Current Trends in Biomedical Engineering & Biosciences
ISSN: 2572-1151

Juniper Publishers
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Research Article
Volume 19 Issue 2 - June 2019
DOI: 10.19080/CTBEB.2019.19.556010

Curr Trends Biomedical Eng & Biosci

α-Amylase Production Using *Aspergilus vadensis* Isolated from Pulverized Cocoa Seeds

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Submission: April 02, 2019; **Published:** June 11, 2019

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Abstract

Aim: α-Amylases are well known for their applications ranging from food and paper industries to pharmaceutical industries.

Materials and Methods: *Aspergillus vadensis* isolated from pulverised cocoa seed was employed in the submerged fermentation of plantain peel. The pH and temperature of the extract were measured at two days (48 hrs) interval using pH meter and sugar concentration was also determined using Fehling test. Protein content and α-amylase activity of the crude extract was determined.

Results: The pH of the crude extract decreased from 4.78 on the first day to 2.51 on day eight (192 hrs) while the temperature of the extract fluctuated between 27 °C and 28 °C. The sugar concentration in the extract increased from 3.36 mg/ml after 48 hrs to 26 mg/ml after 144 hrs and declined to 8.02 mg/ml after 192 hrs. The α-amylase activity also reached its peak (68.86 U/mg protein) on the 6th day (144 hrs) but declined to 50 U/mg protein on the 8th day (192 hrs).

Conclusion: It can be concluded that plantain peel can be employed as a cheap and readily available substrate in the production of α-amylase that is used for various biotechnologically-based industrial applications thereby, adding value to plantain and also decreasing the amount of this agro industrial waste in the environment.

Keywords: Plantain peel; α-amylase; *Aspergillus vadensis*

Introduction

α-Amylases are enzymes that are well known for their applications in starch and food, brewing, distilling, textile, paper and pharmaceutical industries [1-3]. This large range of applications is the triggering factor for the industrial production of this enzyme [4]. Presently the enzyme is one of the mostly sought after as it has great significance in biotechnology; constituting a class of industrial enzymes that controls about 25% of the world's total enzyme market [5,6]. Agro industrial wastes have been reported to be good substrates for the cost effective production of alpha amylases [2] and are thus attracting researchers for using agro industrial waste as a substrate for alpha amylase production. Agricultural wastes include but are not limited to plantain peel, wheat bran, rice husk, banana peel, vegetable waste and citrus waste. A wide range of microbes, such as bacteria and fungi are used for the industrial production of amylases [1]. The use of microorganisms for the production of amylases is economical as microbes are easy to manipulate to obtain enzymes of desired characteristics [7]. However, fungi are preferred over bacteria for enzyme production because of their filamentous nature, which helps in their penetration through solid substrate [8].

Plantain (Musa spp.) occupies a strategic position for rapid food production in Nigeria. It is ranked third among starchy staples [9]. Total world production of plantain is estimated to be over 75 million metric tons [10]. Twelve million metric tons are produced in Africa annually [11]. Nigeria is one of the largest plantain producing countries in the world. It is the largest producer in West Africa with annual production of about 2.4 million metric tons mostly obtained from the Southern States [12]. However, Nigeria does not feature among plantain exporting nations because it produces more for local consumption than for export [13]. Plantain is a major source of carbohydrate for more than 50million people [14]. Besides being the staple for many people in more humid regions, plantain is a delicacy and favoured snack for people even in other ecologies [9]. In Nigeria, the ripe fruit are processed into different forms for consumption either by boiling, frying or roasting. The peel of the fruit is discarded as waste after the inner fleshy portion has been eaten, thereby constituting a menace to the environment, especially where its consumption is common [15]. According to FAO [16] about 17,397,000 metric tonnes of plantain peels are generated in African countries.

The accumulation of the discarded peel may lead to the generation of domestic waste which could constitute health hazards in different parts of the country. Hence, this study aims at generating additional value to plantain by utilizing the peel as a substrate for the production of the enzyme amylase in a view to converting this agricultural and potentially hazardous waste into wealth.

