KWAME NKRUMAH UNIVERSITY OF SCIENCE AND TECHNOLOGY, KUMASI

COLLEGE OF SCIENCE

DEPARTMENT OF BIOCHEMISTRY AND BIOTECHNOLOGY

ENHANCEMENT OF THE NUTRITIVE VALUE OF COCOA (*THEOBROMA CACAO*) BEAN SHELLS FOR USE AS FEED FOR ANIMALS THROUGH A TWO- STAGE SOLID STATE FERMENTATION WITH *PLEUROTUS OSTREATUS* AND *ASPERGILLUS NIGER*

BY

JOSEPH ASANKOMAH BENTIL

MAY, 2012

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DECLARATION

I do declare that, except with references to other peoples work which have been duly cited, this work submitted as a thesis to the Department of Biochemistry and Biotechnology, Kwame Nkrumah University of Science and Technology, Kumasi, for the degree of Master of Science in Biotechnology, is the result of my own investigation and has not been presented for any other degree.

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ABSTRACT

The animal industry is being faced with several challenges one of them is the high cost of feeding as a result of the exorbitant prices of feed ingredients. This challenge facing particularly the poultry industry is being addressed by the use of agro-industrial by-products (AIBPs) as alternative energy sources for animal feeding. However, these by-products are faced with certain limitations such as their low inclusions in animal diet especially poultry and pigs due to their high fibre content and high levels of anti-nutritional factors. One of such agro-wastes is the cocoa bean shells (CBS) produced from the cocoa processing factories and underutilized due to its high fibre content (mainly lignocellulose) and high theobromine content. The application of biotechnology by the use of fungi with GRAS status to improve its feed value for animals was therefore investigated. The dried CBS obtained from the factory was taken through a two-stage solid state fermentation. The CBS was milled into 2mm particle size, composted and pasteurized. The pasteurized substrate was then fermented with the spawn of *Pleurotus ostreatus* for 6 weeks followed by *Aspergillus niger* fermentation for seven days. The fermentation of the CBS with *P. ostreatus* significantly increased ($p<0.05$) the protein content by 25.22% and decreased ($p<0.05$) the cellulose, hemicellulose and lignin by 49.93%, 39.99% and 34.65% respectively at the sixth week, the optimum fermentation period. The mineral content (calcium, phosphorus and potassium) of the *P. ostreatus* fermented CBS was also significantly enhanced ($p<0.05$) at the sixth week of fermentation. The *P.ostreatus* fermented CBS used as substrate for the second stage *A. niger* fermentation had its theobromine content significantly reduced ($p<0.05$) by 72.89% at the end of the seven days of fermentation. The degradation of the complex carbohydrates of the CBS by the fungi increased the level of soluble carbohydrates (from 35.05% to 45.30%) which resulted in a 33.64% increase in Metabolizable Energy (M.E) in the fermented product. These results make the CBS fermented with *P. ostreatus* and *A. niger* more suitable for use as feed material for animals.

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CHAPTER ONE

1.0 INTRODUCTION

Cocoa (*Theobroma cacao)* tree is widely cultivated in Ghana. In addition to the highly flavoured cocoa products, cocoa tree provides by-products such as Cocoa Bean Meal (CBM), Cocoa Pod Husk (CPH) and Cocoa Bean Shell (CBS), among others.

Cocoa bean shell (CBS) is an industrial ligno-cellulosic waste material produced at cocoa and chocolate factories, especially in the industrialized countries and it forms 12-14% of the roasted cocoa bean (Aina, 1998). CBS is a potential tropical feed resource and its utilization in animal feeding will greatly reduce the disposal problem facing the cocoa processing factories (Aina, 1998). The dried CBS contains 13.12% crude protein; 13.00% crude fibre; 8.71% ether extract; 9.15% ash (Olupona *et al*., 2003). Several studies on broilers, cockerel chick finishers and laying hens have established the inclusion rate of CBS in these poultry rations though they reported a low growth performance at higher inclusions due to several factors (Olubamiwa *et al*., 2000; Hamzat and Babatunde, 2006)

Gohl (1981) attributed the limited use of CBS in animal feeds to its theobromine content in spite of its high nutritive value. Theobromine belongs to the same naturally occurring methylated xanthine group as caffeine (Ching and Wong, 1986). When taken in modest quantities, it acts as a stimulant like caffeine but intake of more than 0.0279kg per body weight is injurious to animals (Menon, 1982). Menon (1982) indicated that the anti-nutritional compound could be reduced by heat, sun-drying and boiling.

Poor monogastric utilization of CBS- based diets is partly attributed to the high fibre content of CBS (Hamzat *et al*., 2006)

Most of these by-products, which are often referred to as agro-industrial by-products (AIBPs) contain certain complex carbohydrates termed lignocellulose which are very difficult to degrade naturally into simpler forms (Howard *et al*., 2003). Lignocellulose is the major structural component of woody plants and non-woody plants such as grass and represents a major source of renewable organic matter. Lignocellulose consists of lignin, hemicellulose and cellulose (Howard *et al*., 2003). The chemical properties of the components of lignocellulosics make them a substrate of enormous biotechnological value (Malherbe and Cloete, 2003).

