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Degradation of Theobromine in Cocoa (*Theobroma cacao*) by-products by Fermentation with *Aspergillus niger*

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Authors' contributions

This work was carried out in collaboration between both authors. Both authors read and approved the final manuscript.

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ABSTRACT

Aim: This study was carried out to determine the ability of *Aspergillus niger* to break down theobromine in cocoa by-products, for its possible use in animal feeds.
Place and Duration of Study: Department of Biological Sciences, Wesley University, Ondo, Ondo State, Nigeria between January 2013 and June 2013.
Methodology: Cocoa by-products (combination of cocoa bean shell (CBS) and cocoa bean meal (CBM)) was milled into fine particle size, sterilized and fermented with *Aspergillus niger* for 5 days. The theobromine content was monitored at 24 h interval during fermentation and proximate analyses were carried out before and after the fermentation process.
Results: Significant reduction of theobromine content of 78.13% was observed after 5 days of fermentation. The crude protein and crude ash content increased by 2.7% and 51.9%, while the moisture content and crude fibre reduced by 21.3% and 23.8%, respectively.
Conclusion: The study showed that *Aspergillus niger* has the potential to reduce the theobromine content and enhance the nutritive value of cocoa by-products, for possible use in animal feed.

Keywords: Cocoa by-products; theobromine; Aspergillus niger; fermentation; animal feeds.

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1. INTRODUCTION

The high cost of livestock feed is mainly due to the fact that, the main components of the feeds also serves as food for man as well [1,2]. Due to high human population growth rate over the years, there has been increase in price and need for conventional animal feedstuffs such as groundnut cake, wheat, soybean meal, cocoa meal etc. Therefore, it is important to search for alternative feed ingredients to reduce the competition between man and livestock [3,4]. According to Teguia and Beyen [5], Ayinde et al. [6], sourcing for alternative feed from agro-industrial wastes such as: cassava peels, cocoa husks, cocoa beans shell, cocoa bean meal, maize cobs, and wheat offal could reduce the high cost and enhance the production of compounded animal feed.

Nigeria is one of the leading producers of cocoa in Africa and huge amount of by-products are generated from cocoa processing. The by-products such as cocoa bean shell (CBS) is rich in protein, which is an important component of animal feeds [7].

The presence of theobromine (the most abundant methylxanthine) in cocoa by-products has restricted it utilization as feed component for monogastric and ruminant animals [8,9,10]. Optimizing the use of cocoa pod husk, cocoa bean meal, cocoa shell therefore requires de-theobromination to detoxify the material [11].

The use of cocoa by-products as feed would, therefore, require the development of affordable, efficient procedures for the elimination of theobromine. Chemical methods are available for the removal of theobromine but are inappropriate because they require the use of expensive, sophisticated equipment, solvents and adsorbents [7,12,13]. Although, [14] reported the de-theobromination of CBS as time dependent but prolong boiling could render the shell less nutritive because some sugars and protein would have been lost in the process. Also, alkali treatment could result in significant reduction in protein content [7,14]. Over the years, fermentation has been known to enhance the nutritive value of food products. Bio-processing methods are more affordable and environment-friendly [15]. Highly fibrous agro-waste can be useful to animals by fermentation with certain fungi, capable of improving protein quality and fiber digestibility and aids in degrading anti-nutritional factors [16].

However, *Aspergillus niger* has proven to be capable of degrading alkaloids such as theobromine when used in the fermentation of cocoa by-products. The fungus utilize the theobromine as its sole carbon and energy sources [15].

This research work monitors the level of de-theobromination during fermentation of cocoa by-products with *Aspergillus niger* and the proximate analysis before and after fermentation.

2. MATERIALS AND METHODS

2.1 Collection of Sample and Sample Preparation

The mixed Cocoa bean shells (CBS) and Cocoa bean meals (CBM) in ratio 50:50 were obtained from Stamark Cocoa Industry, Ondo, Ondo State, Southwest Nigeria.

The sample was dried and milled into fine particles using electrical milling machine. The milled samples were then brought into the laboratory for further analysis.

2.2 Isolation and Identification of Fungi Spores

Fungi were isolated from the cocoa by-products by pour plate technique and were plated on potato dextrose agar (PDA) (Difco Lab.). This was done in duplicate and the plates were incubated at 25°C for 6 days. After incubation, the plates were observed for fungal growth. Light microscope was used to identify the fungal isolate based on their colonial and cellular morphological characteristics [7]. The fungus with black spores and conidial production was identified as *Aspergillus niger* and was used for further work.

2.3 Preparation of Pure Cultures of *A. niger* and Spore Suspension

The preparation of pure cultures and spore suspension was carried out according to the method described by Bentil [17]. The identified *A. niger* spores were inoculated into sterile petri dishes containing PDA and duplicated into twenty-one (21) petri dishes. The plates were incubated at 25°C for six days and a sterile scrapper was used to scrape the pure fungi spores from each of the inoculated plates. After

which the spores (*A. niger*), were harvested by flooding each of the cultured plates into 250 ml Erlenmeyer flask, containing 250 ml sterile distilled water.

