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SCREENING AND CHARACTERIZATION OF A BACTERIAL AMYLASE FROM SOME DECAYING ROADSIDE FRUIT SAMPLES IN KADUNA TOWNSHIP, NIGERIA

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ABSTRACT

Amylases have been isolated from various sources owing to their importance in many industrial applications. This current work is also another attempt to further explore cheaper and readily available microbial amylase with simultaneous aim of ridding the environment of some agro wastes which could constitute hazard to the environment. A total of four bacterial isolates from five decaying fruit samples were identified using simple pour plate technique of microbial culturing. They were further tested for their ability to produce amylases and isolates with high enzyme activity were pooled together into one composite sample for characterization studies. An optimum temperature and pH of 55^{O}C and 4 were respectively recorded for this amylase. While incubation period shows that enzyme activity was higher after 96 hours of incubation with enzyme been stable between 40- 65^{O}C. Effect of substrate concentration on enzyme activity showed a Km of 0.02M and Vmax of 7.5µmole/ml/min respectively. Other parameters reported all point to the characteristics of this amylase as possessing a potential for future application in many industrial processes and saving a lot of foreign exchange for a country like Nigeria.

Key words: Amylase, Bacteria, Fruits, Industry, Nigeria

Introduction

Amylases are starch degrading enzymes widely distributed in microbial, plants and animal kingdoms. They degrade starch and related polymers to yield products characteristics of individual amylolytic enzymes. Amylases are present in the saliva of humans and some other mammals, where they begin the chemical process of starch digestion. Foods that contain large amounts of starch but little sugar, such as rice and potatoes, may acquire a slightly sweet taste as they are chewed because amylase degrades some of their starch into sugar [1]. The pancreas and salivary gland make amylase (alpha amylase) to hydrolyze dietary starch into disaccharides and trisaccharides which are converted by other enzymes to glucose to supply the body with energy. Amylases are broadly classified into α, β, and γ subtypes, of which the first two have been the most widely studied. α-Amylase is a faster-acting enzyme than β-amylase. The

amylases act on α-1-4 glycosidic bonds and are therefore also called glycoside hydrolases. The first amylase was isolated by Anselme Payen in 1833. Amylases are distributed widely in living systems and have specific substrates. Amylase substrates are widely available from cheap plant sources, rendering the potential applications of the enzyme more plentiful in terms of costs. Amylases can be divided into endo-amylases and exo-amylases. The endo-amylases catalyze hydrolysis in a random manner within the starch molecule. This action causes the formation of linear and branched oligosaccharides of various chain lengths. The exo-amylases hydrolyze the substrate from the non- reducing end, resulting in successively shorter end products [2]. All α-amylases (EC 3.2.1.1) act on starch (polysaccharide) as the main substrate and yield small units of glucose (monosaccharide) and maltose (disaccharide).

Microbial amylases obtained from bacteria, fungi, and yeast have been used predominantly in industrial sectors and scientific research. The level of amylase production varies from one microbe to another, even among the same genus, species, and strain. Furthermore, the level of amylase production also differs depending on the microbe's origin, where strains isolated from starch- or amylose-rich environments naturally produce higher amounts of enzyme. Factors such as pH, temperature, carbon and nitrogen sources also play vital roles in the rate of amylase production, particularly in fermentation processes. Because microorganisms are amenable to genetic engineering, strains can be improved for obtaining higher amylase yields. Microbes can also be fine-tuned to produce efficient amylases that are thermostable and stable at stringent conditions. Such improvements can also reduce contamination by background proteins and minimize the reaction time and lead to less energy expenditure in the amylase reaction [3]. Among the wide range of microbial species that secrete amylase, its production from bacteria is cheaper and faster than from other microorganisms. Furthermore, genetic engineering studies are easier to perform with bacteria species and they are also highly amenable for the production of recombinant enzymes. A wide range of bacterial species has been isolated for amylase secretion. Most are *Bacillus* species (*B. subtilis*, *B. stearothermophilus*, *B. amyloliquefaciens*, *B. licheniformis*, *B. coagulans*, *B. polymyxa*, *B. mesentericus*, *B. vulgaris*, *B. megaterium*, *B. cereus*, *B. halodurans*, and *Bacillus* sp. Ferdowsicous), but amylases from *Rhodothermus marinus*, *Corynebacterium gigantea*, *Chromohalobacter* sp., *Caldimonas taiwanensis*, *Geobacillus thermoleovorans*, *Lactobacillus fermentum*, *Lactobacillus manihotivorans*, and *Pseudomonas stutzeri* have also been isolated [4]. Halophilic strains that produce amylases include *Haloarcula hispanica*, *Halobacillus* sp., *Chromohalobacter* sp., *Bacillus dipsosauri*, and *Halomonas meridian* [5].

