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Characterization of Amylase from Some Aspergillus and Bacillus Species Associated with Cassava Waste Peels

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Abstract

Cassava peels are generated as waste on soils during cassava processing in many tropical countries. This work set out to isolate some microorganisms associated with cassava peel degradation and characterize amylase enzymes responsible for the degradation under some physiological conditions. A total of 30 bacteria was isolated from the peels with *Bacillus* species occurring the most (46.5%) and *Enterobacter* species (13.3%) being the next. Frequencies of fungal isolations was *Rhizopus* sp. (35%); *Aspergillus niger* (25%); *Aspegillus flavus* (20%) and *Penicillium* species (20%). B*acillus cereus, Bacillus substilis Bacillus pumillus, Aspergillus niger* and *Apergillus flavus* were selected and screened for their abilities to produce amylase. Amylase activity was highest at day 4 for *B. substilis* (39.4 units/ml) and *A. flavus* (66.1 units/ml); at day 3 for *B. cereus* (55.6 units/ml) and *A. niger* (44.6 units/ ml). While maximum amylase activity was obtained at day 6 for *B. pumilus* (80.2 units/ml). Optimum pH for amylases from the two fungal isolate was 6.0 (*A. niger* = 53.5 units/ml and *A. flavus* = 65.4 units/ml). While optimum pH for *B.cereus* (51.7 units/ ml) and *B. pumilus* (44.6 units/ml) was 6.5 and for *B. substilis* (56.1 units/ml) at pH 7.0. Amylase activities increased from 20˚C to 40˚C for amylase from *Bacillus* sp. and 20˚C to 50˚C for amylase from the *Aspergillus* sp. after which there was a decline in activities as temperature increased to 80˚C. Effect of heating duration (at 70˚C for 5 minutes) on the amylase showed that *A. niger* has the highest activity of 127 units/ml. Effect of substrate concentration on amylase activity showed that amylase form *A. flavus* had the highest activity of 72.2 units/ml at 0.4% substrate concentration. The implications of the findings were discussed.

Keywords

Amylase, *Bacillus, Aspegillus*, Enzyme Activity

1. Introduction

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 production. The cost of amylase production is high and the cost of procurement by developing countries can even be higher as a result of importation duties [1]. Due to this, screening for effective and efficient fungal isolates for amylase production will significantly reduce production cost in industries which employ amylase and provide economic opportunities. In Nigeria, the local production of the enzyme will save about 200 million naira that is spent annually for its importation [2].

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 amylase producing microorganisms [2]. Though many microorganisms can grow on a wide range of carbon and nitrogen sources, it is economically more viable to utilize the cheap and easily available resources as substrates for amylase production [3]. Although 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 [4] [5]. Most industrial fermentation leading to the production of amylase use soluble starch. Sani *et al.* [6], however, used cassava peels in place of soluble starch and obtained a yield about 170 times better. Microorganisms that produce amylases could be assayed in the immediate environment especially in places such as soil around mills, cassava farms and processing factories as well as flour markets [7].

Prominent among bacteria used in commercial production of amylases are the *Bacillus* sp. [8] [9] [10] [11]. Others, such as *Escherichia* sp, *Pseudomonas*, *Proteus*, *Serratia* and *Rhizobium* also yield appreciable quantity of the enzyme [12]. *Aspergillus*, *Rhizopus*, *Mucor*, *Neurospora*, *Penicillium* and *Candida* species are some of the fungi that also produce extracellular amylases of commercial value [10] [11]. 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 [13].

A great deal of attention is being given to thermophilic and extremely thermophilic microorganisms and their enzymes [13]. *Bacillus* species produce a large variety of extra-cellular enzymes, such as amylases, which have significant industrial importance [14]. In the same vein, bacterial enzymes are known to possess more thermostability than fungal amylases [15]. Among the species of *Bacillus*, *B. subtilis*, *B. stearothermophilus*, *B. amyloliquefaciens*, *B. licheniformis*, *B. acidocaldarius*, *Bifidobacterium bifidum* and *B. acerans* are important species [16]. The *α*-amylases from *B. licheniformis*, *B. amyloliquefaciens* and *B. stearothermophilus* are among the most widely studied amylases and are highly homologous with respect to primary and tertiary structure [17]. Moulds are capable of producing high amounts of amylases. *Aspergillus niger* is widely used

for commercial production of α–amylase. Studies on fungal amylase especially in developing country have concentrated mainly on *Aspergillus niger* probably because of their ubiquitous nature and non-fastidious nutritional requirement of these fungi [18].