2.4 Determination of Spore Concentration

The determination of spore concentration was as described by Bentil [17]. One milliliter of the spore suspension was taken from the stock into a sterile conical flask containing 100 ml of sterile distilled water. The diluted suspension was thoroughly mixed and 1 ml was taken using sterile pipette and released into the grooves of a haemacytometer and placed under a light microscope for the counting of the spores. The spores were counted within the regions of the haemacytometer and the average taken. The average value was then multiplied by 10^4.

2.5 Fermentation Procedure

Ratio 50:50 ground mixture of the cocoa bean shells (CBS) and cocoa bean meal (CBM) were used as substrate for the fermentation process. One kilogram of the substrate was composted with sterile distilled water for 3 days, thereafter wrapped in aluminum foil and sterilize in an autoclave at 121°C for 15 minutes. One hundred grams of the sterile substrate was inoculated with 100 ml of the spore suspension [17]. A control experiment was set up without inoculation with *A. niger*. The inoculated and the un-inoculated substrates were incubated at 25°C for fermentation for five days. At the end of each day, the temperature and pH was monitored and a portion was taken and dried in an oven at 60°C for two days to inactivate the *A. niger*. The dried substrates were then prepared for theobromine determination. Analysis was carried out in duplicates.

2.6 Theobromine Determination

The theobromine content in the cocoa by-products before and after the fermentation process was carried out as described by Adamafio [7], Bentil [17]. 0.5 gram of each of the samples was weighed and dissolved in 100 ml of distilled water containing 1.5 ml of 0.10N sulphuric acid by heating almost to boiling and the temperature maintained for 5 minutes. The content was then cooled to 40°C and then 1.5 ml of phenol red indicator solution was added. A measured volume of approximately 1.5 ml of 0.10N sodium hydroxide was added until the solution turned pink. The colour was then brought back to yellow by the careful addition of 0.10N sulphuric acid. A measured volume of 40 ml of 0.10N silver nitrate was added which was neutral to phenol red. The solution was then titrated with 0.10N sodium hydroxide until the pink colour returned after the end-point with a drop of the alkali solution. The equivalent amount of sodium hydroxide to the theobromine in the sample was determined by the equation:

> 1 ml of 0.10N sodium hydroxide =18.01 mg of theobromine

The analysis was carried out in duplicate.

2.7 Proximate Analysis

The proximate analysis that was carried out on both the fermented and the unfermented samples include crude protein, crude fiber, ether extract (crude fat), ash and moisture content according to the method describe by AOAC [18].

2.8 Data Analysis

The theobromine breakdown result was presented as mean standard values of duplicates. One-way analysis of variance (ANOVA) was carried out at $P < 0.05$ level of significance.

3. RESULTS AND DISCUSSION

Fig. 1 shows the temperature of the substrates during fermentation with *A. niger*. Increase in temperature was observed after 24 h from 30.6°C to 39°C at 72 h and thereafter reduced to 33°C at 92 h and remain constant. The increase in temperature in the inoculated sample when compared with the un-inoculated sample might be as a result of fermentation processes taking place in the inoculated sample. Constant temperature (33°C) observed after 92 h of fermentation could likely be the optimum temperature for the degradation of theobromine by the fungus because more theobromine was degraded at this temperature. The pH of the substrate during fermentation reduced slightly (Fig. 2). The pH was within the standard pH of 6.5-7.5. The decrease in pH signifies that there is increase in acidity as the fermentation proceeds. The level of theobromine in the mixed sample of CBS and CBM was decreased significantly, when fermented with *A. niger* as

compared to the un-inoculated sample (control) (Fig. 3). There was a reduction of 78.13% theobromine content after fermentation for five days.

This result agrees with the result of Adamafio et al. [15], who reported 71.8% reduction in theobromine of cocoa pod husks fermented with *A.* niger after seven day of fermentation. Muhammed et al. [19] also reported 24.5% reduction of theobromine content of CBS fermented with *Pleurotus pulmonarius* after four weeks of fermentation. Increased growth rate of 12.2% was observed in albino rats fed with P. *pulmonarius* fermented CBS compared with 9.1% growth rate in untreated rats [7,19].

Table 1 shows the proximate composition of the mixed by- products (CBS and CBM) before and after fermentation. The moisture content reduced by 21.3% and there was slight increase in the crude protein content (2.7%). The composition of protein in the CBS is consistent with the report of [7]. At the end of the fermentation process, there was a significant decline in the crude fiber content from 38.60% to 29.40%. The crude ash content increased after the fermentation (51.9%), as compared with the unfermented sample. The moisture content of a feed material gives an indication of the extent to which the nutritive value of the feed material can be maintained i.e. its shelf life. The decline of the moisture content from 7.75% to 6.1% could be due to the ability of the fungi to grow and utilize the water and nutrient content for growth [17]. Low moisture content is therefore required for a longer shelf life and the recommended level for foodstuffs should not be higher than 12% [17]. The decline in the fiber content could be due to reduced fiber components such as cellulose. This is in accordance with the report of [1]. The decrease in fiber content is an indication of *A. niger* having enzymatic system for degradation of polymeric lignocellulose of cocoa by-products (CBP) [20]. The observed increase in ash could be as a result of decrease in some organic fractions of the CBP during the bioconversion process thereby enhancing the substrate [20].