The cost of amylase production is high and the cost of procurement by developing countries like Nigeria can even be higher as a result of importation duties. Littered around some major cities of Northern Nigeria are decaying and decomposing fruits from road side vendors who do not have the capacity to store unsold stock. These decaying fruits constitute a major source of environmental pollution. Since most bacterial amylases are easily screened from this kind of perishing fruits, an effective and efficient amylase production from those agro waste will significantly reduce the danger they pose to the environment and enhance economic opportunities for teeming unemployed citizens of the country. In Nigeria, the local production of the enzyme will save millions of naira that is spent annually for its importation. It is in the light of this therefore that more studies involving the isolation and improvement of novel strains are needed to pave the way towards creating important strains with high enzyme yield. This current study is aimed at isolation and characterization of such amylase from bacterial isolates of decaying abandoned fruit samples found in Kaduna metropolis of Nigeria and study the optimization processes involved in the production.

Materials and Methods

Study Area and Sample Collection

Five decaying fruit samples (Banana, Apple, Carrot, Cucumber, and Water melon) were collected from different locations within kaduna metropolis of Nigeria into sterile plastic bags and maintained in aseptic conditions. Stock samples were stored in a refrigerator at 4˚C until analysis were carried out.

Microbial Isolations

Microbial isolation from the fruit samples were carried out using the pour plate technique. Two grams each of the peeled fleshy endocarp were first blended using a clean blender and mixed with 20 ml of distilled water in a 100 ml conical flask. The mixture were shaken for 20 minutes and to mix thoroughly for another 30 minutes. One milli-litre of the sample was serially diluted by mixing with 9ml sterile deionised water in test tubes up to the 6th dilution. One milli litre each was placed in sterile Petri dish to which nutrient agar HIMEDIA fortified with 20 ml of 1% starch was added for bacterial isolations: incubation was done at 37˚C for 24 hours. The plates were replicated by several streaking to obtain pure cultures from the different peeled fruits. Pure cultures were placed on agar slants (in the refrigerator) until needed for further analysis. Major bacteria present in the peels were identified using the usual laboratory procedures which included Gram's staining, catalase, oxidase, citrate, motility, sugar fermentation tests, spore staining etc [6].

Screening Of Potent Amylase Producing Bacteria By Starch Hydrolysis Test

Bacterial isolates were screened for amylolytic activity by starch hydrolysis test on starch agar plate [7]. The microbial isolates were streaked on the starch agar plate and incubated at 37°C for 48 hours. After incubation, iodine solution in few drops were added with dropper for 30 seconds on the starch agar plate. Presence of blue colour around the growth indicates negative result and a clear zone of hydrolysis around the growth indicates positive result. The isolates producing clear zones of hydrolysis were considered as amylase producers and were further investigated. Major bacteria present in the fruits were identified using the usual laboratory procedures which included Gram's staining, catalase, oxidase, citrate, motility, MRVP, sugar fermentation tests, spore staining etc.

