Amylase production by fungi isolated from Cassava processing site

F.S. Johnson*, A. K. Obeng and I. Asirifi

Department of Biotechnology, Faculty of Agriculture, University for Development Studies, P.O. Box 1350, Tamale, Ghana

ABSTRACT

Amylases are carbohydrases with greatest industrial applications. Twenty (20) fungal strains were isolated from the Aboam Cassava Processing dumpsite soil. Isolates AI020WE, AI017BN and AI009GN with largest clearing zone diameters of 59.75±0.035 mm, 57.75±1.177 mm and 56.25±0.35 mm respectively were evaluated. Submerged fermentation technique was employed for crude amylase and biomass production. There were significant differences (p<0.05) in growth rates between the three isolates. AI020WE recorded the highest growth rate of 5.38±0.21 mg/h followed by AI017BN and AI009GN with growth rate of 2.34±0.37 mg/h and 1.35±0.10 mg/h respectively on dry biomass weight basis. AI020WE, AI017BN and AI009GN attained their optima amylase activities of 4.49±0.398 U/ml, 2.86±0.30 U/ml and 4.51±0.20 U/ml at 60 °C, 55 °C and 65 °C respectively making them thermophilic. Isolates AI020WE and AI017BN attained optima amylase activity of 4.80±0.68 U/ml and 2.54±0.57 U/ml respectfully at pH 4 making them acidogenic. Isolates AI020WE, AI017BN and AI009GN attained their highest activities at the starch concentrations of 2%, 1.5% and 4% and fermentation period of 72 hours, 42 hours and 60 hours respectively. Isolate AI020WE achieved its highest amylase activity of 11.01±0.11 U/ml at 72 hours of incubation. AI017BN and AI009GN also showed their maximum amylase activity of 3.83±0.94 U/ml and 3.24±0.11 U/ml at the 42nd and 60th hours of incubation respectively. The result shows that the cassava processing dump site soil is a repository of amylolytic fungi and some of the isolates are of industrial interest.

Keywords: amylase activity, clearing zone diameter, fungi, isolate

INTRODUCTION

Amylases are group of enzymes that catalyze the hydrolysis of glycosidic linkage of starch and starch derivatives such as dextrans into glucose monomer [1]. Amylases are classified into three types namely α-amylase, β-amylase, and γ-amylase based on their catalytic mechanisms [2]. α-Amylase (EC 3.2.1.1), are enzymes that catalyses the hydrolysis of internal α-1,4-glycosidic linkages in starch into low molecular weight products, such glucose, maltose and maltotriose units [3]. β-amylase (EC 3.2.1.2,) catalysis the hydrolysis of α-1,4-glycosidic linkage of starch and β-anomeric maltose from the non reducing ends [4]. γ-Amylase (EC 3.2.1.3 ), hydrolysis α-1,6 glycosidic linkages, in addition to the last α-1,4 glycosidic linkages at the non reducing end of amylose and amylopectin, yielding glucose. Unlike the other forms of amylase, γ-amylase is most efficient in acidic environments and has an optimum pH of 3 [5].

Amylases have wide range of industrial applications. They are employed mainly in starch processing, brewing, baking, detergent and textile industries [6]. Amylases contribute approximately 25% of the world enzyme market [7]. The global market for industrial enzymes was valued at $3.9 billion in 2011. Business Communication
Company Report (2012) has projected that the global market for enzyme will grow at a compound annual rate of 9.1% and it will reach $6 billion by 2016.

Amylases are derived from several sources such as plants, animals, bacteria and fungi. Microbial sources especially fungal amylases are preferred due to their high accepted GRAS (Generally Recognized As Safe) status and their extracellular means of their enzyme production reference. The cost of amylase production is high and the cost of procurement by developing countries like Ghana can be even higher as a result of importation duties [8]. Therefore screening for efficient amylolytic fungal isolates for amylase production will significantly reduce production cost of industries which employ amylase and provide economic opportunities.

