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Fruit Wastes as Substrate For the Production of Amylase by *Aspergillus niger*

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ABSTRACT

Waste generation demands that measures must be put in place in order to avert their detrimental effect to the environment. Bioconversion of agricultural waste to useful products like enzyme is a welcome development. Amylase production by *Aspergillus niger* via submerged fermentation of fruit wastes such as pineapple, orange, banana, cucumber and watermelon was investigated. Biomass of *A. niger*, amylase produced and pH of the fermenting fruit waste media were determined using standard techniques during submerged fermentation. From the data obtained Banana waste medium produced the highest yield of *A. niger* amylase (259.00 ± 1.23 U/mL) compared to other waste media at 4 d of fermentation. Amylase yield from Banana waste medium was significantly higher compared with the other fruit wastes ($p < 0.05$). Among the various supplemented nitrogen sources in the Banana waste medium, ammonium nitrate gave the highest amylase yield of 881.00 ± 16.97 U/mL while the least (549.5 ± 20.51 U/mL) was from potassium nitrate medium. Thus, the study revealed that *A. niger* can be used for amylase production from fruit waste and optimal yield can be enhanced by supplementing the medium with ammonium nitrate.

Keywords: *Amylase, Banana waste, Aspergillus niger, Submerged fermentation, Optimization*

Introduction

Recent years have seen the global intensification of food production resulting in the production of large quantities of food and generation of a lot of agricultural wastes.[1] Fruit wastes are discarded peels from various fruits to the environment and regarded as no longer important for consumption. However, these discarded peels can cause pollution which can affect the environment and cause various infectious diseases due to growth of pathogenic microorganisms.[2] These fruit wastes are rich in organic matters that are natural substrates for microorganisms. Bioconversion of this cheap available substrate to useful enzymes, a value-added product, will help in reducing pollution caused by the waste.[3]

Enzymes are biological catalysts (protein) produced by living cells and are useful in synthesis and degradative processes.[4,5] The limitations of using chemical catalysts were overcome by the use of enzymes. Enzymes work at milder conditions, are highly specific and catalyze reactions faster than chemical catalysts.[6] Enzymes are used in food and beverage production (e.g, yoghurt, cheese, and wine). Industrially, useful enzymes produced by microorganisms are becoming a major focus for researchers.[5]

Current interest in research has been shifted to the potential of utilizing agricultural wastes for enzyme production.[7] In this study, fruit wastes were exploited for amylase production due to their availability and cost effectiveness. Amylase is a well-known and important industrial enzyme that is used during the hydrolysis of starch and glycogen.[6] Plants, animals and microorganisms can be of several sources for amylase production. Amylases obtained from microorganisms have greater industrial applications and are more stable compared to that produced from plants and animals.[8] Among microorganisms, *Aspergillus* species are the most exploited fungi, especially *A. niger*.[9, 10] The fungus, *A. niger* has been studied extensively for amylase production because of the ubiquitous nature and non-fastidious nutritional requirement.[8] Oshoma and Ikenebomeh [11] reported that *A. niger* had high amylolytic activity in cell biomass production. Sundar *et al*.[8] stated that amylase production capacity of *A. niger* is due to its tolerance to acidic pH that discouraged bacterial contamination. Also, thermal stability of the fungus amylase is a desirable feature for economic viability of enzymatic processes.

Amylases have found their way in so many useful processes such as starch liquefaction, paper production, desizing of textile fabrics, in preparing starch coatings of paints, in removing wall paper, food and brewing industries and pharmaceutical industries.[8, 12] To meet the demands of these industries, low cost medium is required for the production of amylases.[13] Nowadays the potential of using fungi as a biological source of industrially economic enzymes has stimulated interest in the exploitation of extracellular enzymatic activity in several fungi. On this premise, the study was aimed at investigating different fruit wastes, such as Banana, cucumber, orange, pineapple and watermelon peels, as an alternative raw material (substrate) for cost effective fungal amylase production. The effect of various nitrogen sources on amylase yield was also studied, since nitrogen compounds have been found to be an essential macronutrient for nucleic acids and protein biosynthesis.

