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Full Length Research Paper

Production of amylase by bacteria isolated from a cassava waste dumpsite in Minna, Niger State, Nigeria

Oyeleke*, S. B. and Oduwole, A. A.

Department of Microbiology, Federal University of Technology, Minna, Niger State, Nigeria.

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Ten grams (10 g) of soil sample obtained from a cassava waste dumpsite in Kasuwan Gwandabe, Minna, Niger State, was bacteriologically analyzed. One gram (1.0 g) **of the sample inoculated into a liquid soluble starch medium generated reducing sugar with a concentration of 1.65 mg/ml after 72 h. Characterization of the soluble starch amylases revealed an optimum temperature of activity of 70°C. Optimum pH for activity was between 6.5 and 7.5. The most frequently occurring amylolytic bacteria were *Bacillus subtilis* (37.5%), followed by *Bacillus subtilis megaterium* and *Bacillus coagulans* (18.75% each). The least occurring isolates were *Merus* and *Bacillus pumilus* (6.25% each). The mean zone of amylolytic activity for the isolates ranged between 2.1 mm for *B. subtilis* and 1.1 mm for *B. pumilus*.**

Key words: Aminolytic, enzymes, amylase, optimum, *Bacillus* sp.

INTRODUCTION

Enzymes are substances present in the cells of living organisms in minute amounts and are capable of speeding up chemical reactions (associated with life processes), without themselves being altered after the reaction. They accelerate the velocity of the reaction without necessarily initiating it (Pelczar et al., 1986). There are three major sources of enzymes (Burhan et al., 2003), that is derived from a variety of plants, e.g. pappain, animal enzymes – derived from animal glands, e.g. trypsin, pepsin and microbial enzymes – derived from micro organisms (fungal and bacterial) through the process of fermentation, e.g. amylase. Microbial enzymes are preferred to those from both plants and animal sources because they are cheaper to produce, and their enzyme contents are more predictable, controllable and reliable (Burhan et al., 2003).

Members of the genus *Bacillus* are heterogeneous and they are very versatile in their adaptability to the environment. There are various factors that influence the nature of their metabolic processes and enzymes produced (Ajayi and Fagade, 2006). A great deal of attention is being given to thermophilic and extremely thermophilic microorganisms and their enzymes (Ajayi and Fagade 2006). *Bacillus* species produce a large variety of extra cellular enzymes, such as amylases, which have significant industrial importance (Cordeiro et al., 2003). In the same vein, bacterial enzymes are known to posses more thermostability than fungal amylases (Eke and Oguntimehin, 1992).

Amylases are examples of hydrolases and function in the hydrolysis of molecules. Amylases are of the most important enzymes used in biotechnology (Burhan et al., 2003). Their use includes hydrolysis of starch to yield glucose syrup, amylase-rich flour and in the formation of dextrin during baking in food industries. Furthermore, in the textile industry, amylases are used for removal of starch sizing and as additives in detergents (Shaw et al., 1995; FAO, 2001). However, the cost of producing this enzyme is high and the cost of procurement by developing countries can be even higher as a result of importation. In Nigeria, the local production of the enzyme will save about 200 million naira that is spent annually for its importation. Cheap and readily available agricultural waste such as cassava peels, which presently constitutes a menace to solid waste management, may be a rich source of amylolytic bacteria (Ali et al., 1998; Fos, 2003). Therefore, the objectives of the study were to isolate and identify amylolytic bacteria from cassava waste dumpsites, and to perform partial characterization of the enzyme production and its properties with regard to the effect

*Corresponding author. E-mail: droyeleke@yahoo.com.

of temperature and pH.

MATERIALS AND METHODS

Soil samples

Ten grams (10 g) of soil sample obtained from a cassava waste dumpsite in Kasuwan Gwandabe, Minna, Niger State, was collected in sterile bottles and taken to the Enzymology and Genetics Division in the Department of Biotechnology of the Federal Institute of Industrial Research, Oshodi for analysis.

Preparation of media and sugar solutions

Media were prepared according to standard methods described by Cheesbrough (2003) and Oyeleke and Manga (2008). The starch medium was prepared by measuring 8 g of starch into 1000 ml of distilled water (8% [w/v]), as described by Bertrand et al. (2004). Peptone water was prepared according to manufacturer's instructtion by measuring 15 g of peptone into 1000 ml of distilled water. The solution was then sterilized and autoclaved for 15 min. The sugars used in the experiments were prepared by measuring 100 g of each of the sugars, which were then sterilized at 120°C for 15 min. However, solutions of glucose and fructose were sterilized at 115°C for 10 min.

