



Asian Journal of **Biochemistry**

ISSN 1815-9923



Academic
Journals Inc.

www.academicjournals.com

Microbial Detheobromination of Cocoa (*Theobroma cacao*) Pod Husk

¹N.A. Adamafo, ¹F. Ayombil and ²K. Tano-Debrah

¹Department of Biochemistry, University of Ghana, Legon, Ghana

²Department of Nutrition and Food Science, University of Ghana, Legon, Ghana

Corresponding Author: Naa A. Adamafo, Department of Biochemistry, University of Ghana, P.O. Box LG 54, Legon, Ghana

ABSTRACT

Cocoa pod husk, when used in animal feed, exerts certain detrimental effects on animal health at high dietary intake, due to the high concentrations of theobromine. The chemical methods for detheobromination are not feasible in conventional agricultural practice and, therefore, are not used by local farmers. This study investigated the biodegradation of theobromine in cocoa pod husk (bio-detheobromination). Milled cocoa pod husk samples, sterilized and unsterilized, were treated and incubated for 7 days and the theobromine content followed during the period titrimetrically at 24 h intervals. A 54.7% reduction in theobromine content ($p < 0.05$) was observed after 5 days. The microflora of the cocoa pod husk was analyzed to determine the microbial species associated with the detheobromination. Using a 4% selective theobromine medium, in which theobromine was the sole carbon and nitrogen source, *Aspergillus niger* was identified as the only microorganism present capable of metabolizing theobromine. Treatment of sterilized cocoa pod husk with *A. niger* for 7 days resulted in a 71.8% reduction in theobromine content ($p < 0.05$). The observations suggest that fermentation of cocoa pod husk may be a useful procedure for reducing the theobromine content of cocoa pod husk to levels safe for livestock consumption.

Key words: Animal feed, cocoa pod husk, detheobromination, *Aspergillus*, fermentation

INTRODUCTION

Ghana, a major cocoa (*Theobroma cacao*) producing country, generates vast quantities of cocoa pod husk annually. Although a small fraction of it is converted into useful products, most of it is not utilized. In a country where animal production suffers greatly from inadequate dry season feed, it would be prudent to optimize the use of cocoa pod husk as animal feed. The use of cocoa pod husk as a supplementary feed ingredient for ruminants and monogastric animals is constrained by the presence of theobromine, the most abundant methylxanthine in cocoa products (Belscak *et al.*, 2009; Ashihara *et al.*, 2008; Carson, 2006; Olubamiwa *et al.*, 2002). At high dietary concentrations of more than 10% cocoa pod husk, multiple deleterious effects of theobromine are observed in animals including vacuolation within Sertoli cells, abnormally shaped spermatids, foetal malformation, weight loss, increased incidence of spontaneous abortion and death (Gartrell and Reid, 2007; Aregheore, 2002; Funabashi *et al.*, 2000; Ying and Waller, 1994; Wang *et al.*, 1992; Adomako *et al.*, 1984). The European Food Safety Authority (2008) also reported that it causes delayed ossification, a reduction in milk yield, hyperexcitability, sweating and increased respiration and heart rates, growth retardation, diarrhoea and lethargy and liver and

kidney toxicity. Optimizing the use of cocoa pod husk therefore requires detheobromization to detoxify the material (Gartrell and Reid, 2007; Aregheore, 2002).

Chemical methods for eliminating theobromine have been described in the literature (Chiovini *et al.*, 1983). Invariably, these procedures are expensive and cannot be adopted by subsistence farmers in the cocoa industry due to the use of solvents, chemically-treated adsorbents and sophisticated equipment. The development of simple, effective and affordable procedures for the detheobromination of cocoa pod husk is critical to maximum usage of the husk by Ghana's largely illiterate farmer population. A number of reports have suggested that several microorganisms catabolize a related methylxanthine, caffeine, indicating the possibility of the microbial degradation of theobromine in cocoa pod husk (Gokulakrishnan *et al.*, 2007; Mazzafera, 2002). The present study was conducted to investigate the theobromine-degrading properties of the indigenous microorganisms associated with cocoa pod husk.

MATERIALS AND METHODS

Theobromine and nutrient broth No. 3 were purchased from Fluka, Chemie AG, Buchs, Switzerland. This study was conducted from 2006 to 2009. Sabouraud dextrose agar was obtained from Oxoid Ltd. Basingstoke, Hampshire, England. Cocoa pods were obtained from the Cocoa Research Institute, Tafo, Ghana. The pods were sun-dried for 2 weeks, milled to fine powder using a hammer mill and stored at -20°C.