Materials and Methods

Sample Collection and Preparation of Fermentation Medium

Banana, cucumber, orange, pineapple and watermelon fruit wastes were collected from the fruit section of Uselu market in Ugbowo area of Benin City, Edo state, Nigeria. These fruit wastes (peels) were washed several times with sterile, distilled water and sun dried for 5 d. The various sun-dried fruit wastes were weighed and blended using a Blender separately with distilled water in the ratio 1:4 (w/v) according to modified procedure of Krishna *et al*.[6] The blended fruit wastes were passed through muslin

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cloth to trap solids leaving behind filtrate as fruit waste broth. From the fruit waste broth, 100 mL was transferred in to 250 mL Erlenmeyer flasks. The samples thus prepared were autoclaved at 121ºC for 15 min. Samples were prepared in duplicates and were designated Fruit Waste Medium (FWM).

Isolation and Inoculum Preparation of Aspergillus niger

Aspergillus niger was isolated from Onion left at room temperature to undergo spoilage. The fungal isolate was isolated based on cultural and microscopy characterization following standard methods[14,15] and maintained on potato dextrose agar (PDA) slant and stored at 4ºC.

Inoculum was prepared from a subcultured *Aspergillus niger* on potato dextrose agar (PDA) plates and incubated for 5 d. The *Aspergillus niger* cultured plate was flooded with 10 mL of sterile 1% $^{v}/_{v}$ tween 80 solution to dislodge the spores from the hyphae. The solution with spores was filtered with a sterile muslin cloth to remove any hyphal fragments present.[16] The number of spores was counted using a haemocytometer and inoculum size of 10^6 spore/mL was used to inoculate all the media.

Fermentation process

Submerged fermentation was carried out at room temperature of 28 ± 2ºC on an orbital shaker at a speed of 120 rpm using the fruit waste media. The media were designated as Cucumber waste medium (CWM), Orange waste medium (OWM), Banana waste medium (BWM), Pineapple waste medium (PWM) and Watermelon waste medium (WWM).

The effect of different nitrogen supplements on amylase production was investigated. The nitrogen sources employed were ammonium chloride (NH_4Cl), potassium nitrate (KNO_3), ammonium sulphate ($(NH_4)_2SO_4$), ammonium nitrate (NH_4NO_3) and sodium nitrate ($NaNO_3$) in the concentration of 1.6, 3.0, 2.0, 1.2 and 2.6 g/L respectively for each to supply 0.42 gN/L in Banana waste medium according to modified method of Ikenebomeh and Chikwendu.[16]

The initial pH of the different nitrogen supplemented media was adjusted to 4.5 using 1N H_2SO_4 and/or 1N NaOH. Each nitrogen sources medium (100 mL) was transferred into 250 mL Erlenmeyer flask and sterilized at 121ºC for 15 min. The medium in each flask was inoculated with 500 uL of *A. niger* inoculum (10^6 spores/mL). The nitrogen sources media were left to ferment on an orbital shaker at 120 rpm at temperature of 28± 2 ºC followed by determination of fungal biomass, amylase production and pH of the fermenting broth at every 2 d interval for 6 d.

Analytical methods of the fermented media

Determination of fungal biomass, amylase produced and pH of the fermented fruit waste media were carried out after 48 h interval for 6 d. The fermenting broth was pasteurized at 65ºC for 30 min in a water bath at 2 d interval. The fungal mycelia were collected through filtration using a pre-weighed Whatman No 1 Filter paper and washed twice with 50 mL sterile distilled water. The collected fungal biomass on the filter paper was dried at 90ºC in a Genlab hot air oven (YIA 110 model, England) to a constant weight.

Amylase activity was assayed by measurement of glucose released from starch as described by Ramakirshna *et al*. [17] using a reaction mixture comprising of 1 mL of crude enzyme (from the fermented broth), 1 mL of 1% starch solution and 0.1 mL of citrate buffer solution (pH 4.5). The reaction mixture in a 10-mL test tube was incubated at 60°C for 1 h and the reaction was terminated by immersing the reaction tube in boiling water (100°C) for 2 min. The reducing sugars liberated were estimated by the DNS methods.[18] One (01) unit of amylase activity (U) was defined as the amount of enzyme that liberated 1.0 µmole of D-glucose from starch in 1.0 µL reaction mixture under the assay conditions. Determination of pH was through the use of pH meter (3305 Jenway, England).

Statistical analysis

All assays were carried out in duplicates, means and standard deviations (SD) were determined using SPSS version 23. However, t-test was used for statistical comparison of the data for amylase production from the different media.

Results and Discussion

The isolated fungus obtained from Onions was related mainly to the generic nomenclature *Aspergillus* known as *Aspergillus niger*.