In this study we investigated the abilities and some characteristics of amylase produced by some *Bacillus* and *Aspergillus* species isolated from cassava waste peels.

2. Materials and Methods

2.1. Study Area and Sample Collection

Cassava peels were collected from cassava waste dump sites through aseptic measures from two different locations: a dumpsite near a *gari* processing factory in Oyo town, Oyo state, Nigeria and another from a dumpsite in Alaya village, Aiyedire local government, Osun state, Nigeria and transported to the laboratory for immediate analysis. Sampling was carried out twice: during dry season (December, 2014) and early rainy season (March, 2015). Stock samples were stored in a refrigerator at 4˚C.

2.2. Microbial Isolations

Microbial isolation from the cassava samples was carried out using the pour plate technique. The peels were first blended using a clean manual grinding machine. Twenty grammes of each peel were mixed with 180 ml saline solution in a 250 ml conical flask. The mixture was shaken for 20 minutes. One millilitre of the sample was serially diluted by mixing with 9ml sterile deionised water in test tubes up to the 6th dilution [19]. One mililitre each was placed in sterile Petri dish to which molten nutrient agar (20 ml) was added for bacterial isolations: incubation was done at 37˚C for 24 hours. Sterile Saboraud dextrose agar was added for fungal isolations and incubation was done at 25˚C for 72 hours. The plates were replicated. Several streaking was done to obtain pure cultures from the peels. Pure cultures were placed on agar slants (in the refrigerator) until when needed for further analysis.

Major bacteria and fungi present in the peels were identified using the usual laboratory procedures which included Gram's staining, catalase, oxidase, citrate, motility, MRVP, sugar fermentation tests, spore staining etc. Microbial isolates tested for amylase activity were also picked for molecular sequencing of their genes (16s RNA).

2.3. Amylase Production

The medium used was nutrient agar and 1% starch powder. The mixture was sterilized at 121˚C for 15 minutes. Twenty millilitre of the molten starch agar at 45˚C was poured into sterile Petri dishes aseptically and allowed to solidify. The dried medium surface was inoculated with the test organism by streaking and incubated at 37˚C for 72 hours. The inoculated culture was flooded with Gram's iodine. A clear zone around the line of streaking indicated starch hydrolysis

while un-hydrolysed starch formed a blue-black surface with the iodine [20].

2.4. Amylase Preparation

2.4.1. Bacteria

Each isolate was grown in semi-synthetic medium containing (%w/v) soluble starch 1.0%, bacteriological peptone 6%, $MgSO_4 \cdot 7H_2O$ 0.1% and KCl 0.5%. The culture supernatant was obtained by centrifugation at 6000rpm for 10min.

2.4.2. Fungi

The growth medium used for enzyme extraction contained micronutrients and macronutrients ($MnSO_4 \cdot 4H_2O$, $CaSO_4 \cdot 4H_2O$, $FeSO_4 \cdot 7H_2O$, $ZnSO_4 \cdot 7H_2O$, KNO_3, KH_2PO_4, KCl, $MgSO_4 \cdot 7H_2O$) as well as yeast extract in different proportions which was sterilized at 121˚C for 15 minutes. The filtrate (crude enzyme) was collected in a conical flask and examined for amylase activity.

