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Full Length Research Paper

Amylase activity of a yellow pigmented bacterium isolated from cassava waste

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This study investigated the amylase activity of a yellow pigmented bacterium isolated from cassava wastes obtained from a dumpsite near a gari processing factory in Ibadan, Nigeria. Isolate was grown in nutrient broth containing 1% starch and then centrifuged at 5,000 rpm. Amylase activity was assayed using the DNSA method for the detection of reducing sugar. Best enzyme activity was recorded at 30ºC and pH 7.5. The amylase was found to be stable after exposure to temperatures between 20 and 40ºC and pH 6.0 to 9.0, outside which there was a decline in activity. The ability of the isolate to produce amylase explains its colonization of a cassava waste dumpsite. Conditions of activity and stability also agree with the growth condition of the isolate.

Key words: Cassava waste, yellow pigmented bacterium, amylase.

INTRODUCTION

Cassava is a rich source of carbohydrate in form of starch. In Nigeria, it is processed into such foods as gari, fufu, lafun and 'starch' (Ketiku et al., 1978). Cassava wastes generated during processing include the peels which are often disposed on open dumpsites near local processing factories. Domestic animals such as goats and sheep may feed on the peels which contain high level of the toxic cyanide and may be of health hazard to the animals. Peels may also pile up at dumpsites thereby constituting nuisance in the environment and eventually leading to emission of offensive odors (Ubalua, 2007).

Cassava peels consist of the rough, brown outer part normally removed with some fleshy white or yellow part. The peels are therefore rich in starch and can be used in some industrial processes rather than indiscriminate disposal. In view of its high content of starch, microorganisms will utilize cassava peels as growth medium and in the process, produce amylase as extracellular enzyme to digest the starch.

Amylases are of great importance in biochemical processes involving starch hydrolysis. The spectrum of application has gone beyond foods, beverages and pharmaceuticals to other fields such as medical and analytical chemistry (Pandey et al., 2000). In the present day biotechnology, approximately 25% of the enzyme market is dominated by amylases (Rao et al., 1998).

Though plants and animals produce amylases, enzymes from microbial sources are generally used in industrial processes. This is due to a number of factors including productivity, thermostability of the enzyme as well as ease of cultivating microorganisms (Odee et al., 1997; Reddy et al., 1999). Most industrial fermentation leading to the production of amylase use soluble starch. Sani et al. (1992) however used cassava peels in place of soluble starch and obtained a yield of about 170 times better.

Microorganisms that produce amylases could be assayed in immediate environment especially in places such as soil around mills, cassava farms and processing factories as well as flour markets (Fossi et al., 2005). Prominent among bacteria used in commercial production are the *Bacillus* spp. (Amund and Ogunsina, 1987; Olafimihan and Akinyanju, 1999; Pandey et al., 2000; Gupta et al., 2003). Others, such as *Escherichia* spp, *Pseudomonas, Proteus, Serratia* and *Rhizobium* also yield appreciable quantity of the enzyme (Oliviera et al., 2007). *Aspergillus, Rhizopus, Mucor, Neurospora, Penicillium* and *Candida* are some of the fungi that also produce extracellular amylases of commercial value (Pandey et al., 2000; Gupta et al., 2003).

The need to improve the efficiency of amylase has led researchers to investigate the possibility of using wastes such as cassava waste as growth medium for amylase producing microorganisms. This, in addition to other

applications such as biogas and ethanol production, enhances removal of waste from the environment. More microorganisms are also being investigated for the amylase activity particularly for improved productivity and stability. In this study, a cassava waste dumpsite was investigated and an amylase producing yellow pigmented bacterium was isolated. The bacterium was characterized along with the amylase produced.

MATERIALS AND METHODS

Isolation of organism

A sample of cassava waste was collected aseptically from a dumpsite near a gari processing factory in Ibadan, Nigeria. 1 g of the sample was suspended in 9 ml of sterile distilled water and properly shaken to dislodge microorganisms. A serial dilution up to 10^{-4} was made and 0.1 ml of the last two diluents inoculated on nutrient agar. This was incubated at 37ºC for 24 h. Subcultures of a yellow pigmented bacterium isolated were made and maintained on nutrient agar slant at 10ºC every 10 days.