Crude Amylase Production From Bacterial Isolates

Each isolate was grown in semi-synthetic medium containing 1%w/v soluble starch and 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 (this serves as the source of the crude enzyme). Isolate with the highest yields of amylase enzyme were then pooled together for the enzyme assay.

Amylase Assay

To determine the activity of the amylase, the crude enzyme was examined for activity by performing the following activity. Citrate phosphate buffer (0.01 M) at pH 6.5 and added to a prepared Starch solution (5% w/v) which was heated to dissolve. The dissolved starch was made up to 100ml with the citrate phosphate buffer and this served as the substrate. Ten set of test tubes labelled experimental (E) and control (C) were prepared for each of the fruits. Two milliliter of the substrate was pipetted into each of the experimental test tubes containing 0.5 ml of the enzyme and allow to mix for about 5 minutes. The control tube does not contain the substrate. 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 each test tube.. 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 [8].

Characterization of Amylase

Effect of Incubation time on Amylase Production

The duration of incubation plays an important role in the production of a microbial metabolite. The study for optimal incubation time 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 [8] and assayed for amylase activity as described earlier.

Effect of pH on Amylase Activity

The effect of pH on the activity of the crude enzyme was determined by incubating 0.2 ml of the crude enzyme with 1 ml of amylase substrate preparation with citrate phosphate buffer ranging from pH between 2.0 - 8.0 and assayed as stated above.

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 35 minutes at 20˚C, 30˚C, 40˚C, 50˚C, 60˚C, 70˚C, 80˚C, respectively [9], and liberated reducing sugar measured as described in the amylase assay (above).

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 [9]

Effect of Substrate (Starch) Concentration on Amylase Activity

Concentrations ranging from 0.8%, 0.7%, 0.6%, 0.5 %,0.3% 0.2% and 0.1% of starch solution were used as substrates in 0.2 M citrate phosphate buffer (pH 6.0) and assayed as stated above [9].

Results and Discussion

A total of 4 bacteria were isolated and identified from the fruit peels as presented in Table 1 and Table 2. All the identified organisms belong to the Bacillus family of bacteria. The Bacillus species with higher yields were pooled together for amylase assay and characterization. Bacteria isolates were screened for

ability to produce amylolytic enzymes for 7 days. Isolates selected for the screening were those that tested positive to starch hydrolysis and the activity of the crude enzyme was assayed after every 24 hours (on each day of production).

Table 1: Biochemical identification of bacterial isolates from different decaying fruits

S/N	FRUIT WHERE ISOLATE CAME FROM	OXIDASE	METHYLRED	CATALASE	INDOLE	COAGULASE	CITRATE	STARCH HYDROLYSIS	GLUCOSE	LACTOSE	SUCROSE	FRUCTOSE	MALTOSE	SUSPECTED ORGANISM
1	APPLE 1	-	+	+	-	-	+	+	+ -	_ _	_ _	_ _	_ _	
2	APPLE 2	-	+	+	-	-	+	+	_ _	_ _	_ _	_ _	_ _	
3	APPLE 3	-	+	+	-	-	+	+	_ _	+ -	_ _	_ _	_ _	
4	BANANA 1	-	+	+	-	+	+	+	_ _	+ -	+ -	+ -	_ _	
5	BANANA 2	-	+	+	-	+	+	-	+ -	_ _	+ -	_ _	_ _	
6	BANANA 3	-	+	+	-	-	-	+	+ -	_ _	+ -	++	_ _	
7	CARROT 1	-	+	+	-	-	+	+	++	_ _	+ -	_ _	+ -	
8	CARROT 2	-	+	+	-	+	+	+	_ _	_ _	_ _	_ _	_ _	
9	CARROT 3	-	+	-	-	-	+	+	_ _	+ -	_ _	_ _	_ _	
10	W/MELON 1	-	+	+	-	+	+	+	+ -	_ _	+ -	++	_+	
11	W/MELON 2	-	+	+	-	+	+	+	+ -	_ _	+ -	_ _	++	
12	W/MELON 3	-	+	+	-	-	+	+	++	_ +	++	+ -	_+	
13	CUCUMBER 1	-	+	+	-	-	+	-	_ _	_ _	+ -	_ _	_ _	
14	CUCUMBER 2	-	+	+	-	+	+	+	+ -	+ -	+ -	++	+ -	
15	CUCUMBER 3	-	+	+	-	+	+	+	++	++	++	++	++	