2.5. Amylase Assay

To determine the activity of the amylase each day, the crude enzyme was examined for activity. Citrate phosphate buffer (0.02 M) at pH 6.0 was prepared. Starch (0.5 g) was added to 10ml water and warmed to dissolve. The dissolved starch was made up to 100ml with the citrate phosphate buffer and this served as the substrate. Test tubes were labelled experimental (E) and control (C). Two milliliter of the substrate was pipetted into each of the test tubes. To each of the experimental tubes 0.5 ml of the enzyme was added. All the experimental and control tubes were incubated for 1 hour at 35˚C. After incubation, termination was achieved by the addition of 2 ml of 1N HCl into the each test tube. The enzyme (0.5 ml) was added to each of the control tubes only. The tubes were properly shaken. Two milliliters of the content of each tube were pipetted into each new tube and labeled appropriately. Three milliliters of 0.1 N HCl were added to each tube. Iodine mixture (0.1 ml) was added to each tube. Optical densities were read using a spectrophotometer (Jenway 6305 UV/visible) at 670 nm.

One unit of amylase activity is defined as the amount of enzyme in reaction mixture which produced 0.01 reduction in the intensity of the blue colour of the starch-iodine complex under the conditions of the assay.

2.6. Characterization of Amylase

2.6.1. Effect Incubation Periods on Amylase Production

The duration of incubation plays an important role in the production of a microbial metabolite. The study for optimal incubation period for maximum amylase production requires that the flasks containing the production medium (pH 6) be inoculated and incubated at 37˚C. The samples were then withdrawn periodically at every 24 hours up to 144 hours [21] and assayed for amylase activity as described earlier.

2.6.2. Effect of pH on Amylase Activity

The effect of pH on the activity of the crude enzyme was determined by incu-

bating 0.5 ml of the crude enzyme with 2 ml of amylase substrate preparation with citrate phosphate buffer ranging from pH between 4.0 - 8.0 [21] and assayed as stated above.

2.6.3. Effect of Temperature on Amylase Activity

The effect of temperature on the enzyme was determined. The reaction mixture consisted of 2 ml substrate (1% starch in 0.02M citrate phosphate buffer, pH 6.0) and 0.5 ml of enzyme. The reaction mixtures were incubated for forty-five minutes at 20˚C, 30˚C, 40˚C, 50˚C, 60˚C, 70˚C, 80˚C, respectively [22], and liberated reducing sugar measured as described in the amylase assay (above).

2.6.4. Effect of Heating Duration on Amylase Activity

Samples of enzyme preparations were heated at 70˚C for 5˚C, 10˚C, 15˚C, 20˚C, 25˚C, 30˚C and 35˚C minutes respectively. The reaction mixture consisted of 2 ml substrate (1% starch in 0.02 M citrate phosphate buffer, pH 6.0) and 0.5 ml of enzyme and assayed as stated above.

2.6.5. Effect of Substrate (Starch) Concentration on Amylase Activity

Concentrations ranging from 0.7%, 0.6%, 0.5%, 0.4 %,0.3% 0.2% and 0.1% of starch solution were used as substrate in 0.2 M citrate phosphate buffer (pH 6.0) [21] and assayed as stated above.

3. Results

A total of 30 bacteria were isolated and identified from the cassava peels as presented in **Table 1**. *Bacillus* species were the most frequently encountered bacteria (46.5%). This was followed by *Enterobacter* sp. (13.3%). Some of the *Bacillus* sp. were then selected for amylase production and characterization. **Table 2** shows the frequency of occurrence of fungi isolated. Four different species were the major ones isolated with *Rhizopus* sp. (35%) being mostly occurring; folllwoed by *Aspegillus niger* (25%) and *Aspergillus flavus* & *Penicillium* sp each having an occurrence of 20%.

Bacteria isolates were screened for ability to produce amylolytic enzymes for 144 hours (6 days). Isolates selected for the screening had tested positive to starch hydrolysis and the activity for the crude enzyme was assayed after every 24 hours (on each day of production). As shown in **Figure 1**, three bacterial and two fungal isolates (*Bacillus cereus*, *B. subtilis*, *B. pumilus*, *Aspergillus niger* and *A. flavus*) which had the highest activities were picked (with activities of 55.6 units/ml on day 3; 39.4 units/ml on day 4; 44.6 units/ml on day 3; 80.2 units/ml on day 6 and 66.1 units/ml on day 4 respectively). These selected isolates were used for further assay.