Determination of the spontaneous detheobromination of cocoa pod husks: Samples of cocoa pod husk powder sterilized by autoclaving at 121°C for 15 min and unsterilized samples of the powder, 40 g each, were each suspended in 80 mL sterilized distilled water in plastic containers. The containers were sealed and incubated, without aeration or shaking, at 30°C (ambient) for 7 days. Sub-samples of these were taken each day during the 7 day period to determine the residual theobromine contents. The theobromine fractions were extracted as described by Jalal and Collins (1976) and estimated using a titrimetric method (European communities stuff feeding methods, 1978).

Screening of indigenous microorganisms with detheobromination property: Microbial culture was prepared by rinsing cocoa pod husks with sterilized distilled water and using the rinse-water to inoculate a nutrient broth. The broth was incubated at 37°C with shaking for 24 h and was screened for the detheobromination microorganisms.

In the screening, theobromine (4% w/v) infusion plates, containing theobromine as the sole carbon or nitrogen source, were prepared by adding agar-agar (20 g L⁻¹) to theobromine infusion medium, pH 7.31, containing (L⁻¹): 1.3 g K₂HPO₄, 0.16 g MgSO₄·7H₂O, 0.21 g CaCl₂, 0.2 g Na₂HPO₄·12H₂O, 40.0 g theobromine and/or without 2.02 g (NH₄)₂SO₄. The medium was autoclaved at 121°C for 15 min and plates were prepared. Aliquots (1 mL) of the nutrient broth culture prepared were then used to inoculate the theobromine infusion plates and incubated at 37°C without shaking.

Isolation of microorganisms in cocoa pod husk culture: Nutrient agar plates were prepared from 1.3% nutrient broth and 1.5% agar-agar and spread-plated with 0.5 mL of the inoculum and incubated at 37°C for 24 h. Colonies were sub-cultured on same medium to obtain distinct colonies. Isolates were subsequently used to inoculate 6.5 % Sabouraud Dextrose Agar plates which were incubated at 37°C for 3 days. Light microscopy was performed to identify fungi on the basis of their

colonial and cellular morphology (Thom and Raper, 1945; Barnet and Hunter, 1972). For bacterial identification, peptone agar plates (2.5% peptone water broth and 1.5% agar-agar) were inoculated with the distinct colonies from the nutrient agar plates and incubated at 37°C for 24 h. The bacteria isolates were identified on the basis of colony and cell morphology, Gram stain reaction and standard biochemical tests (Harley and Prescott, 1989).

Detheobromination of Cocoa Pod Husk with *Aspergillus niger*: *Aspergillus niger* isolated from the theobromine infusion medium plates was cultured on Sabouraud dextrose agar. Spores of the mould were harvested by flooding 6-day old culture with sterilized distilled water and dislodging the spores by scraping. The spore suspensions were then decanted and pooled to obtain a total volume of 80 mL (Krishnan *et al.*, 1954). Sterile cocoa pod husk powder (40 g) was added to the suspension and incubated over a 7 day period as described previously. The residual theobromine content of the pod husk powder suspensions was then determined at 24 h intervals. Each experiment was conducted at least three times.

Statistical analysis: Analysis of Variance (ANOVA) tests along with Least Significant Difference (LSD) post-hoc comparisons were conducted using Excel Data Analysis Statistical Software (2007 version) and Statgraphics-plus Software Programme (Version 3.0). The level of significance was set to $p < 0.05$. Differences among means with $p < 0.05$ were accepted as representing statistically significant differences.

RESULTS

The results of experiments to test the natural detheobromination of the cocoa pod husks are presented in Fig. 1. After 7 days of soaking and incubation in water, the theobromine content of the unsterilized cocoa pod husk powder decreased by 54.7% ($p < 0.05$), while the theobromine