Isolation and identification of *Bacillus* isolates

One gram (1 g) of the soil sample was weighed into 9 ml of sterile distilled water and then placed into a water bath, maintained at 90°C for 1 h. Serial dilutions were prepared up to the 10^{-2} dilution and then 1 ml of the dilution was added, using the pour plate method, to nutrient agar that had been fortified with 1% starch. The agar plates were incubated at 37°C for 24 - 48 h. *Bacillus* isolates, based on colony characteristics, were purified by subculturing on fresh nutrient agar to obtain pure cultures. The bacterial isolates were characterized and identified based on the following morphological and biochemical tests, gram staining, spore staining, catalase test, sugar fermentation, starch hydrolysis and citrase utilization (Cheesbrough, 2003; Oyeleke and Manga, 2008).

Amylolytic activity of *Bacillus* isolates

The amylolytic activity of the *Bacillus* isolates was determined using the method of Bertrand et al. (2004), with slight modifications. One ml (1 ml) of each *Bacillus* isolate culture was inoculated aseptically into a 2 mm hole on nutrient agar medium, which was supplemented with 1% starch (that is, 2.5 g of starch). The agar plate was then incubated at 37°C for 48 h. After the incubation period, Lugol iodine solution was added to the medium. The diameter of the clearing zone formed around inoculation site, after the addition of iodine, was measured and taken to represent the amylolytic activity of the strain. The isolate displaying the widest diameter was selected further characterization.

Enzyme production and partial purification

The selected *Bacillus* isolate was propagated at 37°C for 22 h in 50 ml of 8% (w/v) of starch medium in a 250 ml flask. The flask was incubated in a shaker incubator, operated at 120 rpm and at 30°C. After the incubation period, the resultant broth was centrifuged at 10,000 rpm for 15 min and the supernatant was collected as the source of crude enzyme (amylase). To partially purify the enzyme extract, a solution of 65% (w/v) of sodium sulphate was added to the clarified supernatant, centrifuged at 10,000 rpm and the pellet was suspended in 0.005 M Na_2HPO_4 (pH 6.0). The purified enzyme extract was used in subsequent assays.

Enzyme activity assay

The activity of the partially purified amylase enzyme was examined by using soluble starch (dissolved) as substrate. A few drops of Dinitrosalicylic acid (DNSA) reagent were added and the absorbance of the reaction was measured on a spectrophotometer at a wavelength of 540 nm. Enzyme activity was defined as the amount of soluble starch hydrolyzed by 1 ml of enzyme extract in 1 min.

Effect of temperature and pH on enzyme activity

The optimal temperature for activity was determined by assaying activity of the enzyme, as described above, between 10 -100°C. Thermo stability of the enzyme extract was determined by maintaining the enzyme extract in a water bath at different temperatures (20 - 100°C) for 30 min. The optimum pH for enzyme activity was determined over a pH range of 2.0 - 10.5 on 1% starch medium, using 0.05 M Na_2HPO_4 as buffer solution. The pH stability was determined by incubating the enzyme extract in a water bath at 70°C and the residual enzyme activity was then measured as described above. The pH was adjusted by the addition of 0.l N HCl and 0.1 N NaOH to achieve acidity and alkalinity, respectively.

Determination of the reducing sugar

The reducing sugar content, following hydrolysis of starch by the enzyme extract, was determined using the method of Bertrand et al. (2003). The DNSA reagent was prepared by adding 1.8 g of 3.5 DNSA T to 20 ml of 1.0 N NaOH and 60 ml of distilled water. Potassium sodium tata rate (60 g) was added and the mixture was diluted to 200 ml with distilled water. The reducing sugar content from hydrolyzed starch by amylase enzyme was assayed by adding 2 ml of 3.5 DNSA reagents to 1 ml of the sample. The mixture was heated in boiling water for 5 min and then cooled under running tap water. The absorbance at 540 nm of the resulting coloured solution (slight brown) was read in a spectrophotometer against a blank, prepared by substituting the hydrolyzed sample with distilled water. The reducing sugar content was subsequently determined by making reference to a standard curve of known glucose concentrations.