The effect of pH on the activity of amylase produced by the selected isolates is shown in **Figure 2**. Optimum pH for amylases from both fungal isolates was observed to be at 6.0 (*A. niger* 53.5 units/ml and *A. flavus* 65.4 units/ml) while optimum pH for *B. cereus* (51.7 units/ml) and *B. pumilus* (44.6 units/ml) was at 6.5 and *B. subtilis* (56.1 units/ml) was at 7.0. Activity was lowest at 8.0 for *A niger*, *A. flavus* and *B. cereus* (8.8 units/ml, 8.1 units/ml and 9.6 units/ml respec-

Table 1. Frequency of occurence of bacteria from both sampling sites and season.

Isolate	Number of Isolates	Frequency of Isolation (%)
Acinetobacter baumannii	2	6.7
Bacillus anthracis	1	3.3
Bacillus cereus	3	10
Bacillus licheniformis	1	3.3
Bacillus olivae	1	3.3
Bacillus pimulus	1	3.3
Bacillus safensis	1	3.3
Bacillus subtilis	4	13.3
Enterobacter sp.	4	13.3
Klebsiella pneumoniae	1	3.3
Lysinbacillus sphaericus	1	3.3
Neisseria sp.	2	6.7
Pseudomonas sp.	2	6.7
Staphylococcus aureus	3	10
Staphylococcus epidermis	2	6.7
Sphingobacterium compostii	1	3.3
Total	30	100

Table 2. Frequency of occurence of fungi from both sampling sites and season.

Isolates	Number of Isolates	Frequency of Isolates (%)
Aspergillus flavus	4	20
Aspergillus niger	5	25
Penicillium sp.	4	20
Rhizopus sp.	7	35
Total	20	100

tively) while the activity for *B. subtilis* and *B. pumilus* (17.0 units/ml and 9.8 units/ml respectively) was lowest at 4.0.

Figure 3 shows the effect of incubation temperature on amylase activity on all of the selected isolates for further assay. The fungal isolates (*A. niger* 73.5 units/ml and *A. flavus* 58.2 units/ml) had higher activities than the bacterial isolates (*B. cereus* 57.1 units/ml, *B. subtilis* 39.8 units/ml and *B. pumilus* 29.5 units/ml). Activity of amylase steadily increased from 20˚C to 40˚C for *B. cereus* and *B. pumilus* but from 20˚C to 50˚C for both fungal isolates and for *B. subtilis* before decreasing gradually up to 80˚C. Enzyme activity was lowest at 80˚C for all the isolates besides *A. niger* (5.7 units/ml) where activity was lowest at 20˚C.

The effect of heat on amylase activity carried out by heating the crude enzyme from all the selected isolates at 70˚C (Figure 4). *Aspergillus niger* had the highest activity (127 units/ml) after 5 minutes. For all the isolates, highest activity

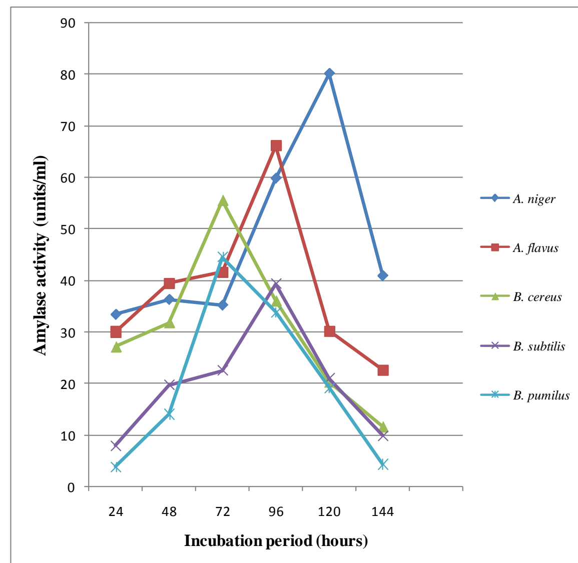

Figure 1. Effect of incubation period on amylase activity by *Aspergillus niger*, *Aspergillus flavus*, *Bacillus cereus*, *Bacillus subtilis* and *Bacillus pumilus.*