Materials and Methods

Isolation of *Aspergillus vadensis* from cocoa

The isolate of *Aspergillus vadensis* used for this investigation was isolated from pulverised cocoa seeds. Potato dextrose agar (PDA) media was prepared, autoclaved and poured in sterile Petri dishes. One gram of the pulverised cocoa seeds was transferred into 20 mL of sterile distilled water. 0.1 mL from the mixture was transferred into the prepared PDA and the plates were incubated at 27 oC for 72 hours. Nine different isolates were observed on the incubated plates, *Aspergillus vadensis* used for this study was identified based on its physical and microscopic (Lactophenol cotton blue) characteristics [17]. This was used as a form of preliminary identification. *Aspergillus vadensis* was further sub-cultured on a freshly prepared PDA and incubated at 27 oC for 72 hours [18].

Processing of plantain peel substrate

The ripe plantain purchased from the local market was peeled off and the pulp (fruit) was separated. The peels were thoroughly washed using sterile distilled water and were cut into small pieces followed by homogenization in a blender. Water was added to facilitate the homogenization.

Sub merged fermentation

Fermentation was carried out using the modified method of Khan & Yadav [4]. Thirty gram (30 g) each of the paste of plantain peel was dispensed into eight different 250 mL conical flasks and about 70 mL of basal medium containing the following in g/l (0.8 g NaCl, 0.8 g KCl , 0.1 g $CaCl_2$, 2.0 g Na_2HPO_4, 0.2g MgSO4, 0.1 g FeSO4, 8.0g Fructose, 2.0 g NH4Cl was added. The contents in conical flasks were autoclaved, cooled at room temperature, inoculated with 1 mL of 72 hours old grown culture and incubated at 27 °C. The contents of one of the conical was not inoculated with *Aspergillus vadensis*, this was used as the control for comparison.

Determination of physical parameters

pH and temperature: The pH of the crude extract was determined using pH meter PHS -3E (Surgifriend Medicals, England) at 2 days (48 hrs) interval.

Determination of sugar concentration

Different concentrations of glucose used as standard were prepared from 5g of D-glucose dissolved in 45ml of distilled water. The sugar contents of the D-glucose and crude extract were determined, using Fehling's sugar test method. 20ml of prepared Fehling solution "A" was mixed with 20 ml of Fehling solution "B". 2ml of the Fehling solution mixture was added to 5 test tubes containing the different concentrations of D glucose used as standard, and the diluted crude. The test tubes were placed in a water bath at 60 °C for 15min after which absorbance was read by spectrophotometer at a wavelength of 500 nm.

Extraction of crude enzyme

Extraction of crude enzyme was done according to the modified method of Adejuwon [19]. Contents of the flask was filtered using Whatman no. 1 filter paper in a cold chamber at 4 °C. The extract was then subjected to cold centrifugation at 10,000 rpm for 10 minutes at 4 °C using a high speed cold centrifuge. This served as the crude enzyme. Amylase activity was determined [20]. The protein content of the preparation was determined [21].

Protein estimation in crude enzyme

Concentration of protein in crude enzyme was determined by the method of Bradford [21] in which crude enzyme was reacted with Bradford reagent and the absorbance obtained was compared with a standard graph plotted by reacting a standard protein with known concentrations with the Bradford reagent and plotting a graph between concentration of standard protein (Bovine Serum Albumin) on X axis and absorbance at 595 nm on Y axis.

α-Amylase assay in crude enzyme

α-Amylase activity was assayed using the modified method of Pfueller & Elliott [20] and Adejuwon et al. [22]. The reaction mixture was 2 mL of buffered (0.02m citrate phosphate buffer pH 6.0), soluble starch (Sigma) and 0.5 mL enzyme. The control consisted of only the prepared substrate. Incubation was at 35 °C for 30 minutes. The reaction was terminated with 3 mL of 1 N HCl. 2 mL of the terminated reaction mixture was added to 3 mL of 0.1N HCl. Colour was developed by adding 0.1ml iodine solution. Controls consisted of only 2 mL of the prepared substrate. One unit of α-amylase activity was defined as the amount of enzyme which produced 0.01% reduction in the intensity of the blue colour of the starch-iodine complex under assay conditions.

