Journal of Medical and Biomedical Sciences (2016) 5(1): 17-22

doi: http://dx.doi.org/10.4314/jmbs.v5i1.3

ORIGINAL ARTICLE

Removal of tannin from Shea nut cake by *Pseudomonas* strain

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The use of microorganisms to remove the anti-nutritional factors in shea nut cake as the most cost effective and environmentally friendly approach to permit its use in agriculture for animal nutrition and soil fertility is long anticipated. Bacteria isolated from shea nut cake polluted soil in Sagnarigu District of Northern Region of Ghana showed that anti-nutritional factors in shea nut cake are biodegradable. The bacteria were grown in mineral salt medium supplemented with 2% shea nut cake as sole source of carbon. The bacteria isolate was identified biochemically as *Pseudomonas aerogenosa* and reduced total tannin concentration in shea nut cake from 54.58 g Kg^{-1} to 8.71 g Kg^{-1} (84%) in 10 days and 92% in 20 days. Boiling of shea nut cake reduced tannin content from 54.58 g Kg^{-1} to 16.36 g Kg^{-1} (70%) and enhanced biodegradation of tannin in the shea nut cake, removing up to 95% of total tannins in shea nut cake in 20 days.
Journal of Medical and Biomedical Sciences (2016) 5(1), 17-22

Keywords: Tannins, biodegradability, bacteria, mineral salt medium

INTRODUCTION

Shea butter is extracted from the kernels of the nuts of the shea tree (*Vitellaria paradoxa)*, traditionally and by agro-food transformation factories such as vegetable oil mills, soap and cosmetic factories, producing an important waste called the shea nut cake (Hall *et al.*, 1996). Shea butter is a natural vegetable oil, which among numerous uses, is a main cooking oil of the people in the shea nut belt. It is a rich source of vitamins A, D, E, F, fatty acids and glycerol (Okorley *et al.*, 2008).

The demand for natural vegetable oils is assuming centre position in human nutrition, being important sources of dietary energy, raw material for important industrial products in the pharmaceutical, soap and confectionary industries (Hall *et al.*, 1996). It is a good cocoa butter replacement in the manufacture of margarine and has been reported to prolong the shelf-life of chocolate (Hall *et al.*, 1996). Cocoa butter is made from oil high in mono-unsaturated symmetrical triglycerides SOS, distearin (StOSt), stearopalmitine (StOP) and Dipalmitin (POP) and can only be replace by oils high in SOS triglycerides, something lacking in cultivated vegetable oil except palm oil which is high in only POP and has to be blended with StOSt for compatibility. Shea butter has both StOSt triglycerides and POSt which make it convenient to replace cocoa butter without loss in product quality (Hall *et al.*, 1996).

The shea plant (*Vitellaria),* belonging to the family Sapotaceae (Ndukwe *et al.*, 2007) and species *paradoxa* consists of sub-species *paradoxa* and *nilotica* which are widely distributed in the Savannah zone of West Africa and Central Africa respectively (Uganda). The shea tree is mainly found in the Northern, Upper East, Upper West and part of Brong Ahafo Regions of Ghana. Northern Ghana, where the tree abounds is one of the poorest areas in the country, depending solely on rain-fed subsistence agriculture with erratic and unreliable rain fall and a greater part of the year wasted as a dry unproductive period. Investment in, and development of the shea butter industry is a promising area that can guarantee jobs and economic empowerment of the people especially the youth and wom-

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en, most of whom become victims of North-South migration with its associated problems such as sexual exploitation.

Resourcing state institutions, including research institutions to expand the shea butter industry and add economic value to the large volumes of waste generated annually will make the sector a powerful source of employment and foreign exchange earner. However, there are challenges of the industry which need serious attention. These include; low extraction efficiency (Abdul-Mumeen, 2013), shea nut cake disposal and how to make use of or add value to the wastes generated from the extraction process. This huge quantity of tannin-rich product, estimated at about 450-600 kg annually (Ofosu, 2011) is discarded into the soil where it is also destroying useful but vulnerable soil microorganisms. The potential of converting the product into biogas, animal feed and degradable fertilizer to increase soil fertility and crop production due to its high protein, carbohydrates, fatty acids and minerals contents has been recognized (Hall *et al.*, 1996; Ofosu, 2011; Abdul-Mumeen, 2013).