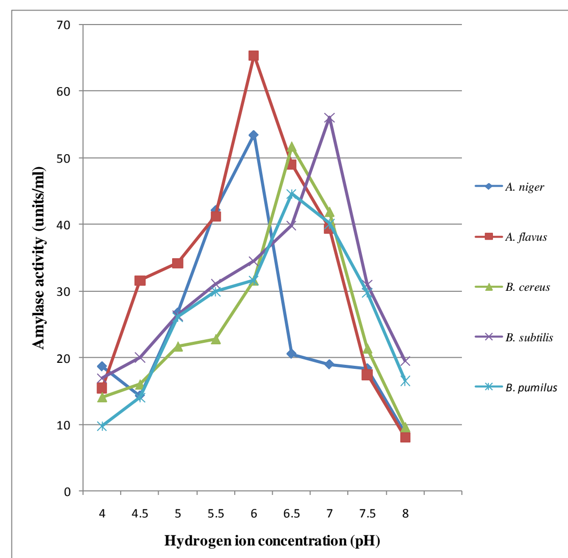

Figure 2. Effect of pH on amylase activity produced by *Aspergillus niger*, *Aspergillus flavus*, *Bacillus cereus*, *Bacillus subtilis* and *Bacillus pumilus.*

was at 5 minutes (*A. niger* 127 units/ml, *A. flavus* 77.6 units/ml, *B. cereus* 24.2 units/ml, *B. subtilis* 19.0 units/ml and *B. pumilus* 59.4 units/ml) and afterwards, there was a rapid decrease in activity to 64.7 units/ml, 40.9 units/ml, 16.5 units/ml, 15.1 units/ml, 22.9 units/ml respectively after 10 minutes of heating the crude enzyme. The values of amylase activity across the heating period for the bacterial isolates were much lower than that of the fungal isolates. The lowest activity for all the isolates was at 35 minutes which was at 34.7 units/ml for *A. niger*, *A. flavus* (20.1 units/ml), *B. cereus* (1.4 units), *B. subtilis* (2.3 units/ml) and *B. pumilus* (4.0 units/ml). The lowest amylase activity was recorded as 1.4 units/ml for *B. cereus* at 35 minutes.

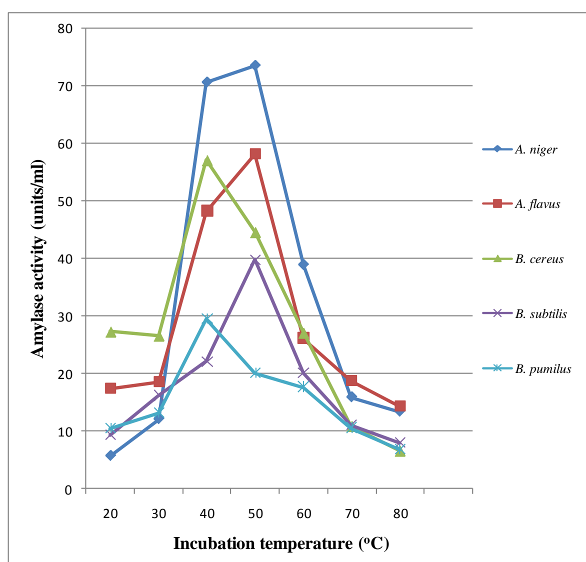

Figure 3. Effect of incubation temperature on amylase activity produced by *Aspergillus niger*, *Aspergillus flavus*, *Bacillus cereus*, *Bacillus subtilis* and *Bacillus pumilus.*

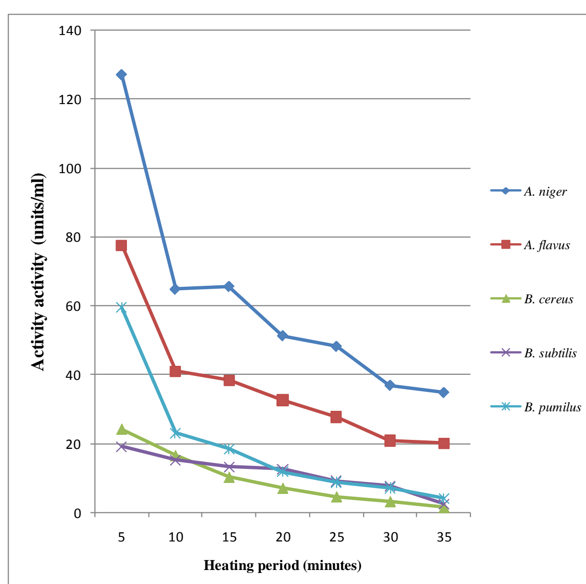

Figure 4. Effect of heating period on amylase activity produced by *Aspergillus niger*, *Aspergillus flavus*, *Bacillus cereus*, *Bacillus subtilis* and *Bacillus pumilus*