Large amounts of lignocellulosic materials are generated through forestry and agricultural practices, paper-pulp industries, timber industries and many agro industries and they pose an environmental pollution problem. Majority of these lignocellulose wastes are often disposed of by biomass burning, which is not restricted to developing countries alone, but is considered a global phenomenon (Levine, 1996). However, the huge amounts of residual plant biomass considered as "waste" can potentially be converted into various different value-added products including biofuels, chemicals, and cheap energy sources for fermentation, improved animal feeds and human nutrients. Lignocellulytic enzymes also have significant potential applications in various industries including chemicals, fuel, food, brewery and wine, animal feed, textile and laundry, pulp and paper and agriculture.

With the advent of fungal biotechnology, highly fibrous agro-wastes can be made useful to animals particularly monogastrics by fermentation with certain fungi such as *Pleurotus spp.* capable of improving protein quality and fibre digestibility (Alemawor *et al.,* 2009). The fermentation process also enhances micronutrient bioavailability and aids in degrading anti-nutritional factors (Achinewhu *et al*. 1998). The use of fungi for the conversion of lignocelluloses into food or feed rich in protein offers an alternative for developing non-conventional sources of proteins as food or feed (Vijay *et al*., 2007). Mushroom is defined by Chang (1980) as a fungus with a distinctive fruiting body which can be either epigeous or hypogenous and has visible growth on lignocellulosic materials used as substrates.

Mushroom forming fungi are therefore amongst nature's most powerful decomposers, secreting strong extracellular enzymes due to their aggressive growth and biomass production (Adenipekun, 2009). They are primary decomposers and saprophytic fungi, which grow easily in clusters on decomposing lignocellulosics, capable of utilizing a wide range of substrate materials with high biological efficiency and good mycelia growth rate (Achio, 2009). They undergo extracellular digestion hence their capability of producing a wide range of enzymes such as laccases, cellulases and hemicellulases that are responsible for the breakdown of complex polysaccharides such as lignin, cellulose and hemicellulose respectively into simpler soluble substances which are highly absorbable for growth and development (Sharma *et al.,* 1999).

Mushrooms therefore grow very well on lignocellulosic substrates containing mainly cellulose and lignin converting them into digestible materials which may be used as animal feed (Sharma *et al.,* 1999). Some of the lignocellulosic materials are sawdust, rice bran, rice straw, corncobs,

cocoa by-products, sugarcane leaves, grasses, rice hull, cassava peels, etc. These agricultural wastes can be used for the cultivation of mushroom which provides not only food and feed at low cost but also help reduce the incidence of environmental pollution (Sharma *et al*., 1999).

Pleurotus ostreatus is a white- rot fungus which forms a sheet of white-like spores on the surface of substrates on which they colonize. A wide range of lignocellulosic substrates are used for cultivation of *Pleurotus* species. Amongst the cereal straws, Garcha *et al*., (1984) reported paddy straw to be the best substrate for the cultivation of oyster mushroom and further reported the use of pearl millet stalks in the cultivation of *P. sajor-caju*. Rice straw waste, lawn grass, maize cobs, banana wastes (Bonatti *et al*., 2004) and maize straw (Bahukhandi and Munjal, 1989) were reported as suitable substrates for cultivation of different *Pleurotus spp*.

A study conducted by Alemawor *et al*., (2009) on fermentation of cocoa pod husk, a cocoa by-product with *Pleurotus ostreatus* reported that the alkaloids in the cocoa pod husk was not affected by the *P. ostreatus* fermentation unlike the polyphenols such as tannins which was significantly reduced. However, *Aspergillus niger* has proven to be capable of biodegrading alkaloids such as theobromine when used in the fermentation of cocoa by-products by using the theobromine as its sole carbon and energy sources (Adamafio *et al*., 2011).

Aspergillus niger is a fungus and one of the most common species of the genus *Aspergillus*. It is known for its black sporulation and ubiquitous nature hence a major contaminant in laboratory cultures. *A. niger* is often cultured for the industrial production of many substances. Various strains of *A. niger* are used in the industrial preparation of citric acid (E330) and gluconic acid (E574) (Papagianni *et al*., 1994) and have been assessed as acceptable for daily intake by the

World Health Organization. *A. niger* fermentation is "generally recognized as safe" (GRAS) by the United States Food and Drug Administration (*US* FDA) (Schuster *et al*., 2002).

1.1 PROBLEM STATEMENT

The poultry industry is faced with several challenges among which is the scarcity of conventional energy source feedstuffs particularly maize, a major energy source in animal feed. This is due to the high demand on such ingredients by humans and by poultry and livestock industries.

The high competition for energy source ingredients between humans and animals has led to escalating prices of these ingredients. This has therefore caused an increase in the cost of feeding which presently accounts for 70-80% of the cost of poultry production (Adebowale, 1981). Currently, the poultry business has become less profitable hence a less fascinating venture for prospective poultry farmers which may eventually lead to economic crisis.