Results

Table 1: pH of the Crude Extract from Fermented Plantain Peel.

Time (hrs)	pH
0	4.78
48	4.02
96	2.83
144	2.54
192	2.51

The pH of the crude extract from the fermenting media was measured using a pH meter after every two days (48 hrs) and as shown in Table 1, the pH decreased as fermentation progresses. The temperature of the crude extract from the fermenting

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media was measured using a thermometer after every two days (48 hrs) and as shown in Table 2, the temperature fluctuated between 27 °C and 28 °C as fermentation progressed. The sugar concentration in the crude extract from the fermenting media was determined using a Fehling test after every two days and as shown in Table 3, the sugar concentration reached its peak on the sixth day after fermentation and declined on the eight day. The crude extract from the fermenting media was assayed for α-amylase activity using the method of Pfueller & Elliott [20]. This was done after every two days (48 hrs) and as shown in Table 4, α-amylase activity reached its peak on the sixth day (144 hrs) after fermentation and declined on the eight day (192 hrs).

Table 2: Temperature of the Crude Extract from Fermented Plantain Peel.

Time (hrs)	Temperature (oC)
0	27
48	28
96	28
144	27
192	27

Table 3: Sugar Concentration of the Crude Extract from the Fermented Plantain Peel.

Time (hrs)	Sugar Concentration (mg/ml)
48	3.36
96	13.5
144	26
192	8.09

Table 4: α-Amylase Activity of the Crude Enzyme from the Fermented Peel.

Time (hrs)	α-Amylase Activity (U/mg protein)
0	0
48	16.39
96	43.9
144	64.86
192	50

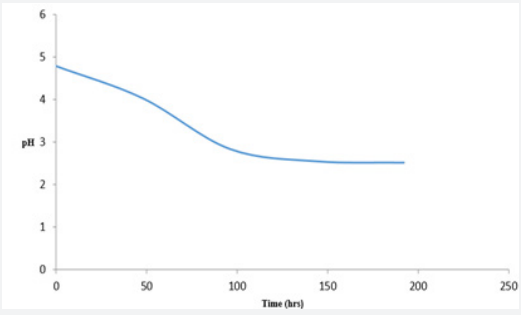

Figure 1: pH of fermented plantain peel.
pH Graph (X axis: Time in hrs; Y axis: pH)

As shown in Figure 1, the initial pH decreased from 4.78 on the first day to 2.51 on day eighth (192 hrs). This may be due to production of acid and alcohol as a result of prolonged fermentation. As shown in Figure 2, the temperature in the crude extract varied between 27 °C and 28 °C. As shown in Figure 3, the sugar concentration in the extract from the fermented peel increased from 3.6 mg/ml after 48 hrs to 26 mg/ml after 144 hrs. It declined to 8.09 mg/ml on the 8th day. As shown in Figure 4, the α-amylase activity of the crude from the fermented peel increased from 0 U/mg protein on the first day to 64 U/mg protein on the sixth day. It declined to 50 U/ mg protein on the eighth day.

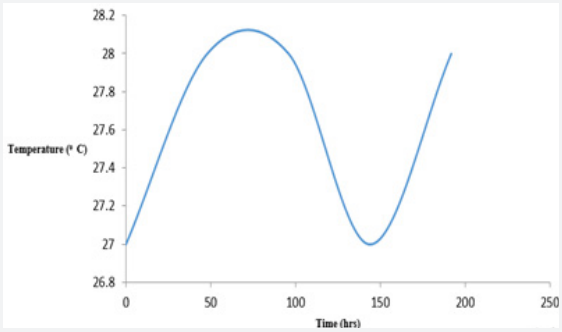

Figure 2: Temperature of fermented plantain peels.
Temperature graph (X axis: Time in hrs; Y axis: Temperature in °C)