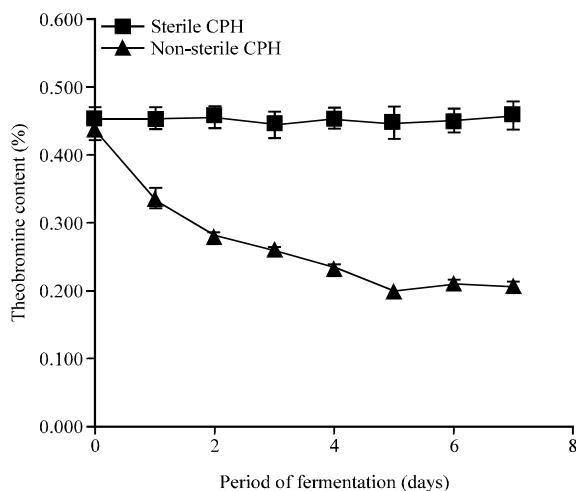


Fig. 1: Theobromine content of cocoa pod husk during natural fermentation. Sterile and non-sterile cocoa pod husk samples were soaked in water and incubated for 1 to 7 days. Theobromine was determined using a titrimetric method. Each value represents mean \pm SEM of at least four determinations. Differences between the values for sterile and non-sterile CPH on each day (1-7) are statistically significant ($p < 0.05$).

content of sterilized samples remained unchanged over the same period. This observation led to the screening and isolation of the indigenous microorganisms.

Table 1 shows the microbial profile of the cocoa pod husk samples. Samples of cocoa husk which were not treated as previously described and samples treated without the pre-sterilization step were screened. Four microbial species were isolated from the untreated samples while seven were isolated from the treated samples. The isolates were moulds and bacteria.

The theobromine infusion studies revealed *Aspergillus niger* as capable of thriving on 4% theobromine infusion medium in which theobromine was the sole source of carbon. Growth on this medium was, however, not as good as that on the Sabouraud dextrose agar. The organism could, however, not grow when theobromine was the sole nitrogen and carbon source (Fig. 2, 3).

Figure 4 illustrates and confirms the detheobromination properties of *A. niger*. In this study, sterilized cocoa pod husk powder samples were treated with *A. niger* for 7 days during which the theobromine contents were monitored. A gradual but significant decrease in theobromine content occurred culminating in a 71.8% reduction ($p < 0.05$).



Fig. 2: *Aspergillus niger* growing on 4% theobromine infusion medium



Fig. 3: *Aspergillus niger* growing on sabouraud dextrose agar

Table 1: Microbial profiles of unfermented and fermented cocoa pod husk

Microorganisms identified	Unfermented CPH	Fermented CPH
<i>Aspergillus flavus</i>	+	+
<i>Aspergillus niger</i>	+	+
<i>Halobacterium</i> sp.	+	-
<i>Klebsiella</i> sp.	-	+
<i>Lactobacillus</i> sp.	+	-
<i>Micrococcus</i> sp.	-	+
<i>Mucor hiemalis</i>	-	+
<i>Planococcus</i> sp.	-	+
<i>Pseudomonas</i> sp.	-	+

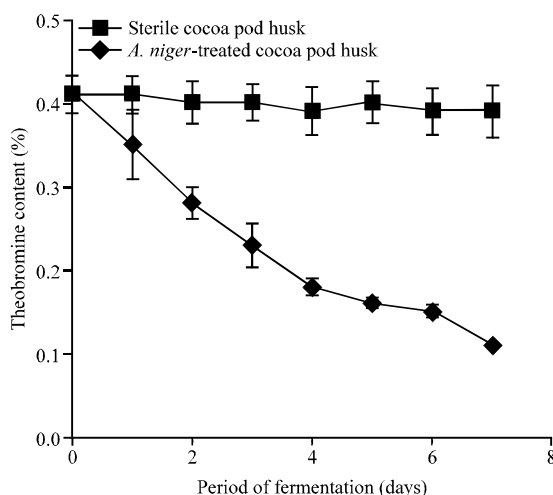


Fig. 4: Degradation of theobromine by *Aspergillus niger*. Sterilized cocoa pod husk samples were treated with *A. niger* for up to 7 days and theobromine was determined using a titrimetric method. Each value represents mean \pm SEM of at least four determinations. Differences between the values for sterile and *A. niger*-treated CPH on days 2-7 are statistically significant ($p < 0.05$).

DISCUSSION

The difference in theobromine content of unsterilized cocoa pod husk and sterilized samples after 7 days of soaking and incubation in water, suggested that microorganisms might be responsible for the degradation of the theobromine, since the difference in the treatments was that, the unsterilized samples contained the indigenous microorganisms associated with the pods.

The findings of the theobromine infusion studies suggest that theobromine essentially serves only as a carbon and energy source for *Aspergillus niger* and not a nitrogen source (Gutierrez-Sanchez *et.al.* (2003). The ability of *Aspergillus niger* to utilize theobromine as an energy source is consistent with observations that *Aspergillus* and *Penicillium* were frequently associated with degradation of methylxanthines (Asano *et al.*, 1993; Hakil *et al.*, 1999). The degradation of theobromine by *A. niger* probably occurs via the demethylase route and might involve the expression of enzymes such as theobromine demethylase, theobromine oxidase, xanthine dehydrogenase, xanthine oxidase, urease and uricase (Yamoka-Yano and Mazzafera, 1999; Dash and Gummadi, 2006; Huq, 2006).