Several attempts have been made by researchers to address this pestering issue facing the poultry industry by the use of alternative and cheaper sources of ingredients to replace the less available and expensive ones. One such alternative source is the use of agro-industrial by-products which are abundantly found in the environment produced by processing industries. Proper disposal of these wastes also increases the production cost of these industries. However, improper disposal of these wastes by the industries that produce them may lead to environmental pollution.

One such agro-industrial by-product is cocoa bean shells (CBS) produced from cocoa and chocolate factories. Several researchers have established alternative use of CBS for animal feed especially in poultry diets (Olubamiwa *et al*., 2000; Hamzat and Babatunde (2006); Hamzat *et al*. (2006). However, the inclusion levels of untreated CBS in poultry diets have been reported to be very limited due to the reason that, CBS is lignocellulosic i.e. contains high cellulose and lignin (Hamzat *et al*., 2006) which cannot be digested by poultry. Moreover, the high theobromine content of the CBS (Gohl 1981) makes it impossible to feed more of the by-products to poultry since it is highly toxic to their digestive system.

1.2 JUSTIFICATION

Agro-industrial by-products are abundant in Ghana especially as the trend of industrialization increases. Among the common ones are brewer's dried grains (BDG), rice bran (RB), palm kernel meal (PKM), corn bran (CB), cassava peels, sheanut meal (SM), cocoa bean shells (CBS), cocoa pod husk (CPH) and many more produced from their respective industries.

Though these agro-industrial wastes are a threat to the environment if not properly disposed, they are biodegradable. These wastes, which are mostly lignocellulosic composed of complex polysaccharides (lignin, cellulose and hemicellulose) become substrates for both bacteria and fungi, which are able to break them down into simple sugars (Iyayi, 2004).

Although cocoa bean shell contains substantial quantities of lignin, cellulose, nitrogen and other substances, it has limited use and its disposal is mainly by dumping or burning. However, a better option may be found in solid-state fermentation with *Pleurotus ostreatus* leading to improved feed material at low cost (Alemawor *et al*., 2009). *P. ostreatus*, a white-rot fungus, is capable of increasing the protein and soluble sugars hence increasing fibre digestibility of agro-industrial wastes as well as capable of degrading polyphenols such as tannins present in these wastes (Alemawor *et al*., 2009). However, *P. ostreatus* is not capable of degrading alkaloids present in cocoa by-products (Alemawor *et al*., 2009).

Additionally, *Aspergillus niger* has also been reported to be capable of biodegrading alkaloids (methylxanthine compounds) such as theobromine in cocoa by-products reducing the theobromine content to an appreciable level when the theobromine was used as a sole carbon and energy source for fermentation by the *A. niger* (Adamafio *et al*., 2011).

1.3 OBJECTIVE

The main objective of this project was to investigate the effect of a two-stage fermentation of cocoa bean shells (CBS) with *Pleurotus ostreatus* and *Aspergillus niger* on its nutritive value for use as animal feed material.

1.4 SPECIFIC OBJECTIVES

To determine the optimum duration for *Pleurotus ostreatus* fermentation of CBS by monitoring changes in proximate, mineral content and level of individual fibre components

To determine the optimum duration of the fermentation process with *Aspergillus niger* on *P. ostreatus* fermented CBS

To monitor the level of detheobromination in the substrate during the *Aspergillus niger* fermentation of *P. ostreatus* fermented CBS

CHAPTER TWO

2.0 LITERATURE REVIEW

2.1 COCOA

Cocoa is a major agricultural commodity grown exclusively in tropical areas in three principal producing regions namely Africa, Latin America and the Caribbean, Asia and Oceania. At the start of the 20th century, Latin America and the Caribbean region accounted for 60% to 80% of the world's supply of cocoa beans (Sukha, 2003). However, Africa became the world's leading

cocoa-producing region soon after the end of the First World War. This was followed by the rapid expansion of cocoa cultivation in Asia in recent years which has caused the share of the Latin America and the Caribbean region to decline to 16% of world cocoa production in 1996/97, compared with 18% for Asia and 66% for Africa (Sukha, 2003).

Cocoa is vital to the economies of some countries in the regions that produce it. Cocoa exports comprise a significant proportion of the foreign exchange earnings generated by these countries and are used to service external debt obligations. This is a great challenge when one considers that approximately 86% of the world production of cocoa is grown by farmers with small land holdings of generally a few hectares. Average production from these farms varies between 400 to 800kg/ha (Sukha, 2003).

2.1.1 PRODUCTION

Cocoa is grown principally in West Africa, Central and South America and Asia. In order of annual production size, the eight largest cocoa-producing countries at present are Côte d'Ivoire, Ghana, Indonesia, Nigeria, Cameroon, Brazil, Ecuador and Malaysia. These countries represent 90% of world production (Sukha, 2003).