RESULTS AND DISCUSSION

Sixteen strains of *Bacillus* species were isolated and identified from the cassava dumpsites. The morphological and biochemical characterization revealed the presence of *Bacillus subtilis* (A_1, A_3, A_7, A_8, A_9, A_{10}, and A_{16}). *B. macerans* (A_6), *B. megaterium* (A_2, $A_{11}$and A_{14}), *B. polymyxa* (A_5 and A_{13}) and *B. coagulans* (A_{14}, A_9 and A_{15}). All the *Bacillus* isolates were Gram-positive, rod-shaped, spore formers and hydrolyzers of starch. The amylolytic activity of *Bacillus* isolates according to their halos is presented in Table 1. *Bacillus subtilis* (A16) had the highest halo (3.1 mm), and *B. macerans (*A6) and *B. coagulans* (A9) had the smallest (0.3 mm). Since the distinction between the strains lies in the soil origin and taxonomic characteristics of isolates, it was not possible to define whe-

Table 1. Amylase activity of *Bacillus* isolates.

Isolate codes	Probable identity	Zones of amylase activity (mm)
A_1	*B. subtilis*	2.3
A_2	*B. megaterium*	2.15
A_3	*B. subtilis*	1.8
A_4	*B. coagulans*	2.4
A_5	*B. polymyxa*	1.1
A_6	*B. macerans*	0.3
A_7	*B. subtilis*	1.3
A_8	*B. subtilis*	2.8
A_9	*B. coagulans*	0.3
A_{10}	*B. subtilis*	1.3
A_{11}	*B. megaterium*	1.0
A_{12}	*B. pumilus*	1.1
A_{13}	*B. polymyxa*	2.8
A_{14}	*B. megaterium*	2.3
A_{15}	*B. coagulans*	2.3
A_{16}	*B. subtilis*	3.1

Table 2. Frequency of *Bacillus* isolates.

Bacterium	Frequency (%)
B. subtilis	37.5
B. megaterium	18.75
B. coagulans	18.75
B. polymyxa	12.50
B. macerans	6.25
B. pumilus	6.25

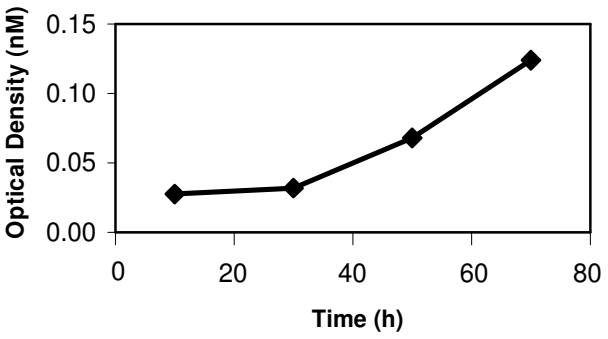

Figure 1. Changes in Growth of *Bacillus subtilis* in starch medium.

ther amylolytic variation between the strains was the consequence of species variability or environmental effect on the same micro organisms.

Similar observations were made by Bertrand et al. (2004) from amylolytic haloes produced by different yeast strains isolated from starchy soil. Thus, the strain A_{16} identified as *Bacillus subtilis*, which showed the highest amylolytic halo, was selected for further analysis. The isolated *B. subtilis* had the highest frequency (37.5%), while *B. pumilus* had the lowest frequency (6.25%) (Table 2).

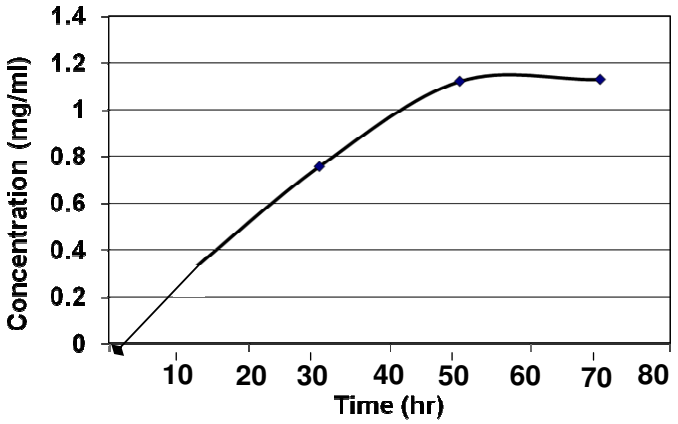

Figure 2. Changes in reducing sugar content.