Production of amylase by *Aspergillus niger* was carried out in a prepared fruit waste media. The time course of growth, pH and amylase produced were analyzed during fermentation. Five fruit (Banana, Cucumber, Orange, Pineapple and Watermelon) wastes filtrate media were used as the fermentation substrate. The various fruit waste media were found to support the growth of *A. niger* as shown in Fig. 1. *A. niger* biomass was observed to be highest (2.29 ± 0.02 g/L) when BWM was used at 6 d of fermentation and the lowest value (1.13 ± 0.02 g/L) was from CWM.

The composition and concentration of medium affect growth of microorganisms which invariably affect enzyme production.[19] For maximum utilization of microbes, focus should be optimization of media conditions such as carbon sources, nitrogen sources, pH, temperature and incubation period.[19, 20] Fermentation medium is believed to be an ideal one if production of specific metabolite is enhanced. In this study, *A. niger* was found to produce maximum yield of dry fungal biomass and amylase when Banana waste medium was used as substrate.

The levels of amylase production were observed to be dependent on the fruit waste medium used. Amylase produced was found to have the highest yield at 4 d of fermentation for all the media (Fig. 2). The highest amylase of 259.00 ± 1.23 U/mL was produced from BWM followed by OWM (235.00 ± 5.57U/mL) while the least (191.40 ± 1.07 U/mL) was from CWM at 6 d.

The result in amylase production by the fruit wastes revealed that the five fruit waste media with their chemical composition had potentials as substrate for *A. niger* and production of amylase. Among the fruit waste media screened, Banana waste medium demonstrated to be the best substrate for *A. niger* growth and amylase production. Banana waste has been reported as a promising substrate for microbial growth,[6] and the probable reason may be that the medium is rich in nutritional composition such as carbohydrate and protein than other fruit wastes.[21] Since this agricultural waste is a cheap raw material, it should be utilized for the production of amylase.

Sundarram *et al*.[22] reported that duration of fermentation is one of the critical factors in the process of amylase production. Short fermentation duration offers a potential for inexpensive enzyme production.[23] The enzyme production increases as the growth time progresses, until it reaches the optimum duration and decline afterward due to depletion of nutrient in the medium.[6] The study agreed with the report of Ali *et al*.[5] that *Aspergillus flavus* gave a highest yield of amylase after incubation for 96 h. Maximum amylase production was recorded after 5 d of fermentation.[24] Decline in amylase yield after 4 d of fermentation could be nutrient exhaustion, production of other metabolites and enzyme denaturation due to possible interaction with other medium components used.[6, 19]

During fermentation, the pH was found to increase during time course in some media while there was a drop in some media at initial fermentation duration (Table 1). The pH values of OWM and PWM increased to 5.8 ± 0.07 and 6.60 ± 0.00 at 2 d, there after decreased to 3.30 ± 0.00 and 3.80 ± 0.00 respectively. In studying the effects of various nitrogen supplements on amylase production, pH of the supplemented Banana waste media was also analyzed at 4 d of fermentation. The pH values from the nitrogen sources showed an increased from the initial pH of 4.5 (Table 2). The highest and lowest final pH recorded at 4 d of fermentation were 5.5 ± 0.21 and 4.8 ± 0.07 for NH_4NO_3 and $(NH_4)_2SO_4$ media respectively. One of the critical factors for enzyme production stability is an optimum pH of a medium known to induce fungi morphological changes and

Table 1: pH value of the different fruit waste medium during fermentation.

Day	BWM	CWM	OWM	PWM	WWM
0	4.2 ± 0.14	6.4 ± 0.00	4.6 ± 0.00	5.4 ± 0.14	5.3 ± 0.14
2	5.1 ± 0.07	6.7 ± 0.14	5.8 ± 0.07	6.6 ± 0.00	5.7 ± 0.07
4	5.6 ± 0.07	8.3 ± 0.07	3.9 ± 0.14	3.9 ± 0.14	6.7 ± 0.07
6	5.7 ± 0.00	8.5 ± 0.07	3.3 ± 0.00	3.8 ± 0.00	5.2 ± 0.07

Values are means ± standard deviations of duplicate determination. BWM = Banana waste medium, CWM = Cucumber waste medium, OWM = Orange waste medium, WWM = Watermelon waste medium and PWM = Pineapple waste medium.

Table 2: Changes in pH values of nitrogen sources media at 4 d fermentation period using Banana waste media.