Table 2.1 World Production of cocoa beans (2008-2010) (thousand tonnes)

Country	**2007/08**	**2008/09**	**2009/10**

Africa	**2692**	**72.1%**	**2520**	**69.9%**	**2459**	**68.4%**
Cameroon	185		227		200	
Cote d'Ivoire	1382		1223		1190	
Ghana	729		662		650	
Nigeria	230		250		260	
Others	166		158		159	
America	**451**	**12.1%**	**487**	**13.5%**	**505**	**14.0%**
Brazil	171		157		155	
Ecuador	113		134		150	
Others	167		196		200	
Asia & Oceania	**591**	**15.8%**	**598**	**16.6%**	**632**	**17.6%**
Indonesia	485		490		535	
Papua New Guinea	52		59		50	
Others	54		49		47	
World Total	***3734***	***100%***	***3605***	***100%***	***3596***	***100%***

***Source**: ICCO Quarterly Bulletin of Cocoa Statistics (2009/10)*

In the early 1970s production was concentrated in Ghana, Nigeria, Côte d'Ivoire and Brazil, but it has now expanded to areas such as the Pacific region, where countries like Indonesia have shown spectacular growth rates in production (Sukha, 2003).

2.1.2 CONSUMPTION

Although cocoa is largely produced in developing countries, it is mostly consumed in industrialized countries. For cocoa, the buyers in the consuming countries are the processors and the chocolate manufacturers. A few multinational companies dominate both processing and

chocolate manufacturing (Sukha, 2003). The main consumers of cocoa, based on the apparent domestic cocoa consumption, is calculated as grindings plus net imports of cocoa products and of chocolate products in beans equivalent (Table 2.2).

2.1.3 TRADE

2.1.3.1 EXPORTS

It can be realized from tables (2.1, 2.2) that the main exporters are also the main producers of cocoa beans. Although countries like Brazil and Malaysia are main producers, they are not necessarily large exporters due to the size of their processing industry, which absorbs local production. In Latin America for example, the Dominican Republic exports more cocoa beans than Brazil (Sukha, 2003).

Table 2.2 World Consumption/Grindings of cocoa beans (thousand tonnes)

	2003/04		**2004/05**		**2005/06**	
Europe	**1346**	**41.6%**	**1375**	**41.1%**	**1462**	**42.1%**
Germany	225		235		302	
Netherlands	445		460		470	
Others	676		680		690	

Africa	**466**	**14.4%**	**494**	**14.8%**	**507**	**14.6%**
Cote 'Ivoire	335		364		360	
Others	131		130		147	
America	**852**	**26.3%**	**853**	**25.5%**	**856**	**24.6%**
Brazil	207		209		223	
United States	410		419		426	
Others	235		225		207	
Asia &Oceania	**575**	**17.7%**	**622**	**18.6%**	**651**	**18.7%**
Indonesia	120		115		120	
Malaysia	203		250		250	
Others	252		257		281	
World total	***3239***		***3344***		***3476***	

Source: *ICCO Quarterly Bulletin of Cocoa Statistics, (2005/06)*

2.1.4 PROCESSING OF COCOA BEANS

Cocoa processing and chocolate manufacturing are two different processes that, although linked, require different procedures to obtain the products wanted. Cocoa processing is basically a means of converting the beans into nib, liquor, butter, cake and powder (Ng, 2007). Much emphasis is laid rather on the by-products produced from the processing of the beans especially the production of the shells which occurs at the initial stage where the beans are deshelled before or after the roasting of the nib as shown in Fig. 2.1