KEY

A= Apple, B= Banana, CA= Carrot, CU=Cucumber, WM= Water Melon and Apple, + = positive, - = negative, ++ =presence of color change and gas production, + - = presence of color change and absence of gas production, - - = Absence of color change nor gas production, - + = Absence of color change and prsence of gas production

Table 2: Frequency of isolation of bacteria from different fruit samples

Isolate	Fruit Source	Number of Isolate	Frequency of Isolation (%)
B. *cereus*	Apple	2	10.4
	Banana	1	6.2
	Carrot	1	4.1
	Cucumber	3	2.0
	Water Melon	4	1.4
B. *anthracis*	Apple	5	12.4
	Banana	1	6.1
	Carrot	1	3.0
	Cucumber	3	2.5
	Water Melon	2	0.8
B. *subtillis*	Apple	3	9.6
	Banana	2	7.8
	Carrot	1	5.3
	Cucumber	2	3.5
	Water Melon	6	1.9
B. *olivae*	Apple	2	6.5
	Banana	2	4.2
	Carrot	3	3.1
	Cucumber	1	2.8
	Water Melon	5	1.7

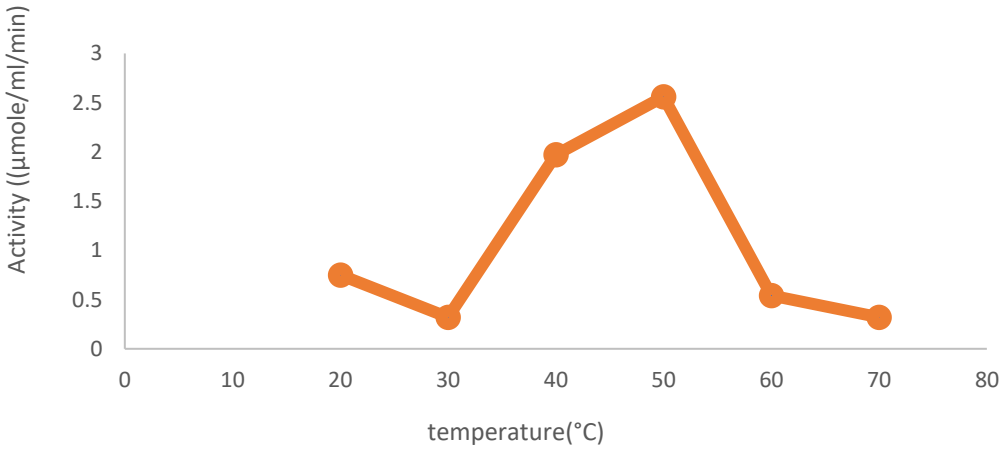

Figure 1: Effect of temperature on amylase activity

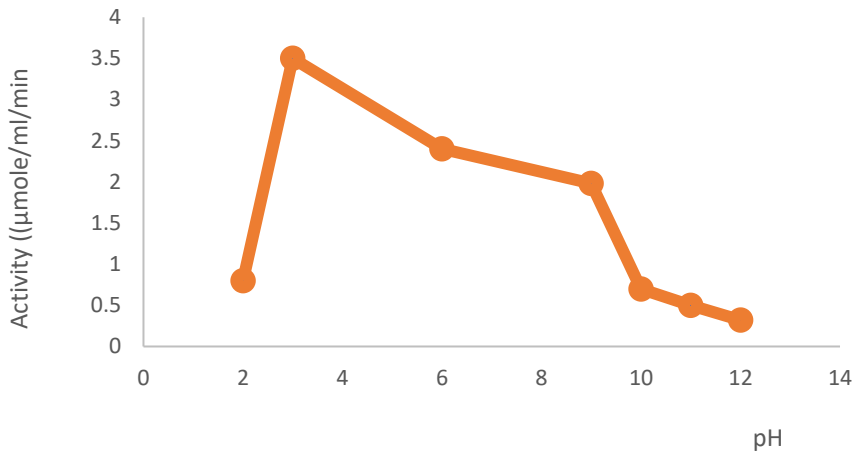

Figure 2: Effect of pH on amylase activity

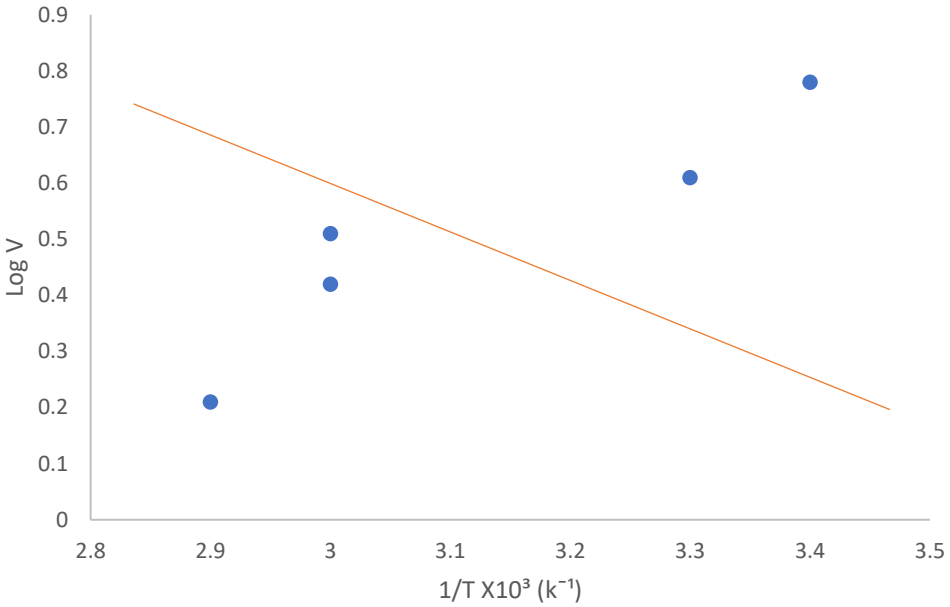

Figure 3: Arrhenius plot of partially purified amylase from *bacillus spp of some decaying fruit peels*