However, the presence of some anti-nutritional factors such as tannins, theobromine and saponins make shea nut cake unacceptable to animals as feed, toxic and recalcitrant to biodegradation by most soil bacteria (Hall *et al.*, 1996; Oddoye *et al.*, 2012). Abdul-Mumeen (2013), reported the following composition (gKg^{-1}) of first extraction cake total ash (3.17), crude protein (13.32); crude fat (17.42); fibre (10.57) and carbohydrate (66.13). Tannins form indigestible complexes with proteins and some carbohydrates (Nitiema *et al.*, 2010). Tannins are toxic to bacteria by inhibiting the absorption of iron and forming complexes with and inactivating metabolic enzymes and membrane phospholipids (Nitiema *et al.*, 2010).

Various strategies for the removal of anti–nutritional factors such as tannins and theobromine include boiling of SNC in water which was reported to remove 70% of tannins from shea nut cake and sodium hydroxide, a chemical treatment was recommended as the best strategy for the removal of both tannins and theoromine (Oddoye *et al.*, 2012). However the negative effects of chemical treatments such as residual effects on SNC as feed, cost of the chemical and removal of the chemical in terms of affordability to the ordinary farmer were immediately recognised.

With increasing volume of toxic pollutants and agricultural/agro-industrial wastes on the environment, microorganisms are being investigated to either add value to these nutrient – rich wastes by removing anti-nutritional factors (Mazzafera, 2002) or degrading completely the wastes or pollutants into harmless substances in the environment (Gordon, 1994). The use of microbes to degrade wastes and pollutants rests greatly on the degradability of the product. The most obvious pollutant encountered in the environment has been hydrocarbons and vegetable wastes. A hydrocarbon is said to be biodegradable when about 60% or more is degraded by microorganism in 28 days and vegetable oil is classified as biodegradable when between 70-100% is degraded by microorganism (Aluyor *et al.*, 2009). Soil has a myriad of bacteria that can degrade all forms of natural products with *Pseudomonas* reported to produce plasmids that code for substrate–induced enzymes which make it the most versatile, capable of degrading any form of organic substrate (Bhatta *et al.*, 2012).

Crude plant material has been reported to contain higher amounts of condensed tannins than hydrolysable tannins (Ullhyan *et al.*, 2012). However shea nut cake has been reported to contain higher amounts of hydrolysable tannins than condensed tannins, with condensed tannin constituting less than 0.1% (Bhatta *et al.*, 2012). Some bacteria are reported to degrade hydrolysable tannins more easily than condensed tannins. Though the use of microorganisms was recommended as the most environmentally safe and cost effective method of removal of anti-nutritional agents in shea nut cake (Okai *et al.*, 2011; Oddoye *et al.*, 2012) and disposal of environmental pollutants in general (Gordon, 1994; Chaillan *et al.*, 2004), available literature has not indicated the use of microbes to ascertain the biodegradability of shea nut cake and to remove anti-nutritional agents from shea nut cake. This

study was designed to use bacteria to demonstrate biodegradability of shea nut cake by microorganism and remove tannins from shea nut cake.

MATERIALS AND METHODS

Isolation and identification of shea nut cake degrading bacteria

Shea nut cake degrading bacteria were isolated by the enrichment method. A mineral salt medium for the isolation of soil bacteria was used. One gram (1 g) each of the one hundred and sixty two (162) shea nut cake polluted soil samples was weighed into 100 ml of sterile mineral salt shea nut cake medium in a 250 ml Erlenmeyer flask, mixed and incubated at 25^{o}C for 72 hours. The inoculated medium was subcultured into similar mineral salt shea nut cake medium after every 72 hours. After a third successive subculture, a final subculture was made onto mineral salt medium supplemented with 2% shea nut cake to which 15 grams of agar was added as a solidifying agent. Colonies perceived to be different were isolated and sub-cultured on 5% and 10% shea nut cake agar. GUR/09 gave good growth on 5% shea nut cake agar within 48 hours and was identified biochemically as a strain of *Pseudomonas aerogenosa* as described by Cheesbrough (2006) and with the help of Bergy's Manual of Determinative Bacteriology (Williams *et al.*, 1994).