Cassava tubers are rich in starch: Amylopectin 83% and Amylose 17% [9]. Ghana currently produces about 12,260,000MT of cassava annually. Cassava tubers are processed traditionally into products such as “gari”, “agbelima” and “kokonte” [10]. Cassava has been processed at Aboam Cassava Processing Site located in the Kwahu East district in the Eastern Region of Ghana for over 30 years. In the process, cassava wastes and effluents are disposed at areas of this processing site. The objectives of this work was to isolate and screen for amylase-producing fungi from this cassava processing dumpsite soil and to determine the optimal conditions for their enzyme activity.

MATERIALS AND METHODS

Sample Collection
About 50 g each of top soil were sampled from 10 locations of cassava waste dumpsite and squeezing spot from Aboam cassava processing site located at Kwahu South district in the Eastern Region of Ghana (6°65N, 0°45W). They were put in sterile rubber envelopes and transported in ice pack to Laboratory. All the works were carried out under aseptic conditions. All chemicals used were of standard analytical grade.

Isolation and Screening for Amylase Activity
A selective media, starch-peptone agar consisting of the following chemicals in g/L (starch, 20; peptone, 10 streptomycin 0.05 and agar, 20) was used in isolating the amylolytic fungi. The soil samples were bulked together and 10 g was weighed into a beaker containing 90 ml of sterilized distilled water. The suspension was serially diluted and inoculated onto starch-peptone agar plate using spread plating method and incubated at 30 °C for 4 days. Conspicuous and distinct colonies were randomly sampled from the mixed cultures and subcultured to obtain pure cultures. The pure isolates were kept on starch peptone agar and stored in refrigerator at -4 °C.

A cock borer with diameter of 4 mm was used to transfer a 7 day old pure culture of each of the pure isolates and placed on the middle of replicate starch peptone agar plate and incubated at 30 °C. After 4 days of incubation, the isolates were flooded with Lugol’s iodine solution. The diameter of the clear zone formed around the fungal colonies were measured and taken to represent the amylolytic activity of the fungal isolates.

Production and Assay of Crude Amylase
Fermentation broth consisting of the following chemicals g/L (starch 20, peptone 2, NH4SO4 3, KH2PO4 1, MgSO4.7H2O 0.3 and CaCl2 0.3) was used. Tenmillilitres of sterile distilled water was poured onto a 7 days old sporulating slants cultures of AI020WE, AI017BN and AI009BN and agitated to obtain spore suspensions. Haemocytometer (MarienfeldNeubauer) was used to count the spores and determine the spore concentration of the suspension. Inoculum of 1.2 x 10^7 spores were used to inoculate100 ml of fermentation broth and incubated at 30 °C for 4 days in a water bath with shaker (Unitronic Orbital Selecta- J.P) at 100 rpm. Aliquot of 6 ml were sampled at regular interval of 6 hours and centrifuged at 10 000 rpm for 10 minutes at -4 °C in centrifuge (Centroli-H Selecta- J.P ). and supernatants stored at -20 °C for amylase analysis.

The enzyme assay was carried out as described by [11] with some modifications. To 0.1 ml of 1% starch solution in an assay test tube, 0.1 ml of crude enzyme extract was added and incubated for 5 minutes. After the incubation period, 2 ml of acidified iodine reagent (0.28g KI + 0.03g I2 in 500ml of water + 6mls of conc. HCl) was added to terminate the reaction. To the control tube 0.1 ml of the 1% starch solution was mixed with 0.1 ml of 0.02 M sodium phosphate buffer, pH 7. The control was also incubated for 5 minutes together with the assay tube after which 2ml of the acidified iodine solution was added. They were then cooled on ice for 5 minutes before measuring the absorbance at A590 after the UV spectrometer (model) was zeroed with distilled water. The amount of starch
NaOH buffer for pH 9 and 10 was used. A volume of 0.1 ml of crude enzyme solution was added to stop the reaction and the absorbance at A590nm was measured and recorded using spectrophotometer.