Characterization of Isolate

Isolate was characterized based on basic microbiological techniques following a laboratory manual of Microbiology (Fawole and Oso, 2004). The morphological characters examined include appearance of colony, Gram's reaction, spore stain and motility test. The biochemical characteristics tested include catalase, methyl red, Voges Proskauer, gelatin hydrolysis, starch hydrolysis, sugar fermentation, oxygen relation, citrate utilization, urease production, casein hydrolysis, indole production, oxidase test as well as a test to determine oxidative or fermentative metabolism.

Preparation of culture filtrate

A portion of the isolate was transferred from the agar slant into a 250 ml Erlenmeyer flask containing 100 ml nutrient broth and incubated overnight at 30ºC on a gyratory shaker. An aliquot (5 ml) of this overnight culture was used to inoculate a nutrient broth medium containing 1% starch (Adeyanju et al., 2007). This was also incubated at 30ºC on a gyratory shaker at 150 rpm for 24 h. Culture was thereafter centrifuged in a MSE refrigerated centrifuge at 5000 rpm for 30 min. The supernatant obtained was stored at temperature of about -10ºC.

Assay of amylase activity

The reaction mixture was made up of 1 ml of 1% soluble starch in 0.02 M phosphate buffer and an aliquot (1 ml) of culture filtrate. This was incubated for 1 h. The reducing sugar content of the reaction mixture was determined using the Nelson/Somogyi method as outlined by Agu et al. (1997). Di-nitro salicylic acid (DNSA) reagent (3 ml) was added to the reaction mixture in a test tube. Mixture was boiled for 45 min in a water bath. Absorbance was taken at 450 nm and amount of reducing sugar read from a standard curve prepared from various concentrations of maltose. Boiled enzyme filtrates served as control. A unit of amylase activity was defined as the amount of amylase in 1 ml of culture filtrate which releases 1 mg of reducing sugar from soluble starch under assay condition. Activity was determined for different temperatures between 10 and 40ºC and different pH values ranging from 4.5 to 8.0.

Stability of crude amylase

Temperature

The method of Kainuma et al. (1985) was adopted in testing the thermostability of the amylase. An aliquot (0.4 ml) of crude enzyme filtrate was added to 0.2 ml of 0.1 M acetate buffer pH 7.0 and incubated at different temperatures between 20 and 80ºC for 1 h. Activity was determined at 30ºC. Boiled enzyme filtrate served as control. Percentage relative activity was calculated using the following formula:

Relative activity (%) = (Remaining acrivity / Initial activity) x 100

The percentage relative activity was plotted against temperature.

pH

The method of Kainuma et al. (1985) was also employed. An aliquot (0.4 ml) of the enzyme filtrate was added to 0.2 ml of 0.1 M buffer solution of various pH values ranging from 3 to 10. This was incubated at 30ºC for 1 h. Activity was determined at 30ºC and percentage relative activity calculated. Boiled enzyme filtrate served as control. Percentage relative activity was plotted against pH.

Analysis of product of crude amylase activity on starch by paper chromatography

The crude enzyme filtrate was first dialyzed against distilled water at 10ºC for 48 h. This was necessary to remove any sugar in the enzyme filtrate. An aliquot (1 ml) of crude enzyme was then added to 1 ml of 1% starch solution. A few drops of toluene were added to prevent growth of microorganisms. The mixture was incubated at 35ºC for 24 h and then analyzed for sugar by paper chromatography (Adeyanju et al., 2007).

About 20 µl of the hydrolysate and a 0.01% (w/v) ethanolic mixture of the standard sugar, that is, maltose, glucose and fructose were spotted onto Whatman no.1 filter paper. Starch solution (1%) was spotted as control. The paper was developed for 24 h using n-butanol: acetic acid: water (4:1:1, v/v/v). The air dried chromatogram was located by the method of Trevelyon et al. (1950).

RESULTS AND DISCUSSION

Morphological characteristics

On nutrient agar, the isolate is a round, transparent, yellow colony with smooth and glistening surface. Edge is entire, elevation, low convex and is about 2 mm in diameter. The cells are Gram positive, non motile, non sporulating, short rods, occurring singly. Isolate produces negative results from catalase, methyl red, oxidase, indole production, urease production and citrate utilization tests, positive results from Voges Proskauer, gelatin liquefaction, starch hydrolysis and casein hydrolysis tests. The isolate is strictly aerobic and exhibit oxidative metabolism.

As compared to the other chromogenic bacteria such as *Cytophaga, Flexibacter* and *Pseudomonas*, the isolate exhibited more similarities of morphological and biochemical characters with the *Flavobacteria* (Table 1).