Day	$(NH_4)_2SO_4$	NH_4Cl	$NaNO_3$	NH_4NO_3	KNO_3
0	4.5 ± 0.07	4.5 ± 0.07	4.5 ± 0.00	4.5 ± 0.07	4.5 ± 0.14
4	4.8 ± 0.07	4.9 ± 0.21	5.0 ± 0.00	5.5 ± 0.21	5.1 ± 0.07

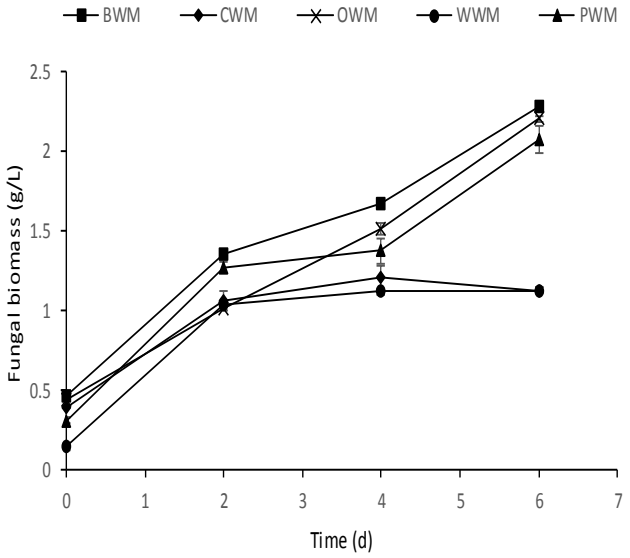

Figure 1: Biomass yield of *Aspergillus niger* in the various fruit waste media in submerged fermentation at 120 rpm on a time course basis. (BWM = Banana waste medium, CWM = Cucumber waste medium, OWM = Orange waste medium, WWM = Watermelon waste medium and PWM = Pineapple waste medium).

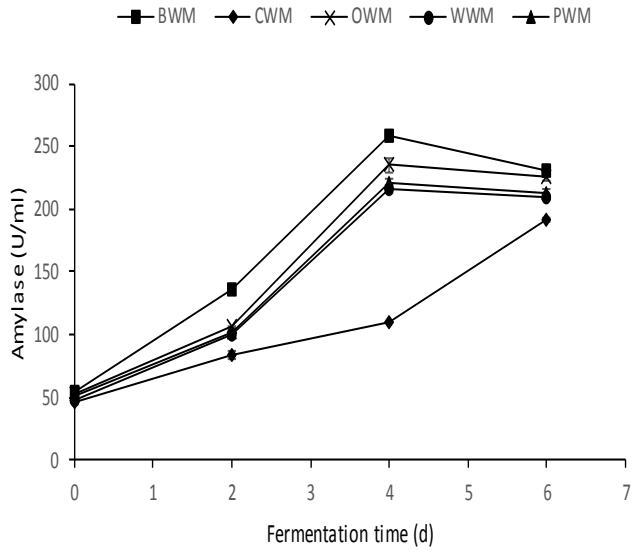

Figure 2: Amylase yield of *A. niger* using various fruit waste media in submerged fermentation at 120 rpm on a time course basis. (BWM = Banana waste medium, CWM = Cucumber waste medium, OWM = Orange waste medium, WWM = Watermelon waste medium and PWM = Pineapple waste medium).

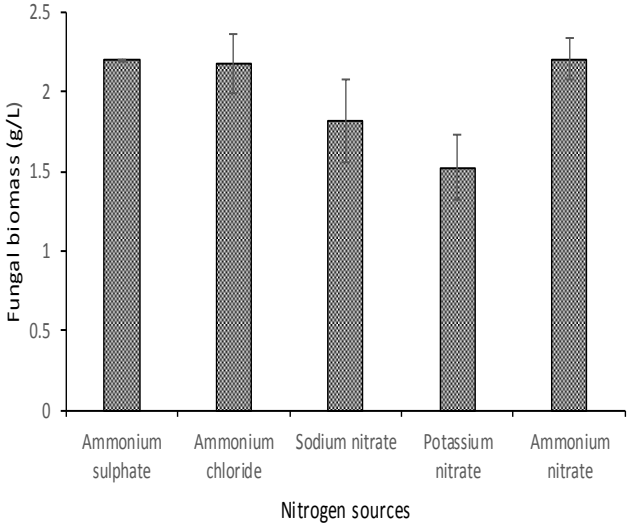

Figure 3: Biomass yield of *A. niger* using various nitrogen supplemented Banana waste media at 4 d fermentation period at 120 rpm and 28 ± 2ºC.