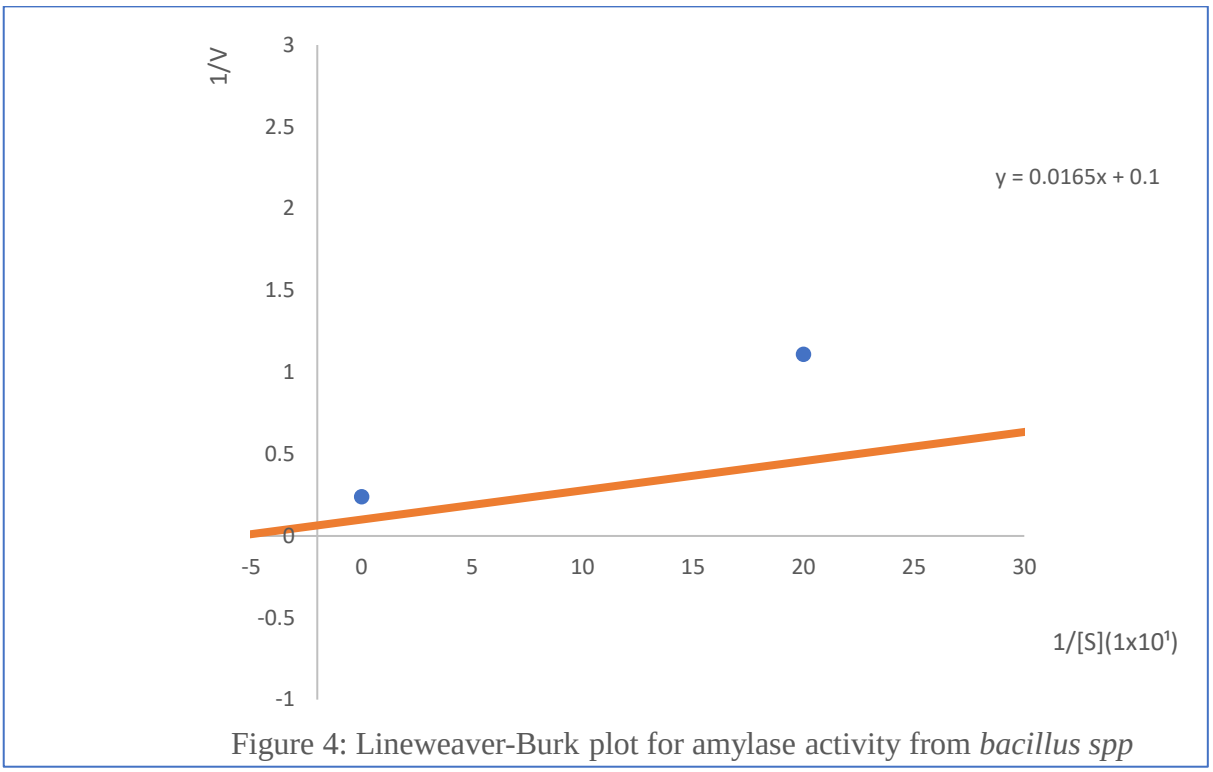

Figure 4: Lineweaver-Burk plot for amylase activity from *bacillus spp*

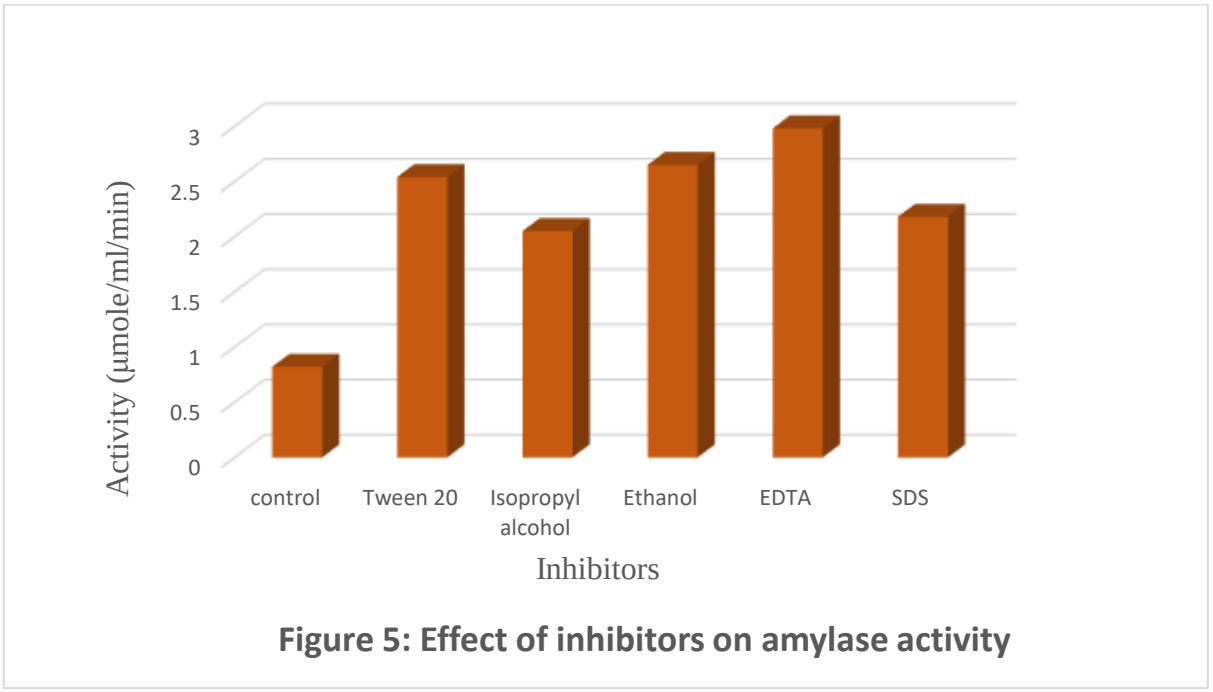

Figure 5: Effect of inhibitors on amylase activity

Discussion

Among microorganisms, Bacillus species have been discovered to be great producers of α-amylases because amylolytic enzymes are widely distributed in the bacterial kingdoms [10]. Maximum amylase production as seen from this work varies amongst the isolates. However, incubation time also influence 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 [11] who reported that maximum production of amylase occurred at 96 hours of incubation. Growth decreased in most of the bacterial isolates after 72 hours of incubation most likely due to substrate inhibition.

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 begins to decrease with further temperature increase. The amylase activity of the mixed isolate in this study had optimum temperature range of 55˚C (Figure 1). This value nearly agrees with the work of Natasa et al [12] who reported an optimum temperature of 50˚C for amylase activity. However it slightly differs from the findings of Nisa et al[13] who got maximum enzyme production at 60˚C from his work. The reduction in enzyme activity recorded at temperatures between 60˚C and above might be attributable to the denaturation of proteins by heat. However, the high activities obtained at 40˚C - 55˚C in this current work confers industrial advantage to this particular amylase. 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 [13].

The pH of a growth medium plays a major role by inducing morphological changes in the organisms and enzyme secretion. The pH change observed during the growth of the organisms also affects stability of products in the medium [14]. It's generally known that amylases are generally stable over a wide range of pH (between 4 and 11). In this study, amylase from the combined samples shows optimum activity at pH 4.0 (Figure 2). This differs