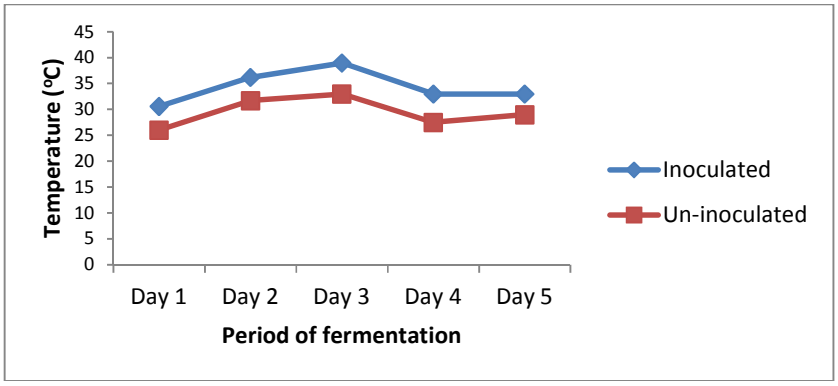

Fig. 1. Temperature of substrate during fermentation

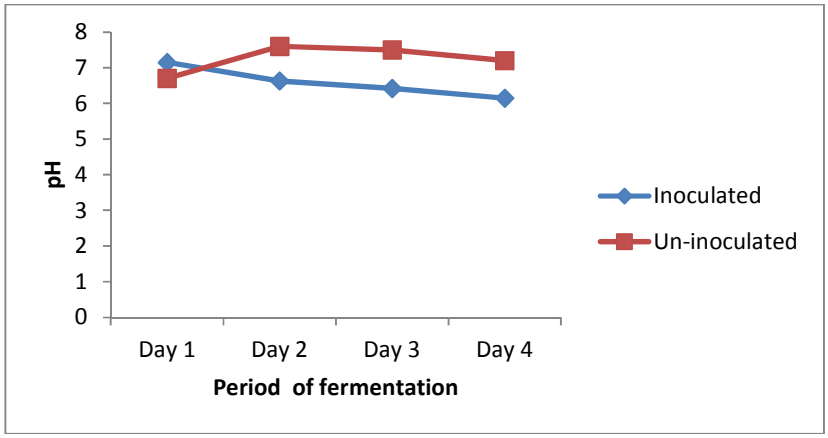

Fig. 2. pH of substrate during fermentation

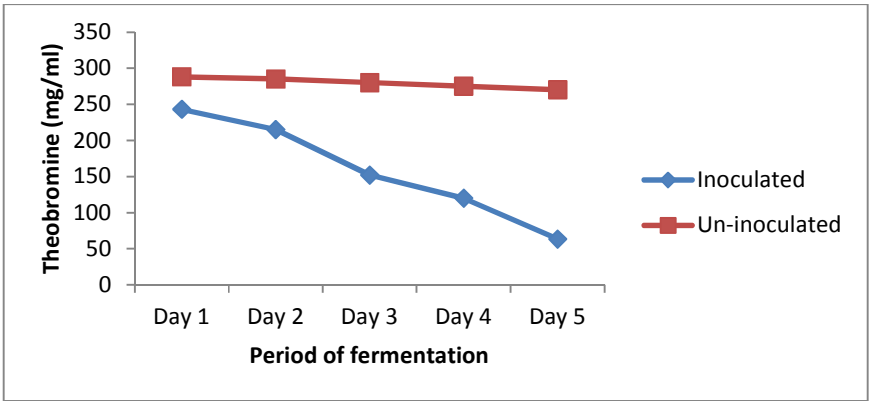

Fig. 3. Degradation of theobromine by *Aspergillus niger*

Table 1. Proximate analyses of the Cocoa by-products

Proximate	Un-inoculated substrate (%)	Inoculated substrate (%)
Moisture content	7.75	6.10
Crude protein	14.60	15.00
Ether extract	20.43	16.40
Crude fibre	38.60	29.40
Ash content	8.10	12.30
Nitrogen –free extract	10.52	20.80

The difference in the theobromine content of the treated and untreated sample after 5 days of fermentation, suggested that *A. niger* might be responsible for the degradation of theobromine, because there is significant difference between the treated and the untreated sample.

4. CONCLUSION

This study showed that the theobromine content in cocoa by-products (cocoa bean shell and cocoa bean meal) inoculated with *Aspergillus niger* was reduced by more than 78% compared with the un-inoculated sample. The isolate was also able to increase the crude protein and crude ash contents during the fermentation process. Therefore, the isolate can be further improved, for it possible use in the breakdown of theobromine in cocoa by-products for use in animal feed.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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