The growth and amylase activity of A_{16} (*B. subtilis)* in 8% starch fermented broth is shown in Figures 1 and 2. There was a gradual increase in growth of *B. subtilis* in the starchy medium, probably as a result of its ability to utilize the starch following its amylolytic activity to glucose. This agrees with the report of Oguntimehin (1993) regarding the use of *Bacillus licheniformis* isolated from cassava processing waste for the production of amylase. In this study, the activity of amylase produced increased from 0 to 72 h with a reducing sugar content of 1.13 mg/ml (Figure 2). Kocher and Katgal (2003) found a comparable trend in amylase production by *Shwanniomyces occidentalis*, which was isolated from potato fermentation. The result also agrees with Oguntimehin (1993) for amylase production in *B. licheniformis*, which was isolated from cassava during fermentation.

The temperature stability result of amylase obtained from *Bacillus subtilis* (A_{16}) is shown in (Figure 3). The figure revealed that the enzyme remained stable at 20 and 70°C. The enzyme stability declined at temperatures above 70°C. The maximum activity was displayed at 70°C with a reducing sugar concentration of 0.57 mg/ml. The stability of the amylase enzyme obtained from *B. subtilis* (A_{16}) may probably be a result of their spore-forming ability. The enzyme stability trend, as reported in the present study, agrees with the behaviour of amylases from *Bacillus* spp. investigated by Cordeiro et al. (2002), in which a soluble starch medium was used. The result obtained in this study also agrees with earlier investigations by El-Aassan (1992) regarding *Bacillus lentus*, Heinen and Heinein (1992) and Fatima and El-Rufai (1991) regarding *Bacillus caldolyticus*, and Morgan and Priest (1999) in their characterization of α - amylase produced by *B. licheniformis.*

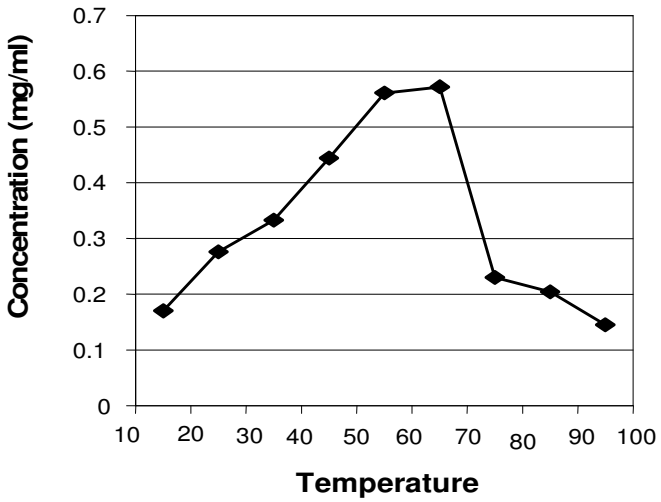

Figure 3. Temperature reading of optical density of reducing sugar at 540 nm.

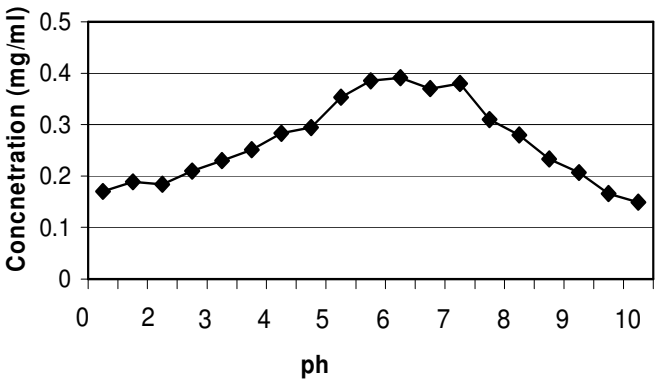

Figure 4. pH reading of optical density of reducing sugar at 540 nm.

The optimum pH activities were 6.5 and 7.5 with reducing sugar contents of 0.39 and 0.38 mg/ml, respectively (Figure 4). Amund and Ogunsina (1987) and Anthrin et al. (1990) reported a lower optimum pH range of 5.5 - 6.0 for *B. licheniformis*. In contrast, Oguntimehin (1993) reported maximum activities at a pH range of 4.8 and 9.2 for an amylase obtained for *B. licheniformis* isolated from a cassava processing waste. In conclusion, this study revealed that cassava waste harbours amylolytic *Bacillus* species and that the amylase produced by these bacteria may in future, be used to treat this agricultural waste material.

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