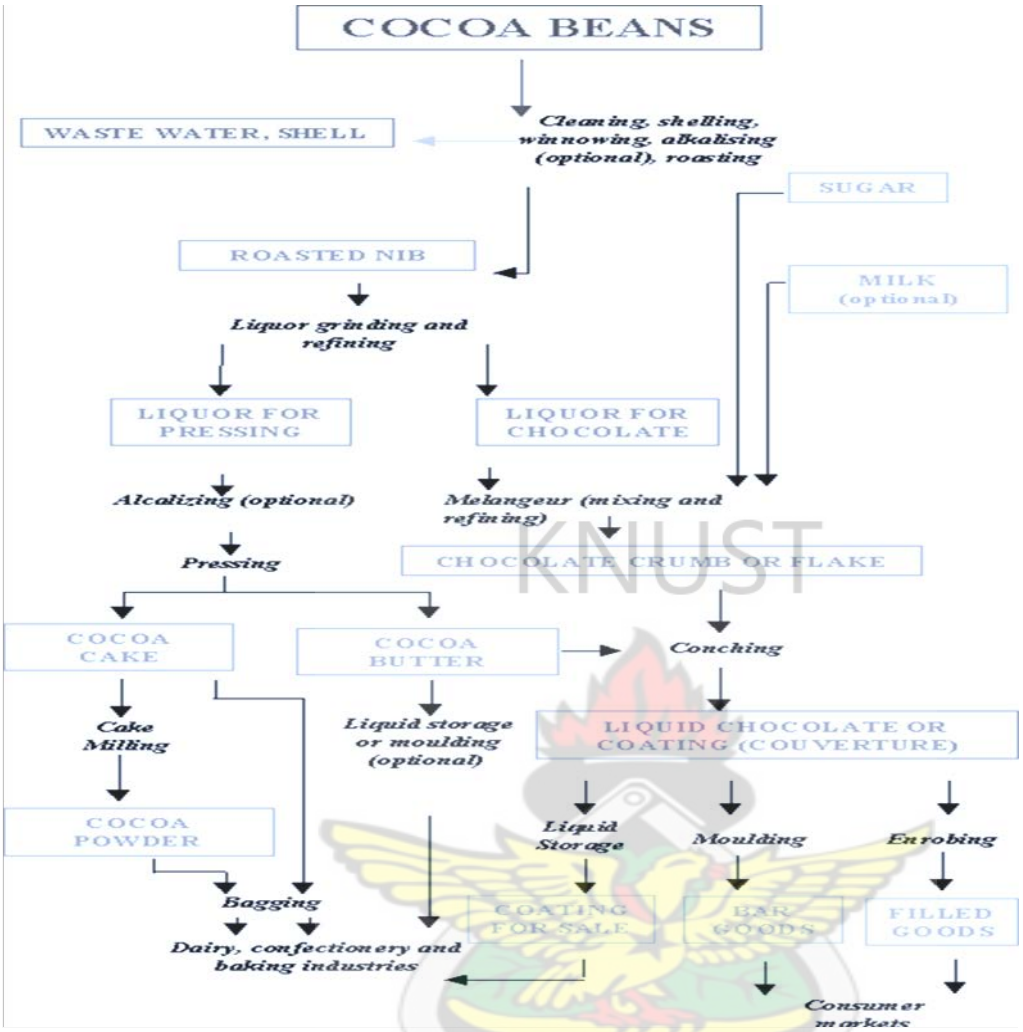


Fig 2.1 Flow chart of the processing of cocoa beans (Source: Ng, 2007**)**

2.2 AGRO-INDUSTRIAL BY-PRODUCTS (AIBPs)

Agro-industrial by-products (AIBPs) refer to the by-products derived from agricultural-based industries as a result of processing of the main products. AIBPs are less fibrous, more concentrated, highly nutritious and less costly as compared to crop residues (Aguilera, 1989). The processing cocoa beans into cocoa and chocolate products has led to the generation of vast quantities of cocoa by-products which can alternatively be used as feed material for animals in order to salvage a threatening problem to the environment when improperly disposed. The main

cocoa by-products produced by the cocoa processing industries are cocoa pod husk (CPH), cocoa bean meal (CBM) and cocoa bean shells (CBS).

2.2.1 COCOA BY-PRODUCTS AS ANIMAL FEED

In developing countries such as Ghana, there is a scarcity of protein and energy rich food and feed plants. Those that are available are used as food by man, and will be too expensive for use as animal feed. As it is only the seeds of the cocoa fruit that are used for human food production, there are abundant amounts of agricultural by-products available for alternative use. In many places, what is left over when the cocoa beans have been removed from the cocoa pod (cocoa pod husk) is left to rot on the plantation or is used as fertilizer (Oladokun, 1986).

However, by-products of cocoa manufacture, including cocoa husk (cocoa pod), cocoa bean shell (cocoa hull), cocoa seed meal and cocoa germ have been investigated for use as feed (Alemawor *et al.*, 2009, Olubamiwa *et al.*, 2002, Muhammed *et al.*, 2000) . Whereas the last three may become available in the country where cocoa beans are processed, the cocoa husk is not usually exported from the country where the cocoa tree was grown. Within the European Union most of the processing takes place in The Netherlands, Germany, Belgium, France and the United Kingdom (Sukha, 2003). The chemical composition of cocoa husks, shells and meals has been widely reported and summarized in Table 2.3.

Table 2.3 Proximate composition (% dry matter) of by-products from the manufacture of cocoa

Proximate	Cocoa pod husk	Cocoa bean shell	Cocoa bean meal
Moisture	5.4-15.3	4.9-12.0	2.1-16.9
Crude protein	6.3-10.4	13.2-20.1	17.8-28.6
Crude fibre	23.4-36.2	9.3-20.5	5.3-22.0
Ether extractable components	0.5-2.4	1.9-22.0	1.1-17.8
Nitrogen-free extract	31.8-61.4	40.2-52.5	25.9-51.1
Ash	7.0-15.3	6.0-10.8	3.0-15.1

Source*: *The EFSA Journal (2008)

The variation in proximate content could be due to year-to-year variation, different varieties being studied (Alvarado *et al.*, 1983), and differences in the preparations of the samples for analysis and the analytical method used.

2.2.1.1 COCOA HUSK

It takes between twenty and twenty-four weeks for cocoa pods to develop from the time the flowers are fertilized until the fruits are fully ripened, naturally depending on the climatic conditions and where the tree is growing. Growth of the pods is generally slow at the beginning and then speeds up. When the beans are removed from the pod after harvest, they are covered by a mucilage pulp. Both the initial colonization of the pulp and subsequent microbial activity on the cocoa husk is largely dependent upon the nature of the pulp surrounding the beans. Pulp of mature fruit generally contains 82-87% water, 10-13% sugar and 1% pectin (Hardy, 1960).