from[15] who reported that Bacillus species used for the production of alpha amylases by sub-merged fermentation have an optimum pH between 6.0 and 7.0 for best growth and enzyme production. There was a drastic decline in enzyme activity in the mixed isolates which indicates that the enzyme loses activity as alkaline concentration is approached. This finding agrees with [15] 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.
Effect of heating period signifies the thermo-stability of an enzyme (Figure 3). After heating at 65˚C, the

amylase in this work was gradually becoming inhibited. The enzyme stability in this study correlates with the findings of Oyeleke and Oduwole [16] who reported that the enzyme stability of most amylases declined at temperatures above 70˚C. From this study, enzyme stability trend agrees with the behaviour of amylases from most Bacillus species. It has been widely 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 (figure 4). Popovic et al [17] assumed 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 decaying fruit peels scattered in different parts of the globe which constitute a nuisance and environmental pollution to the community may well be converted to very useful raw materials in the industries [18]. Studies on the effect of inhibitors as seen in figure 5 also explains the nature of this enzyme and its susceptibility to different substances that can inhibit its activity.

In conclusion, this work shows that bacteria isolated from peeled endocarps of decaying fruits dumped by fruit vendors in some Nigerian urban centers are capable of producing amylases which can be exploited for large scale industrial production. Since amylases are important in many industrial processes and are the most widely used enzymes in food, beverages, and recently detergent industries, it becomes imperative that novel sources are exploited as the need and opportunities for amylase is continuously increasing.

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