Effect of substrate concentration on the selected isolates was also carried out. The substrate used was soluble starch as it was added at different concentrations. As shown in **Figure 5**, *A. niger* had its highest activity at 0.5% concentration (54.8 units/ml) while *A. flavus*, *B. cereus* and *B. pumilus* had their highest activity (72.2 units/ml, 18.1 units/ml, 12 units/ml respectively) when 0.4 g/ml of starch was added while *B. subtilis* had its highest activity(15.9 units/ml) at 0.3% starch concentration. Activity was least for *A. niger* (7.3 units/ml) and *A. flavus* (16.2 units/ml) at 0.6% concentration while for all the bacterial isolates least activity occurred at 0.1% starch concentration. *Bacillus cereus* was recorded as 5.3

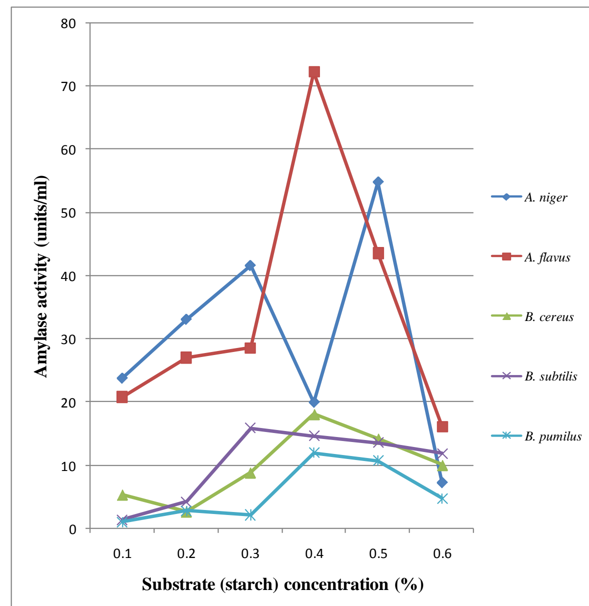

Figure 5. Effect of substrate concentration on amylase activity from *Aspergillus niger*, *Aspergillus flavus*, *Bacillus cereus*, *Bacillus subtilis* and *Bacillus pumilus*

units/ml, *B. subtilis* as 1.4 units/ml, *B. pumilus* as 1.1 units/ml. In all, the least activity was recorded by *B. pumilus* as 1.1 units/ml.

4. Discussion

Amylolytic enzymes are widely distributed in bacteria and fungi although a few selected strains of these organisms meet the criteria for commercial production. Maximum amylase production varied amongst the selected isolates. However, the incubation range was between 72 and 120 hours. After growth of culture in the medium, the biomass yield was found to decrease with an increase in incubation period. This study is in line with Ashwini *et al.* [23] who reported that maximum production of amylase occurred at 96 hours of incubation. Growth decreased in *B. subtilis* after 72 hours of incubation most likely due to substrate inhibition [24].

The pH of growth medium plays an important role by inducing morphological changes in the organisms and enzyme secretion. The pH change observed during the growth of the organisms also affects product stability in the medium [25]. Hamilton *et al.* [26] and Kirti [24] reported that amylases are generally stable over a wide range of pH from 4 to 11. In this study, amylase from *Aspergillus* sp. had its optimum activity at pH 6.0 while *Bacillus* sp. had its activity at pH 6.5. This differs from Ayansina & Owoseni [27] who reported that *Bacillus* species used for the production of alpha amylases by sub-merged fermentation have an optimum pH between 6. and 7.0 for best growth and enzyme production. There was a drastic decline in most of the isolates which indicates that the enzyme loses activity at alkaline concentration. This finding agrees with Senthilkumar *et al.* [28] who reported that *Bacillus* sp. used commercially for the production of bacterial α-amylases by submerged fermentation have an optimum pH between