It is highly improbable that treatment of cocoa pod husk with *Aspergillus niger* would render it unsuitable for animal consumption since *A. niger* is widely used in the food industry and has been found to be particularly effective in upgrading low nutrient value materials such as palm kernel cake and rice bran during solid state fermentation (Pramod and Lingappa, 2008; Abd-Aziz *et al.*, 2008; Hardini, 2010). However, the presence of *Aspergillus flavus*, noted for the production of aflatoxins under certain conditions, in the non-sterile husk samples, raised the possibility that spontaneous fermentation of cocoa pod husk might lead to unacceptably high aflatoxin levels. This necessitated investigation into the production of aflatoxins during the 7 day incubation period. Preliminary investigations were conducted by high performance liquid chromatography (Pons, 1979), using an HPLC (Waters 1525) with a scanning fluorescence detector. The levels of aflatoxins B1, B2, G1 and G2 in samples of the treated unsterilized samples were below the limits of detection (Aflatoxins B1 and B2 = 0.04 $\mu\text{g kg}^{-1}$; Aflatoxins G1 and G2 = 0.06 $\mu\text{g kg}^{-1}$). Further studies should however be carried out to confirm the atoxigenic properties under the treatment conditions.

The observation that *A. niger* could biodegrade theobromine is very important, as is the simplicity of the method of treatment. Although procedures for chemical detheobromination have been described, they require the use of solvents, chemically-treated adsorbents and sophisticated equipment (Chiovini *et al.*, 1983), making their use by subsistence farmers in Ghana impracticable. The magnitude of reduction observed in the present study is much greater than that reported by Oduns and Longe (1998), who investigated the effect of boiling on the theobromine content of cocoa pod husk. Moreover, the requirement of an energy source makes boiling unattractive for subsistence farmers in Ghana. The relative simplicity of the biological treatment process makes it a preferred alternative.

CONCLUSION

The present study provides, for the first time, unequivocal evidence of the efficacy of microbial detheobromination of cocoa pod husk. A very appreciable reduction in theobromine content could be accomplished using this relatively simple treatment process that could be adopted by subsistence farmers. The predominant indigenous microorganism involved was identified to be *Aspergillus niger*.

ACKNOWLEDGMENT

The assistance of Mr. Foster Mensah, of the Food Research Institute, Council for Scientific and Industrial Research, is greatly appreciated.

REFERENCES

- Abd-Aziz, S., H.G. Siew, H.M. Ali, A.K.M. Ismail and S. Noraini, 2008. Indirect method for quantification of cell biomass during solid-state fermentation of palm kernel cake based on protein content. *Asian J. Scientific Res.*, 1: 385-393.
- Adomako, A.D., E.O. Amaning and E.B. Frimpong, 1984. Utilization of cocoa pod husk by pigs. Proceedings of the 9th International Cocoa Research Conference, (ICRC'84), Lome, Togo, pp: 449-456.
- Aregheore, E.M., 2002. Chemical evaluation and digestibility of cocoa (*Theobroma cacao*) by-products fed to goats. *Trop. Anim. Health Prod.*, 34: 339-348.
- Asano, Y., T. Komeda and H. Yamada, 1993. Microbial production of theobromine from caffeine. *Biosci. Biotech. Biochem.*, 57: 1286-1289.