The sugar constitutes mainly glucose and fructose which are metabolized by microorganisms such as yeasts and lactic acid bacteria during fermentation, and traces of sucrose. The parts of the

cocoa pod left over, the cocoa husk, represents between 2/3 and ¾ of the total weight of the fruit (average fruit weight about 400 g) and is usually discarded by local farmers after harvest when the beans have been taken care of (Ashun, 1973). Studies have shown that cocoa husk is rich in fibre and is poor in Metabolizable energy and crude protein, in particular for non-ruminants. Moreso, cocoa husk in addition to the nutrients shown above contain high amounts of soluble phenolics and condensed tannins, and a high content of uronic acids (Vadiveloo and Fadel, 1992). The theobromine level has been reported to be around 1.5-4.0g/kg dry weight (Barnes *et al.*, 1985; Abiola and Tewe, 1991; Falaye and Jauncey, 1999; Falaye *et al.*, 1999).

2.2.1.2 COCOA BEAN SHELL

The shell is a dry, crisp, slightly fibrous brown husk with a pleasant odour resembling that of chocolate. The fibre content is equivalent to medium quality grass hay in feeding value (Betts *et al.*, 1991). When the shell is removed, it may contain 2-3% of an unseparated cocoa nib. In Samoa and other Pacific Island countries where cocoa is produced, the shell that covers the cocoa bean is used as mulching material on the farm (Aregheore, 2002).

The chemical composition of cocoa shell indicates it might be a useful ingredient for ruminant feeding. Studies showed cocoa shell to be a useful ingredient in cattle feeding (for meat or milk production) (Flachowsky *et al.*, 1990). Studies on rumen dry matter degradability, and apparent digestibility of cocoa bean shells, as well as the influence of the shells on the rumen fermentation and performance of sheep and dairy cows allowed Flachowsky *et al.* (1990) to conclude that cocoa bean shells may be used as roughages in ruminant diets up to 5% of dry matter intake.

Hutagalung and Chang (1978) observed that the amino acid profile of CBS compares favourably with palm kernel cake suggesting that it could be utilized as a medium protein source to substitute grain protein in livestock diets. A previous study (Olubamiwa *et al*., 2002) has shown that higher dietary replacement of maize by CBS beyond 10% resulted in lower performance of layers.

The high fibre content and the content of the cell wall constituents (neutral and acidic detergent fibre and lignin) suggest that cocoa bean shells are more suitable for ruminants than monogastrics (Flachowsky *et al.*, 1990). Another factor limiting the use of cocoa bean shell in feed is the theobromine level which is dependent on the way the cocoa bean is prepared for the market (Olubamiwa *et al*., 2002; Olubamiwa and Hamzat, 2005). Originally the shell contains a limited amount of theobromine. This is acquired from the nib during fermentation. The shell of most well-fermented commercial cocoa beans contains over 1% theobromine. Abiola and Tewe (1991) reported a level of 1.9%. In another study Alvarado *et al.* (1983) measured the theobromine and caffeine content of five different shell fractions collected over the whole growing season and observed 14.0 g/kg (7.5-21.0 g/kg) theobromine and 1.4 g/kg (0.8-2.3 g/kg) caffeine, respectively.

2.2.1.3 COCOA BEAN MEAL

When excess cocoa is produced surpluses may be sold for livestock feeding under the name of cocoa bean meal hereafter sometimes referred to as cocoa meal. Cocoa meal may also be prepared from discarded cocoa beans, press cake of cocoa beans, or residues from cocoa

factories. The composition of the meal varies considerably depending on the amount of shell fragments incorporated in the meal and the degree of oil extraction (EFSA, 2008)

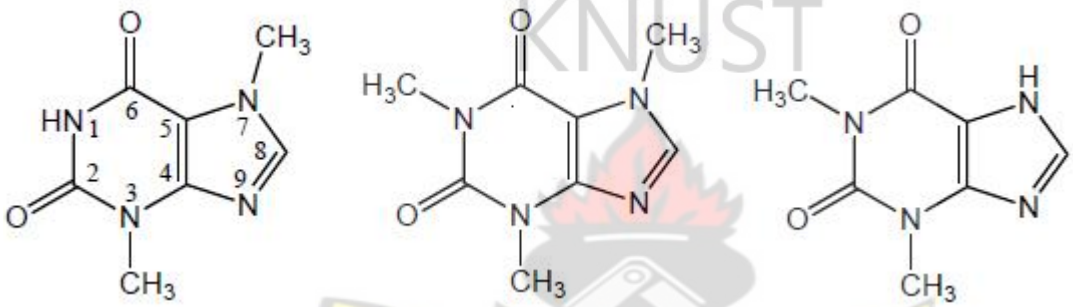
A drawback of the cocoa meal is its high theobromine content, typically 20-33 g/kg. The caffeine content is lower, around 1-4 g/kg (EFSA, 2008). Adegbola and Omole (1973) studied the influence of treating ground cocoa meal with various concentrations of sodium hydroxide or warm water of various temperatures to improve the usefulness of cocoa meal as a grower-fattener ration for swine. Water treatments for a few hours above 60^{o}C efficiently extracted theobromine. The hot water treatment retained nutritional quality of the product better than an alkali treatment.