A forty (40) kilogram shea nut cake sample was obtained from Tungteiya Women Association Shea Butter Extraction Centre in Jisonayili, divided into two portions of 20 kg each. One of the 20 kg portion of shea nut cake was boiled at 75^{o}C for 1hr, cooled overnight and used (Oddoye *et al.*, 2012). The other part was treated fresh (unboiled). Okai *et al.* (2011), and Oddoye *et al.* (2012), reported that boiling of shea nut cake significantly reduces tannin content, hence the need to investigate whether boiling shea nut cake will enhance biodegradation of shea nut cake by bacteria. Each of the twenty kilogram parts was thoroughly mixed and divided into treatments of one 1 kg each and assigned the following identifications: T_0 = Unboiled (fresh, without bacteria serving as Control), T_1 = Boiled at 75^{o}C for one hour (Without bacteria), T_2= Unboiled shea nut cake inoculated with *Pseudomonas* strain GUR/09, incubated for 10 days, T_3 = Unboiled shea nut cake inoculated with *Pseudomonas* strain GUR/09, incubated for 20 days, T_4 = Unboiled shea nut cake inoculated with *Pseudomonas* strain GUR/09, incubated for 30 days, T_5 = Unboiled shea nut cake inoculated with *Pseudomonas* strain GUR/09, incubated for 40 days, T_6 = Boiled shea nut cake inoculated with *Pseudomonas* strain GUR/09, incubated for 10 days, T_7 = Boiled shea nut cake inoculated with *Pseudomonas* strain GUR/09, incubated for 20 days, T_8 = Boiled shea nut cake inoculated with *Pseudomonas* strain GUR/09, incubated for 30 days and T_9 = Boiled shea nut cake inoculated with *Pseudomonas* strain GUR/09, incubated for 40 days. Each treatment slot was replicated three times and mean of results recorded.

Substrate preparation for each treatment slot was a modification of that described by Mazzafera (2002) for the removal of tannin from coffee pulp. To each kilogram treatment lot was added to 5% yeast extracts and 1% glucose, since bacteria use nutrients to synthesize enzymes to degrade pollutants (Gordon, 1994). The pH was adjusted to 7.0 with N/10 NaOH and buffered with 1.0 L of disodium hydrogen phosphate and sodium dihydrogen phosphate (Cheesbrough, 2006). The preparation was then homogenized with one hundred millilitres (100 ml) of fresh 24 hour nutrient broth culture of *Pseudomonas* strain GUR/09, incubated at room temperature and sampled for polyphenol assay at the end of the incubation period for each treatment. Phenolic compounds were extracted by a method described by Makkar (2000).

Air-dried shea nut cake was ground to fine powder to pass through 0.5 mm sieve. To 200 mg of the powder was added 10 ml of 70% aqueous acetone (70:30) in a 50ml flask with lid, vigorously shaken and subjected to ultrasonic treatment for 20 min at room temperature. The contents of the flasks were then transferred to centrifuge tubes, cooled on ice and then centrifuged at 3,000 g for 10 minutes. The supernatant was collected. The brown colour of the supernatant was removed by evaporating acetone at 50^{o}C and washing residue with diethyl ether con-

taining 1% acetic acid until no brown colour appeared. The diethyl ether was evaporated at 50°C and the deposit dissolved in 10 ml of 70% aqueous acetone and used for polyphenol estimation. Folin-Ciocalteu method described by Makkar *et al.* (1993) was used for determination of total phenols.

Determination of total phenolics is based on the fact that phenols are reducing agents (Makkar, 2000). Tannin extract (0.05 ml) was added to 0.45ml of distilled water followed by addition of 0.25ml of 1N Folin-Ciocalteu reagent. After three (3) minutes, 1.25ml of 20% aqueous solution of Sodium carbonate ($Na_2CO_3.10H_2O$) was added, vortexed and allowed to stand at room temperature for forty (40) minutes. The absorbance representing the intensity of the blue colour was measured at 725 nm after 40 minutes against a reagent blank. Total phenol concentration was estimated from using 0.1 mg/ml solution prepared from standard tannic acid reagents as directed by Hach, Loveland CO., USA. Reduction in total phenolic contents expressed as tannic acid equivalent (TA) in Percentage Dry Matter (% DM) was a measure of biodegradation of shea nut cake. Percentage Dry Matter (%DM) was determined by weighing air-dried ground shea nut cake at 100°C to a constant weight for three consecutive times, and calculated using the formula below:

$$\%\,DM = \frac{Constant\ dry\ weight}{Total\ (initial)\ weight} \times 100$$

1. Standard (STD) tannic acid = 0.1 mg/ml
   1ml = 0.1mg
   $0.05ml = \frac{0.05}{1} \times 0.01 = 0.005\,mg = 5.0\,\mu g$
   Absorbance of STD = 0.540
1. $\frac{AbT}{AbSTD} \times$ Concentration of STD