- Ashihara, H., H. Sano and A. Crozier, 2008. Caffeine and related purine alkaloids: Biosynthesis, catabolism, function and genetic engineering. *Phytochemistry*, 69: 841-856.
- Barnet, H.L. and B.B. Hunter, 1972. *Illustrated Genera of Imperfect Fungi*. 3rd Edn., Burges Publishing Company, Minneapolis, pp: 331.
- Belscak, A., D. Komes, D. Horzic, K.K. Ganic and D. Karlovic, 2009. Comparative study of commercially available cocoa products in terms of their bioactive composition. *Food Res. Int.*, 42: 707-716.
- Carson, T.L., 2006. Methylxanthines. In: *Small Animal Toxicology*, Peterson, M.E. and P.A. Talcott (Eds.). 2nd Edn., Elsevier, St. Louis, MO, USA., pp: 845-852.
- Chiovini, J., M. Blanc and G. Margolis, 1983. Detheobromination of cocoa. United States Patent 4390698. <http://www.freepatentsonline.com/4390698.html>.
- Dash, S.S. and S.N. Gummadi, 2006. Catabolic pathways and biotechnological applications of microbial caffeine degradation. *Biotechnol. Lett.*, 28: 1993-2002.
- European Communities Staff Feeding Methods, 1978. Determination of theobromine. S.I. No. 250, pp: 59-60.
- European Food Safety Authority, 2008. Theobromine as undesirable substance in animal feed. Scientific opinion of the panel on contaminants in the food Chain. *EFSA J.*, 725: 1-66.
- Funabashi, H., M. Fujioka, M. Kohchi, Y. Tateishi and N. Matsuoka, 2000. Collaborative work to evaluate toxicity on male reproductive organs by repeated dose studies in rats. Effects of 2- and 4-week administration of theobromine on the testis. *J. Toxicol. Sci.*, 25: 211-221.
- Gartrell, B.D. and C. Reid, 2007. Death by chocolate: A fatal problem for an inquisitive wild parrot. *New Zealand Vet. J.*, 55: 149-151.
- Gokulakrishnan, S., K. Chandraraj and S.N. Gummadi, 2007. A preliminary study of caffeine degradation by *Pseudomonas* sp. GSC 1182. *Int. J. Food. Microbiol.*, 113: 346-350.
- Gutierrez-Sanchez, G., S. Roussos and C. Augur, 2003. Effect of the nitrogen source on caffeine degradation by *Aspergillus tamari*. *Lett. Applied Microbiol.*, 38: 50-55.
- Hakil, M., F. Voisinet, G.V. Gonzalez and C. Augur, 1999. Caffeine degradation in solid-state fermentation by *Aspergillus tamari*: Effects of additional nitrogen sources. *J. Process Biochem.*, 35: 103-109.
- Hardini, D., 2010. The nutrient evaluation of fermented rice bran as poultry feed. *Int. J. Poultry Sci.*, 9: 152-154.
- Harley, P.J. and M.L. Prescott, 1989. *Laboratory Exercises in Microbiology*. W.C. Brown Publishers, Toronto, Canada.
- Huq, F., 2006. Molecular modelling analysis of the metabolism of caffeine. *Asian J. Biochem.*, 1: 276-286.
- Jalal, M.A.F. and H.A. Collins, 1976. Estimation of caffeine, theophylline and theobromine in plant material. *New Phytol.*, 76: 277-281.
- Krishnan, P.S., V. Bajaj and S.P. Damle, 1954. Some observations on the growth of *Aspergillus niger* from spore inoculum. *Applied Microbiol.*, 2: 303-308.
- Mazzafera, P., 2002. Degradation of caffeine by microorganisms and potential use of decaffeinated coffee husk and pulp in animal feeding. *Sci. Agric.*, 59: 815-821.
- Oduns, A.A. and O.G. Longe, 1998. Nutritive Value of hot water on cocoa pod ash solution-treated cocoa bean cake for broilers chicks. *Br. Poult. Sci.*, 39: 519-523.
- Olubamiwa, O., A.R. Otun and O.G. Longe, 2002. Dietary inclusion rate of cocoa husk for starter cockerels. *Int. J. Poult. Sci.*, 1: 133-135.

- Pons, W.A., 1979. High pressure liquid chromatographic determination of aflatoxins in corn. *J. AOAC Int.*, 62: 586-594.
- Pramod, T. and K. Lingappa, 2008. Immobilization of *Aspergillus niger* in polyurethane foam for citric acid production from carob pod extract. *Am. J. Food Technol.*, 3: 252-256.
- Thom, C. and K. Raper, 1945. *A Manual of the Aspergilli*. 1st Edn., Williams and Wilkins Co., Baltimore, USA.
- Wang, Y., D.P. Waller, A.P. Hikim and L.D. Russell, 1992. Reproductive toxicity of theobromine and cocoa extract in male rats. *Reprod. Toxicol.*, 6: 347-353.
- Yamoka-Yano, D.M. and P. Mazzafera, 1999. Catabolism of caffeine and purification of a xanthine oxidase responsible for methyluric acids production in *Pseudomonas putida* L. *Rev. Microbiol.*, 30: 62-70.
- Ying, W. and D.P. Waller, 1994. Theobromine toxicity on sertoli cells and comparison with cocoa extract in male rats. *Toxicol. Lett.*, 70: 155-164.