Table 1. Colonial, Cellular and Biochemical Characters of Isolate; Colonial morphology , Cellular morphology, Biochemical characteristics.

Shape	Round	Gram stain	Gram positive	Catalase test	Negative
Edge	Entire	Shape of cell	Short rod	Oxygen relation.	Aerobic
Surface	Smooth and glistening	Arrangement of cell	Occur singly	Oxiferm. Test	Oxidative Metabolism
Elevation	Low convex	Spores	Absent	Metyhyl red	Negative
Pigmentation	Yellow	Motility	Non motile	Voges Proskauer	Positive
Opacity	Transparent			Gelatin liquefaction	Positive
Diameter	2mm			Starch hydrolysis	Positive
				Casein hydrolysis	Positive
				Oxidase Test	Negative
				Indole production	Negative
				Urease production	Negative
				Citrate utilization	Negative

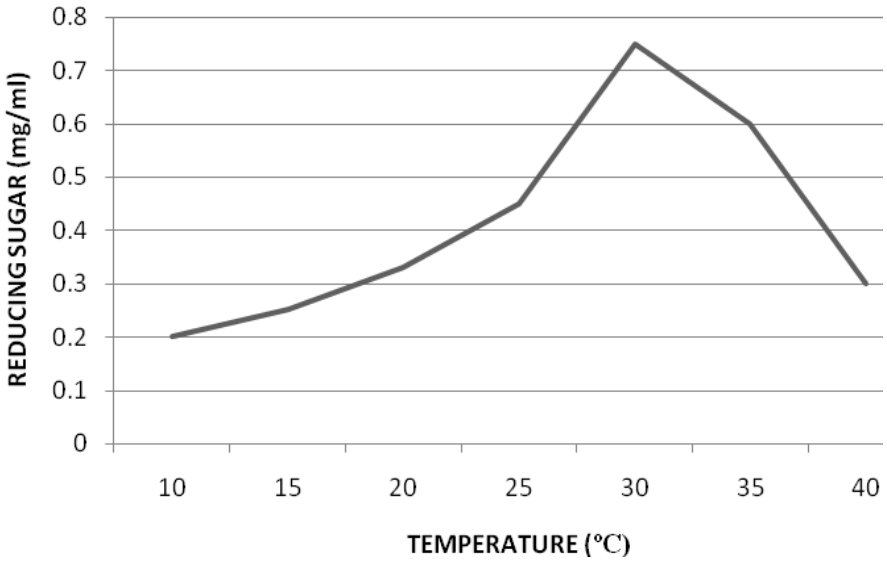

Figure 1. Effect of temperature on the activity of crude amylase obtained from the bacterium isolated from cassava waste.

Rubin et al. (1985) described the genus *Flavobacterium* as Gram negative bacilli that produce chromogenic colonies. Weeks (1974) also described the organism as a genus of pigmented and rod shaped non fermentative bacteria. Some members of the genus share the same characteristics with the isolate. Most, except *F. aquatile, F. meningosepticum* and *F. devorans* do not produce catalase. Indole production occurs in *F. breve* and *F. indolthecum*. Most do not produce urease. Starch is also hydrolysed by *F. lutescens* and *F. indolthecum*. Casein hydrolysis was recorded for *F. aquatile, F. meningosepticum* and *F. devorans*. Utilization of gelatin was observed in *F. aquatile, F. meningosepticum, F. helmophillum, F. uliginosum, F. lutescens, F. rigense, F. indolthecum* and *F. devorans* (Weeks, 1974).

Effect of temperature and pH on amylase activity

Figure 1 shows the pattern of amylase activity at different temperatures. Activity increased with temperature and reached optimum at 30ºC after which there was a decline. This is in agreement with enzyme's reaction to temperature reported by Foster (1980).

A relatively low level of activity was observed at pH values 4.5 to 6.0 (Figure 2). Activity increased significantly from pH 6.0 to 7.5 and dropped at pH values above 7.5 indicating that the enzyme's activity was best at neutral pH. Amund and Ogunsina (1987) recorded pH and temperature optimum for enzymes obtained from *Bacillus subtilis, B. licheniformis* and *B. cereus* as 7.0, 5.5 7.5 and 30ºC, 37ºC and 80ºC, respectively.