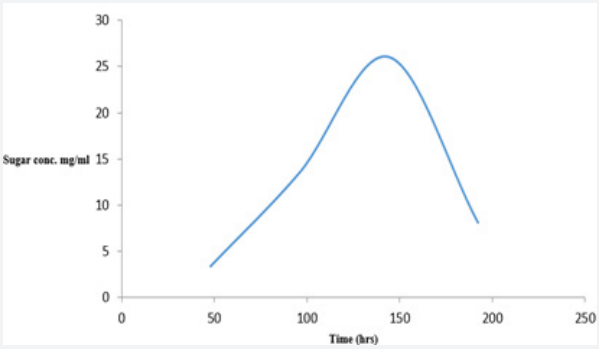

Figure 3: Sugar concentration graph.
(X axis: Time in hrs; Y axis: sugar conc. in mg/ml)

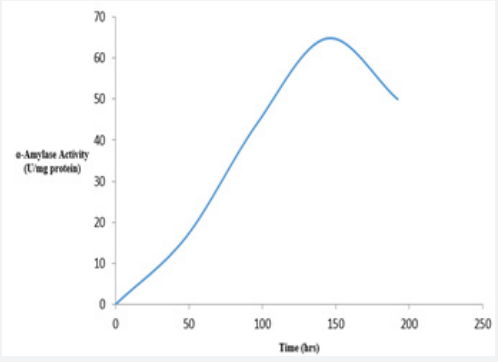

Figure 4: Amylase activity of fermented plantain peel.
Amylase activity graph (X axis: Days; Y axis: amylase activity in u/mg/protein).

Discussion

Plantain peels are by-products of the plantain-processing industry, which are normally dumped in landfills, rivers or un-

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regulated grounds [23]. The bio-accumulation of this waste may pose serious environmental problem; hence, there is a need for effective utilization of this waste. Agro industrial wastes such as banana peel and cassava peel have been reported to be good substrate for amylase production [4,24]. The expression of α-amylase activity by Aspergillus vadensis with plantain peel as the sole substrate is an indication of constitutive expression or induction of the enzyme in the fungus. α-Amylase production by microorganisms may be constitutive or inductive [25,26]. The pH in the fermenting sample declined as the fermentation progressed; this could be as a result of acid and alcohol production due to prolonged fermentation.

The sugar concentration attained its peak 26 mg/ml on day six after fermentation (144 hrs) and declined to 8.09 mg/ml on the eight day, this showed that the fungus is able to optimally utilize the sugar present in the fermenting sample on the 6th day due to the complete hydrolysis of the substrate, this reflected in the α-amylase activity which also reached its peak 64.86 U/mg protein on the 6th day.

The decline in α-amylase activity from 64.86 U/mg protein on the 6th day to 50 U/mg protein on the 8th day (192 hrs) could be attributed to the decline in the sugar concentration in the fermenting sample. It could also be attributed to depletion of nutrient and also accumulation of by-products such as toxins and inhibitors [27,28].

Elmarzugi et al. [29] reported that amylase contributed about 25-30% to a US$ 2.7 billion world enzyme market as at 2012 which is estimated to increase by 4% annually. Considering the fact that Nigeria is one of the largest producers of plantain in Africa [12], this study is important as it will not only add value to plantain and help reduce environmental problems but could also be a source of revenue for Nigeria as it looks to diversity in its economy.

Conclusion

It can be concluded that plantain peel can be employed as a cheap and readily available substrate in the production of α-amylase that is used for various industrial applications thereby, adding value to plantain and also decreasing the amount of this agro industrial waste in the environment. Future prospects of the present study include optimization of pH and optimization of the sugar concentration in order to maintain a constant production for a longer period.

Acknowledgements

Authors are grateful to the Institute of Bioorganic Chemistry and Petrochemistry of the National Academy of Sciences (NAS) of Ukraine, East Europe for Research Supports.

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