed to the presence of two or more active site per protein molecule each of which has a distinctive and separate optimum pH (Robyt and Whelan, 1968). In this study, the α-amylases from actinomyces cultured at room temperature showed maximum activity at 30°C and retained about 61% of activity at 40°C, respectively, as shown in Fig. 2.

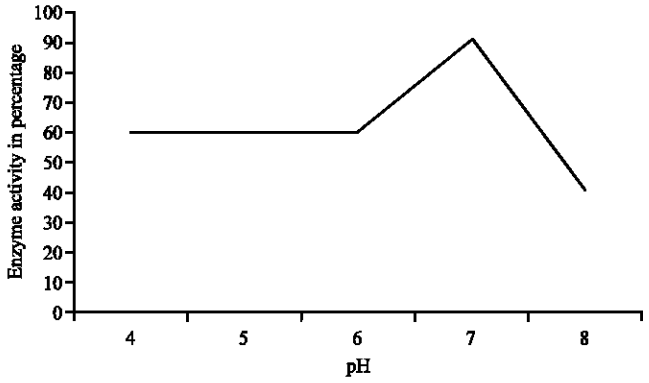

Fig. 1: Effect of pH on enzyme activity

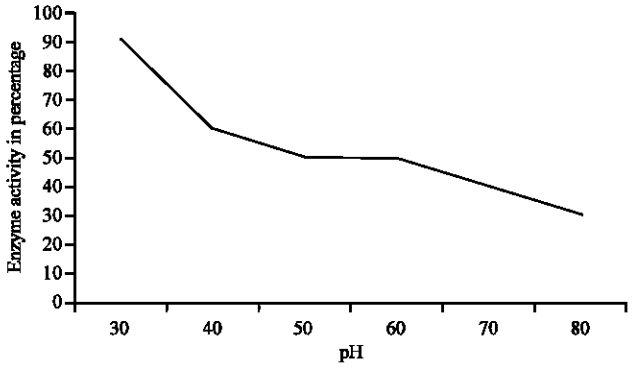

Fig. 2: Effect of temperature on enzyme activity

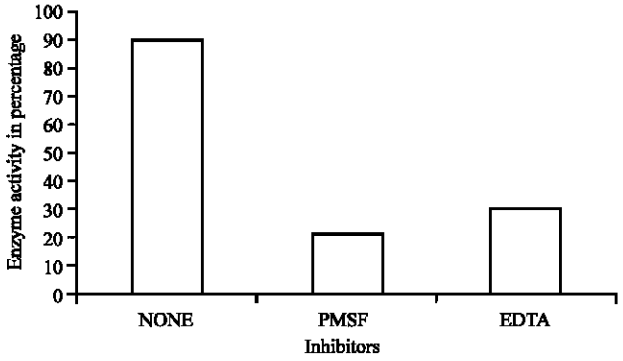

Fig. 3: Effect of inhibitors on enzyme activity

The temperature for maximum activity is lower than that reported for α-amylase of thermoactinomyces strain, (*Thermoactinomyces vulgaris* 70°C) by Shimzu *et al.* (1978) and for *Thermonospora curvata* (65°C by Glymph and Stutzenberger (1977). Similar work done by Amutha and Jaja (2011), showed optimum activity for α-amylase of *Bacillus substilis* at pH 6.0 and temperature of 40°C.

Two inhibitors used EDTA-ethylene diamine tetra acetic acid and PMSF-phenyl methyl sulphonyl floride were both strongly inhibitory to the enzyme. PMSF has a more inhibitory effect than EDTA as observed in Fig. 3. EDTA is known to chelates Ca^{2+} which is required as a cofactor for α-amylase. The denial of Ca^{2+} to the α-amylase makes the active sites of the enzyme less catalytically efficient, hence low activity. The PMSF is known to be a serine protease inhibitor, complexing specifically with the free OH group of serine on the active site to form an irreversible complex called enzyme-PMS complex. This complex occupies and blocks access of the substrate to the active site thereby leading to inhibition. Since the binding of PMSF is specifically to the serine at the active site to bring about inhibition, it means that serine present at active site of the enzymes and its free OH group is essential for catalysis Amutha and Jaja (2011).

CONCLUSION

The crude enzyme extract showed optimum activity at 30°C and reversion to thermophilic behavior at 60°C. The pH optimum for the enzyme was demonstrated at pH 7 but is acid stable between pH 4-6.

The inhibitory effect of EDTA and PMSF which is a serine protein inhibitor showed that ca^{2}+ is required as cofactor for enzyme activity since EDTA chelates ca^{2}+ thereby reducing enzyme activity and again the hydroxyl group of serine at the active site of the enzyme is required for catalysis since PMSF reacted with it and inhibited the enzyme.

The indication of the results is the possibility that actinomyces cultured at room temperature produces amylases capable of liquefying starch with hydroxyl group at room temperature and at thermophilic temperature at 60°C. This is a good exploitation for local industries where the cost of culturing the organism at high temperature is high due to unavailability of high temperature regulating instrumentation. A new channel has been opened for further research.

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