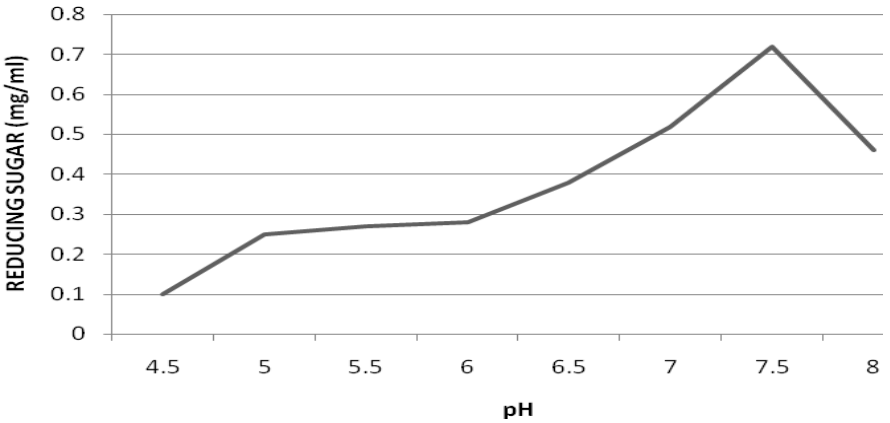

Figure 2. Effect of pH on the activity of crude amylase obtained from the bacterium isolated from cassava waste.

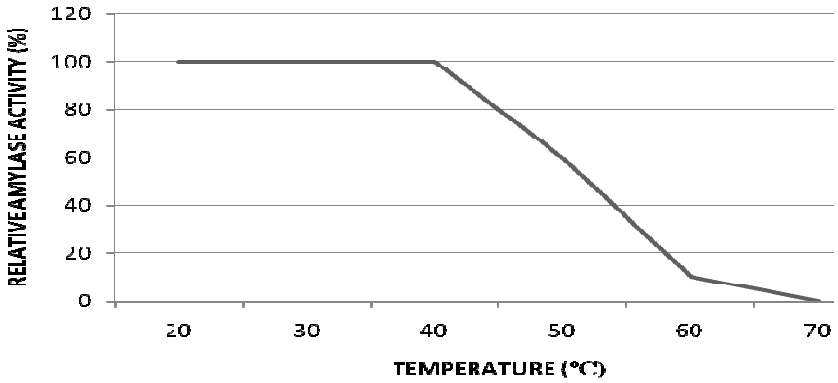

Figure 3. Activity of crude amylase at various temperatures.

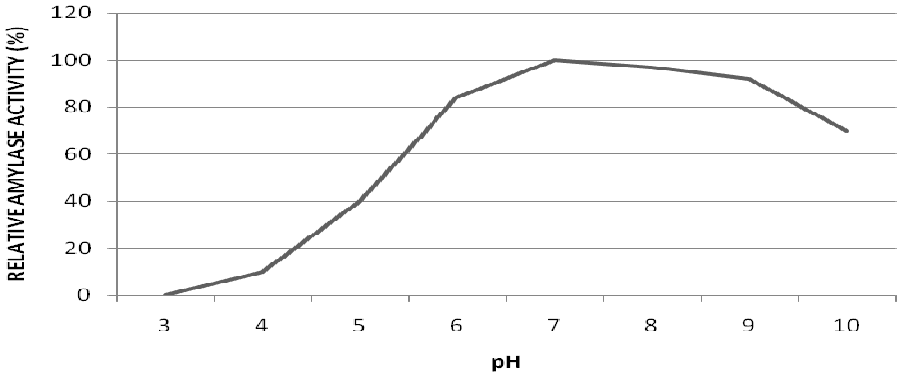

Figure 4. Activity of crude amylase at various pH.

Stability of the crude amylase at varying temperature and pH values

Full activity was obtained after holding the enzyme for 1 h at 20, 30 and 40ºC. Only 60% of the activity remained after heat treatment at 50ºC, 10% at 60ºC and no activity at 70ºC (figure 3). Over 90% of the initial activity was retained between pH 6 and 9. At pH 10, about 30% of the initial activity has been lost. At lower pH values of 3. 4 and 5, activity was very low (Figure 4).