Optimization test
Optimum temperature for the enzyme activity was determined by incubating the enzyme-substrate reaction mixture of 0.1 ml of starch, and control containing 0.1 ml of starch and phosphate buffer solution at different temperatures of 35°C, 40°C, 45°C, 50°C, 55°C, 60°C, 65°C, 70°C, 75°C and 80°C for 5 minutes. A volume of 2 ml of the iodine solution was used to stop the reaction. The absorbance was measured at 590nm after it was cooled to room temperature.

One percent starch solutions of pH 3 to 10 were prepared: citrate phosphate buffer for pH 3 to 6, phosphate buffer for pH 7 and 8, and glycine-NaOH buffer for pH 9 and 10 was used. A volume of 0.1 ml of crude enzyme solution and 0.1 ml of phosphate buffer pH 7 was added to replicate assay and control tubes respectively containing 0.1 ml of starch solution each. The reaction was incubated for 5 minutes. A volume of 2 ml of acidified iodine solution was added to stop the reaction and the absorbance at A590nm was measured and recorded using spectrophotometer.

Varying concentrations of starch solutions of (0.5-5%) were prepared. To 0.1 ml of starch in a test tube, 0.1 ml of phosphate buffer was added and 0.1 ml of crude enzyme was added and 2 ml of acidified iodine solution was added and kept on ice for 5 minutes. The absorbance values at A405 nm were measured and recorded.

Estimation of growth rate
Aliquot of 6 ml were sampled from triplicate fermentation flasks of the isolates at regular interval of 6 hours and centrifuged at 10,000 rpm for 10 minutes at -4°C in centrifuge (Centrolit-II Selecta- J.P). The supernatants were decanted and the filtrates transferred onto Whatman No.1 filter papers. They were kept in hot air oven at 80°C for 2 hours, after which their dry biomass weights determined with electronic balance.

Experimental Design
Genstat discovery (4th edition) was used to compute the significance difference between isolates at a significance level of 5% and Microsoft excel was also used to compute the various graphs

RESULTS

Fig. 1: picture of clearing zone diameter of an isolate
Fig. 2: Clearing Zone Diameter of Fungi Isolates after 4 Days Incubation Period.

Fig. 3: Growth Rate of the Isolates over 96 hours of submerged fermentation at 30 °C, pH 7
Fig. 4: A Time Course of amylase activity over 96-hours incubation period at 30 °C and pH7.

Fig. 5: Effect of Temperature on Amylase Activity
Fig. 6: Effect of pH on Amylase Activity of the isolates.

Fig. 7: Effect of Substrate Concentration on Amylase Activity.
DISCUSSION

Isolation and Screening of Fungi
Twenty amylotic fungal isolates were picked at random from starch-peptone agar plates based on their distinct colony morphologies. There is a positive correlation between radial growth of fungal colony on media containing starch as sole carbon and energy source, and amylase activity. Since amylase is an inducible enzyme produced in presence of starch as sole carbohydrate and energy sources, amylase activity is to a large extent growth related. The twenty isolates were screened on starch-peptone agar, selective media was carried out to select the efficient amylotic isolates. Clearing zone diameter of the isolates showed significant difference (p<0.05). All the isolates exhibited amylase activity but isolates AI020WE, AI017BN and AI009GN exhibited the largest diameters of 59.75±0.04 mm, 57.75±1.18 mm and 56.25±0.35 mm respectively as illustrated in Fig 2. Cassava is a starchy root crop and it has been processed into products like “gari”, a local staple food, cassava dough and flour at Aboam cassava processing site located at Kwahu East District in the Eastern Region of Ghana for so many years where the soil samples were taken. For this reason, the soil at the site is enriched with starchy wastes making it a suitable repository of amylase producers. This result supports result report by [12].