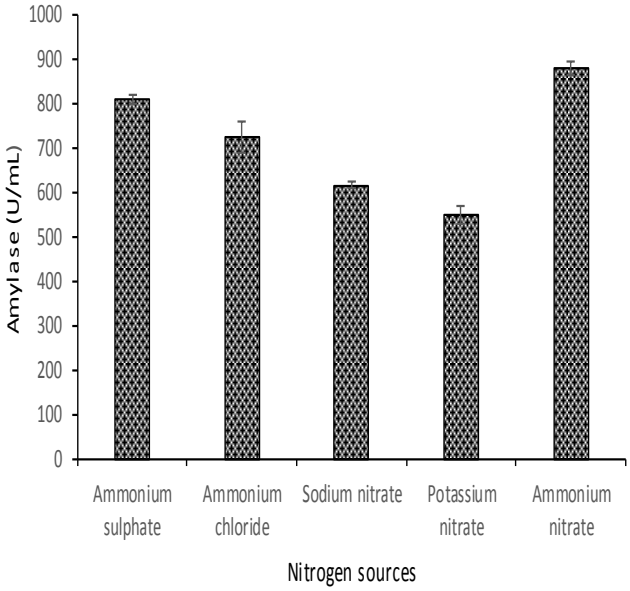

Figure 4: The effects of various nitrogen supplemented Banana waste media on *A. niger* amylase yield at 4 d fermentation period at 120 rpm and 28 ± 2ºC.

enzyme secretion.[5] Care must be taken to get the right pH during production due to enzyme sensitivity to pH.[22] Optimal pH for amylase production by *Aspergillus* species is in the range of 5 to 6.[10, 22] However, Ali *et al*.[5] reported that optimum pH for amylase production by *Aspergillus* spp. was in the range of 4.5 – 5.0 for a duration of 4 – 5 d of fermentation. Hernandez *et al*.[9] reported that acidic pH favours amylase production by *A. niger*. A deviation from optimal pH of an organism can result to poor microbial growth. Thus, the pH value that favours the highest amylase production was found to be within the stated optimal.

The effect of media supplemented with nitrogen sources on *A. niger* growth and amylase production was studied using BWM, which previously showed the highest amylase yield than other fruit waste media investigated. In this study, various nitrogen sources such as KNO_3, NH_4NO_3, NH_4Cl, $NaNO_3$ and $(NH_4)_2SO_4$ were investigated. Dried *A. niger* biomass cropped among the nitrogen sources is shown in Fig.3 at 4 d of fermentation. In all the nitrogen supplementation, NH_4NO_3 medium cropped the highest biomass yield of 2.21 ± 0.13 g/L followed by $(NH_4)_2SO_4$ medium (2.20 ± 0.01 g/L) but, statistically not significant ($p > 0.05$). Medium supplemented with KNO_3 gave the least biomass yield of 1.53 ± 0.20 g/L and statistically significant ($p < 0.05$) to that of NH_4NO_3 and $(NH_4)_2SO_4$ media. The highest amount of amylase produced with the various nitrogen sources was the medium supplemented with NH_4NO_3 followed by $(NH_4)_2SO_4$ and the least was KNO_3 with values of 881.00 ± 16.97, 808.00±9.89 and 549.5±20.51 U/mL respectively as shown in Fig. 4.

Media supplementation by nitrogen sources is known to enhance enzyme production such as amylase[10] which is one of the optimization parameters for a cost effective amylase production.[25] Commonly used inorganic nitrogen sources are $(NH_4)_2SO_4$, NH_4NO_3 and NH_4Cl.[22] High yields of *A. niger* biomass were obtained with the different nitrogen sources and NH_4NO_3 gave the highest biomass yield. Banana waste is poor in chemical composition such as protein content,[26] supplementation of media with nitrogen would enhance fungal growth, worthy of note is that ammonium salts are stimulators for microbial growth and amylase production.[19]

Several authors had reported that medium supplemented with NH_4NO_3 as nitrogen source gave the highest yield of biomass and amylase by *A. niger* in submerged fermentation. [8-10, 25] Thus, confirming our findings that medium supplemented with NH_4NO_3 enhanced fungal growth which in turns increased biomass cropped and amylase activity in the food waste medium.

Conclusion

This study suggests that Banana waste could be utilized as a promising substrate for *A. niger* growth and amylase production. Hence, the Banana waste medium should be employed as a substrate for the production of amylase rather than allowing it to cause environmental pollution. Supplementation of the Banana waste medium with ammonium nitrate is found to be beneficial for enhancing enzyme production yield.

Conflict of interest

The authors declare no conflict of interest.

Authors' declaration

The authors hereby declare that the work presented in this article are original and that any liability for claims relating to the content of this article will be borne by them.

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