2.3 ANTI-NUTRITIONAL FACTORS IN COCOA BY-PRODUCTS

2.3.1 THEOBROMINE

Theobromine is a colourless and odourless substance (melting point 357^{o}C) with a slightly bitter taste that is naturally present in all parts of the seed and in small quantities in the pod, most likely as a component of the chemical defence mechanism of the cocoa plant (IARC 1991; Windholz, 1983; Aneja and Gianfagna, 2001). It is therefore present in cocoa products and by-products of cocoa production. Theobromine, and to some extent caffeine, contribute to the typical bitter taste of cocoa and chocolate. The shells represent 8-10 percent of raw cocoa bean by weight (EFSA, 2008)

Though there are not much data on theobromine levels in feed materials, it has been reported that, cocoa husk meal contains 1.5-4.0 g theobromine per kg material, cocoa bean shell contains 8.0 – 16.9 g/kg (and 1g caffeine/kg) and cocoa bean meal 20 – 33 g/kg material (1-4 g caffeine/ kg). Chocolate waste is expected to contain more variable contents of theobromine (EFSA, 2008).



Theobromine **Caffeine** **Theophylline**

Fig 2.2 Molecular structure of theobromine (3,7-dimethylxanthine) [Mw 180.2], caffeine (1,3,7-trimethylxanthine) [Mw 194.2], and theophylline (1,3-dimethylxanthine) [Mw 180.2]

The fresh unfermented cocoa beans contain 14-38 g theobromine and 1-8 g caffeine per kg seed material on a dry weight basis (Senanayake and Wijesekera, 1971; Chevalley, 1976; Fincke, 1989; Sotelo and Alvarez, 1991; Naik, 2001). Thus, the caffeine content is usually around 10 - 15% of the theobromine content. Traces of theophylline may be found. However, the amount of the individual methylxanthines is dependent on the genotype of the cocoa tree.

African cocoas contain less caffeine and more theobromine than cocoas from South America (Matissek, 1997). Senanayake and Wijesekera (1971) followed the theobromine and caffeine

contents during a cycle of growth of the cocoa pod. They noted that the level of both compounds were negligible until the 3½-month stage when seeds were still mucilaginous. Subsequently, when the seeds started to become hard, both methylxanthines started to increase in amount but the increase was much higher for theobromine than for caffeine. The increase was much higher in seeds than in the hull.

2.3.1.1 EFFECT OF THEOBROMINE ON ANIMALS

Theobromine, an anti-nutritional factor or toxin found mostly in cocoa products and by-products has deleterious effect on animals (Menon, 1982) particularly monogastric animals due to the inability of these animals to detoxify this compound which is alkaloid in nature. The presence of this compound in the diet of poultry or pigs affects several physiological and developmental factors, making the by-products of the cocoa less useful to these animals though highly nutritive especially in protein and other micronutrients (Gohl 1981).

Broiler chickens that received the cocoa meal had reduced feed intake and weight gain, and increased mortality with dose as the lowest inclusion of cocoa bean meal, 15%, corresponds to 3.4 g theobromine/kg diet (estimated to be 340 mg/kg b.w. per day) (Odunsi and Longe, 1998). The most pronounced effects were reduced feed intake, reduced daily weight gain, reduced haemoglobin levels and increased creatinine levels. These negative effects were not observed in chickens given the hot-water or alkali-treated cocoa bean meal feeds at an inclusion rate of 15% of the diet, reducing theobromine exposure to 1.5 and 0.95 g theobromine per kg feed (estimated to be 150 and 95 mg/kg b.w. per day) respectively (Odunsi and Longe, 1998). Researches conducted concluded that feeding 15% and above of untreated cocoa meal to laying birds is

extremely harmful as it decreased appetite and egg production, and caused scouring and high mortality (Odunsi and Longe, 1995a,b).

2.4 EFFECT OF FUNGAL FERMENTATION ON THEOBROMINE

The use of cocoa by-products as animal feed has limited attention due to the relatively high amount of theobromine present in them (Gohl, 1981). Most of the studies conducted using untreated cocoa by-products as ingredients in poultry and pigs diets reported a low growth performance with some mortalities compared to the treated counterparts either by physical means (boiling and sun-drying), chemical means (alkali treated) or biological means (fermentation with microbes) (Olubamiwa *et al*., 2002; Olubamiwa and Hamzat, 2005) .

Several physical methods have been used in detheobrominizing cocoa by-products to maximize their use for animal feed such as sun-drying, heating and boiling which significantly decreased the theobromine level (Menon, 1982). However, these methods may affect the nutritive quality of the by-products. Detheobromination by fungal fermentation (Bio-detheobromination) which has gained little attention has proven to be more reliable and less expensive since it significantly reduces the methylxanthine contents in cocoa by-products such as cocoa pod husk (CPH) when fermented with a fungus, *Aspergillus niger* (Adamafio *et al*., 2011). *Aspergillus niger* is capable of metabolizing theobromine using the theobromine as the sole carbon and energy source without affecting the integrity of the feed material (Adamafio *et al*., 2011). The degradation of theobromine by *A. niger* probably occurs by utilizing the demethylase pathway which involves 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).

Natural fermentation of freshly harvested cocoa beans also causes a reduction in the level of methylxanthines with increasing duration of fermentation since the most available methylxanthine-degrading fungi such as *Aspergillus niger* and *Penicillum* are able to grow on the beans causing breakdown of the methylxanthine compounds (theobromine, caffeine, theophylline) (Aremu *et al*., 1995).