Growth and Effect of Incubation Period, pH, Temperature and Substrate concentration on Amylase Activity
As indicated in Fig. 3, there were significant difference (p<0.05) in growth rate between the three isolates in submerged fermentation. AI020WE recorded the highest growth rate of 5.38±0.21mg/h during the 96 hours of incubation followed by AI017BN and AI009GN with growth rate of 2.34±0.37mg/h and 1.35±0.10 mg/h respectively on dry biomass weight basis. The composition and activity of amylase consortia as well as the sensitivity of the isolates to catabolite repression by glucose might account for the differences in growth rates observed. In submerged fermentation, if amylase composition and activities are kept constant, the physiological adaptation to fermentation factors such bulk oxygen transfer, uniformity of nutrient distribution by agitation, altering pH and excreted metabolites also significant affect microbial growth.

Effect of incubation period on amylase activity over 96 hours of submerged fermentation period is shown in Fig. 4. Isolate AI020WE achieved its highest amylase activity of 11.01±0.11U/ml at 72 hours of incubation. AI017BN and AI009GN also showed their maximum amylase activity of 3.83±0.94U/ml and 3.24±0.11U/ml at the 42nd and 60th hours of incubation respectively. Generally, there was an increase in amylolytic activity with incubation period up to the optimal after which activity decreased with incubation period. Activation of genes which codes for amylases might account for the increase whilst glucose repression and instability of the enzyme might cause the decreased with the incubation period. The optima activities of the isolates achieved at different incubation periods might be attributed to the intrinsic abilities of the isolates. With cost of commercial amylase production in mind, isolate(s) which achieve high amylase activity within a reasonably shorter incubation period is preferable. From this perspective, AI020WE is the best because though its maximal activity occurred at 72 hour, its activity is significantly higher (p<0.01) than maxima activity of the other isolates which achieved at relatively shorter incubation periods.

The influence of temperature on amylase activity of the crude amylase extract was studied. As seen in Figure 5, enzyme activity generally increased progressively with temperature up to the optima after which it begun to decrease with increased in temperature. AI020WE, AI017BN and AI009GN attained optima amylase activities of 4.49±0.40U/ml, 2.86±2.31U/ml and 4.51±0.20U/ml at 60 °C, 55 °C and 65 °C respectively showing that their amylases are thermostable. [13] also reported optimal temperature of 60 °C for amylase extracted from fungi isolates. Isolates AI020WE and AI017BN attained their optima amylase activity of 4.80±0.68 U/ml and 2.54±0.57 U/ml respectively at pH 4 making them acidogenic. As seen in Fig. 6, they began to decrease with increase in the pH after pH of 4. [14] reported of a low pH of 4.8 for amylase obtained from cassava processing waste soil. The optimal pH for AI009GN was at 7. [15] also observed a maximum fungal amylase production at pH 7 from bagasse. This result compares favourable to previous reports that amylases are generally stable over a wide range of pH from 4 to 11 [16] and [17].

As seen in Figure 7, there was a general increase in the amylase activity with increase in starch concentration until the optimum concentration was attained. AI020WE gave the highest amylase activity of 1.96±1.31 U/ml at 2% starch level. [18], reported on fungal amylase with optimal starch concentration of 2%. AI009GN recorded its optimum substrate concentration at 4% which is in support of the report by [19]. The amylase activity of isolate AI017BN reached a plateau after obtaining the maximum value 0.87±0.013 U/ml at 1.5% of starch.
CONCLUSION

From the results and discussions, it is concluded that the Aboam cassava processing dumpsite soil harbors amylotic fungi which have several industrial application. Isolates AI020WE, AI017BN and AI009GN studied had their optimal temperatures at 55 °C, 60 °C and 65 °C respectively making them thermoactive. AI020WE and AI017BN are acidotolerant fungi because they performed best at pH 4 and AI009GN at a neutral pH of 7.

Acknowledgements

We are grateful to Prof. A. K. Quainoo, Head of Department of Biotechnology Department and Vice-Dean of Faculty of Agriculture, and the entire Department of Biotechnology for providing chemicals and equipment. Our gratitude also go to the technical staff of Spanish Laboratory at University for Development Studies, Nyankpala Campus.

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