6.0 and 7.0 for growth and enzyme production. However, this differs from the work carried out by Slivinski *et al.* [29] and Nyamful [30]. In Nyamful's experiments [30], crude enzyme from *A. niger* had the highest amylase activity of 3.34 U/ml at pH 5.0, and when pH was increased beyond 5.0, activity declined to 2.60 U/ml at pH 6.0 and further down to 2.24 U/ml at pH 7.0. Also, Amund and Ogunsina [8] and Anthrin *et al.* [31] reported a lower optimum pH range of 5.5 - 6.0 for *B. licheniformis*.

The influence of temperature on amylase production is closely related to the growth of the organism. Enzyme activity generally increased progressively with temperature up to the optimum after which it began to decrease with further temperature increase. The amylase activity of the isolates in this study had optimum temperature range of 40˚C (*B. cereus* and *B. pumilus*) and 50˚C (*A.niger*, *A. flavus* and *B. subtilis*). This agrees with the work of Martinez *et al.* [32] who reported an optimum temperature of 50˚C for amylase activity. Similarly, Sani [6] reported that microbial amylase was found to be stable after exposure to temperature between 20˚C and 40˚C but enzyme activity declined when exposure to temperature increased. However it slightly differs from the work carried out by Nyamful [30] in which culture filtrate of *A. niger* produced optimum activity at 40˚C. It also differs from the findings of Senthilkumar *et al.* [28] who reported that maximum enzyme production was at 60˚C (3.9 U/ml) and minimum at 30˚C (2.9 U/ml). The reduction in enzyme activity recorded at temperatures between 45˚C and 60˚C might attributable to the denaturation of proteins by heat. However, the high activities obtained at 30˚C - 45˚C confers industrial advantage on the amylases. The temperature range also suggests that the enzyme can be employed in food and beverages to convert starch into maltose where value has been placed on the thermo-stability and thermo-activity of the enzyme because of their high temperature operating conditions [33].

Effect of heating period signifies the thermo-stability of an enzyme. After heating at 70˚C, the amylase in this work was greatly inhibited. This agrees with that of Dutta *et al.* [34] in which they reported that amylase activity was inhibited by heat. The enzyme stability in this study correlates with the findings of Oyeleke and Oduwole [1] who reported that the enzyme stability of *B. subtilis* declined at temperatures above 70˚C. They also reported that the stability of the amylase enzyme obtained from *B. subtilis* (A16) may probably be a result of their spore-forming ability. The enzyme stability trend, as reported in the present study, also agrees with the behaviour of amylases from *Bacillus* sp. investigated by Cordeiro *et al.* [13], in which a soluble starch medium was used. Due to the valid amylase activity after 5 minutes, it can be inferred that the enzymes are moderately temperature-stable which will give rise to more industrial uses and opportunities.

The amylase activity of *A. niger* and *B. subtilis* relative to starch concentration had optimum values of 0.3% while *A. flavus*, *B. cereus* and *B. pumilus* was 0.4% and beyond this optimum, a gradual decrease in enzyme activity was observed. Roskoski [35] reported that the reaction velocity of an enzyme decreases after its

maximum velocity has been attained. Additional amounts of substrate added to the reaction mixture after this point actually decreased the reaction rate [35]. Nyamful [30] explained this by assuming that there are so many substrate molecules competing for the active sites on the enzyme surfaces such that they block the active sites and prevent any other substrate molecules from occupying them.

Recent research has found out that cassava peels and soil from cassava dumpsites which form a nuisance to the community may well be converted to very useful raw materials in the industries [36] [37].

In conclusion, this study shows that microorganisms isolated from peels and soils from cassava dumpsites are capable of producing amylase and can be exploited for large scale production. Amylases which can be harvested from the sources as mentioned earlier are important in many industrial processes and are the most widely used enzymes required for preparation in food and industrial sector. It is important that this advancement is exploited as the need and opportunities for amylase is continuously increasing as more industrial applications of amylases from these microbial sources are found out.

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