1. $\frac{5}{0.540} \times AbT$
   $9.259 \times AbT$
1. 1.0 ml extract = $(\frac{1}{0.05} \times$ concentration of tannin in 0.05ml of extract determined in test) ÷ 1000 = X mg TA. If 200mg shea nut cake sample was extracted in 10ml solvent

1. 100 mg shea nut cake will have $(\frac{100}{250} \times 10) \times$ Xmg (from equation 2 above) 100 mg shea = Y mg TA
2. Shea nut cake contains 45% dry matter (DM)
   $\%DM = \frac{Y}{0.45}$

All tannin and lignin test reagents were obtained from Hach, Loveland Co., USA.

Data Analysis

Gram staining results were expressed as percentage. The study of shea nut cake degradation by indigenous soil bacteria was set up as 10 lots of boiled and fresh (not boiled) inoculated with *Pseudomonas* strain GUR/09. Boiled and unboiled lots without bacteria served as controls. Treatments were labelled T_o to T_9. The inoculated samples taken at ten days interval for forty days from the different slots and one spot sample from the fresh and boiled and not inoculated served as the treatments. Tests on treatments were done in triplicates and their means calculated. Means of treatments were subjected to analysis of variance (ANOVA) using GENSTAT Version 4 (2015) software. Significantly different ($P < 0.05$) means were separated using the Least Significant Difference (LSD) method.

RESULTS

Reduction of total tannins of shea nut cake by *Pseudomonas* strain GUR/09

Boiling of shea nut cake reduced polyphenol content in shea nut cake, from 54.58 g Kg^{-1} to 16.36 g Kg^{-1} (70%) and up to 92% in 10 days when combined with biodegradation (Table 1).

Table 1: Tannin reduction in shea nut cake

Time (Days)	Tannin reduction (g Kg-1)
T0	54.58[a]
T1	16.36[b]
T2	8.71[c]
T3	4.36[d]
T4	2.67[e]
T5	1.78[f]
T6	4.44[g]
T7	2.67[e]
T8	1.69[h]
T9	1.69[h]
LSD	0.012

[a-h] Means with the same superscript letter are not significantly different (P > 0.05) from each other

DISCUSSION

The study found no significant difference ($P > 0.05$) between T_4 and T_7. This could be due to boiling in T_7 which reduced the tannins concentration and enhanced biodegradation, achieving the same result as T_4 within a shorter time. Boiling shea nut cake in this study has reduced the phenolic content from 54.58 g Kg^{-1} to 16.36 g Kg^{-1} (70%). This is in agree-

ment with Okai *et al.* (2011) and Oddoye *et al.* (2012) who reported a reduction in tannin content in shea nut cake from 10% to 3% (70%) and 22.22 g Kg^{-1} to 6.157 g Kg^{-1} (70%) respectively after boiling for one hour.

Pseudomonas strain GUR/09 in the degradation exercise degraded 84% total phenols from fresh shea nut cake in 10 days and 96% in 30 days. This is in agreement with Aluyor *et al.* (2009) who reported that 60% or more degradation of hydrocarbon in 28 days classifies that hydrocarbon as biodegradable while vegetable oils are biodegradable when degradation by microorganisms is within 70-100% in a period of 28 days.

The high biodegradability of shea nut cake, above 90% in 10 days, observed in this study is also supported by Bhatta *et al.* (2012) who reported that shea nut cake has a higher amount of hydrolysable tannin phenol which are more easily degraded by bacteria, than condensed tannin which, according to him is less than 0.1%. It was observed in this study that boiling of shea nut cake accelerated bacterial degradation of shea nut cake where over 90% reduction was achieved in 10 days with boiled shea nut cake, probably by reducing significantly the amount of tannins in shea nut cake by 70%.

CONCLUSION

Shea nut cake was biodegradable. *Pseudomonas euregenosa* can be used to remove anti-nutritional agents from shea not cake. Tannin degrading enzymes can be extracted and used to free the remaining components for other uses. Boiling shea nut cake removed about 70% of tannin in shea nut cake and enhanced bacteria biodegradation of tannin in shea nut cake.

COMPETING INTERESTS

The authors declare that they have no competing interests.

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ISSN 2026-6294