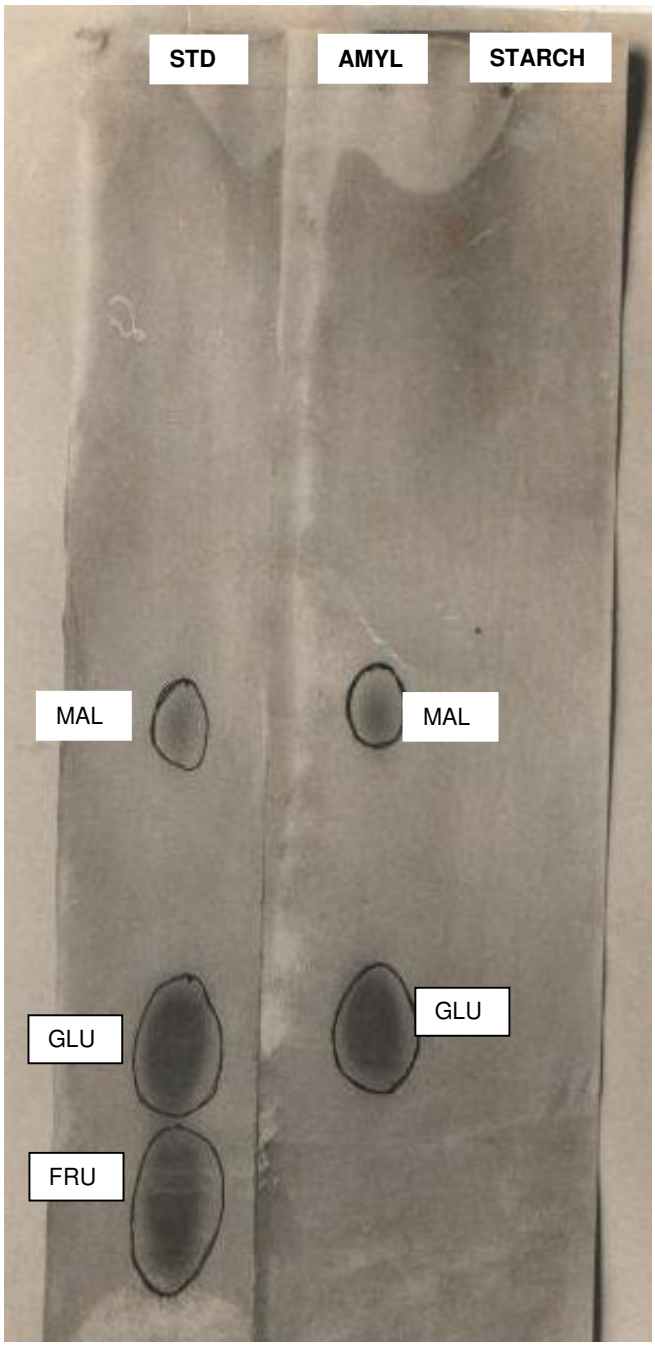

Figure 5. Chromatograph of the products of amylase activity on starch. AMYL = Starch hydrolysate, STD = standard [comprise of ethanolic mixture of maltose, glucose and fructose (0.01%, w/v)], STARCH = starch suspension (1%, w/v), MAL = maltose, GLU = glucose, FRU = fructose.

This is a non thermostable enzyme in line with enzyme from *Lactobacillus coryneformis* and *Lactobacillus delbruckii* (Oboh, 2005) but unlike the amylase obtained from *Bacillus licheniformis* with optimum temperature at 90ºC (Adeyanju et al., 2007) and *B. subtilis* which was stable at 60ºC (Kennedy and White, 1979). On the other hand, the enzyme tends towards alkalinity in consonance with amylase from *B. subtilis* (Kennedy and White, 1979) and *B. licheniformis* (Chiang et al., 1979; Adeyanju et al., 2007).

Paper chromatogram of the product of amylase activity on starch

The product of starch hydrolysis by the enzyme filtrate is shown in a chromatograph (Figure 5). The presence of maltose and glucose in the hydrolysate confirms the amylolytic properties of the enzyme filtrate. This further explains the ability of the isolate to grow in a cassava peel dump. Amylase acts on starch breaking it down to maltose which is a disaccharide comprising of two glucose molecules. The maltose is then broken down to glucose. This indicates the presence of a mixture of enzymes including maltase and glucoamylase. Chiang et al. (1979) characterized the sugar obtained from amylase hydrolysis of corn starch as maltose, maltopentaose and maltohexaose.

Conclusion

This study has shown that a yellow pigmented, Gram negative, rod shaped bacterium is able to synthesize amylase. Evidence of this, is in the hydrolysis of starch from cassava waste by the bacterium. This is very important in biotechnology especially as regard production of enzymes using simple and cost effective materials. Most commercial production of amylases depends on the use of *Bacillus* species. A look into the use other bacteria may yield enzymes of different qualities that may be of value in the modern day biotechnology.

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