Aspergillus niger is widely used in the food industry during fermentation processes and particularly effective in upgrading low nutrient value materials such as palm kernel cake and rice bran during solid state fermentation (Mirnawati *et al*., 2011). These attributes render *A. niger* treated cocoa by-products suitable for animal consumption. However, there may be the presence of *Aspergillus flavus*, noted for the production of aflatoxins under stress conditions during fungal fermentation which may pose a serious threat to the health of the monogastric animals particularly poultry (Sobiya *et al*., 2009).

2.5 LIGNOCELLULOSIC MATERIALS

Lignocelluloses are the most abundant materials present on earth, comprising 50% of all biomass with an estimated annual production of 5×10^{10} tonnes (Goldstein, 1981). According to Han and Smith (1978), the most abundant renewable biomass on earth consists of cellulose, with between 5 and 15 tonnes per person being synthesized annually by photosynthesis. Much of the cellulose in nature is bound physico-chemically with lignin. Lignin, due to its highly resistant nature, protects cellulose against attack by most microbes, and it must be degraded by chemical or biological means before the cellulose can be utilized (Salvagi and Kaulkarnis, 2001). The use of the polysaccharides in the lignocellulosic complex is limited due to their high lignin content

(Hadar *et al*., 1992). The handling and disposal of these lignocellulosic residues are often problematic due to their chemical structure and decomposition properties (Philippoussis *et al*., 2001).

2.6 NATURE OF LIGNOCELLULOSIC MATERIALS

2.6.1 CHEMICAL COMPOSITION

Cellulose, hemicellulose and lignin are the main constituents of lignocellulosic materials (Deobald and Crawford, 1997) and this chemical composition of plants may differ considerably by the influence of genetic and environmental factors (Breen and Singleton, 1999). Plants may comprise not only of these primary polymers, but also other structural polymers such as waxes and proteins (Leonowicz *et al*., 1999). Lignocelluloses vary from source to source both in terms of their chemical constituents and relative ratios (Brownm, 1983).

2.6.2 TERTIARY ARCHITECTURE OF LIGNOCELLULOSE

A variety of covalent and non-covalent linkages between the constituents of lignocellulose gives the lignocellulose structures their tertiary architecture (Eriksson, 1995). Cellulose is usually complexed with hemicellulose, lignin and other compounds, which complicates the extent of hydrolysis hence comparable to reinforced concrete (Leonowicz *et al.,* 1999; Tomme *et al*., 1995). Cellulose microfibrils are stabilized by intra- and intermolecular hydrogen bonds and surrounded by hemicellulose polysaccharides (mannans and xylans) linked to cellulose by covalent and hydrogen bonds (Heredia *et al*., 1995). These covalent bonds are extremely resistant to chemical and biological hydrolysis (Leonowicz *et al.,* 1999). The amorphous regions with the cellulose crystalline structure, on the other hand, have a heterogeneous composition characterized by a variety of different bonds (Eriksson, 1995).

2.7 STRUCTURE AND PROPERTIES OF LIGNOCELLULOSIC CONSTITUENTS

2.7.1 CELLULOSE

Cellulose was first isolated in 1834 by the French Chemist Anselme Payen. While studying different types of wood, Payen obtained a substance that he knew was not starch, but which still could be broken down into its basic units of glucose just as starch can. He named this new substance 'cellulose' because he had obtained it from the cell walls of plants (Brownm, 1983).

Structure of Cellulose

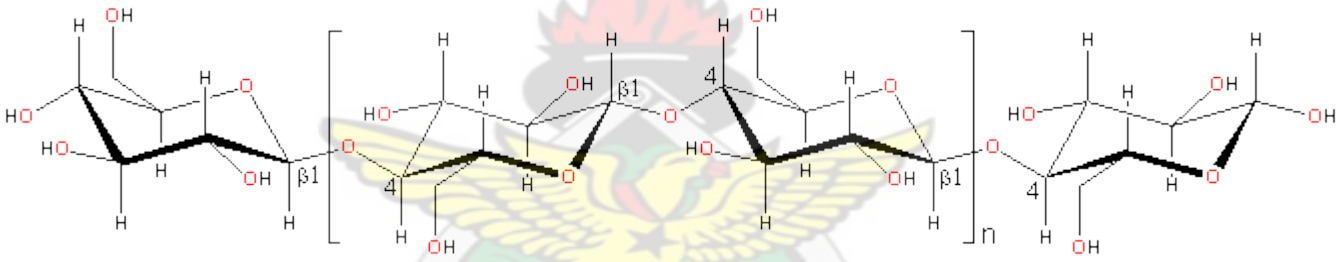


Fig 2.3 Structural units of cellulose

Source: Brownm, 1983

Cellulose (a polymeric β-glucoside) is the substance that makes up most of a plant's cell wall (Boxer, 1997). It is a polysaccharide consisting of tightly packed extended chains of D-glucopyranose molecules joined by β-1, 4 glycoside bonds and it is the most abundant of all polysaccharides (Schmid, 1996). Since it is made by all plants, it is also probably the most abundantly synthesized building material out of which plants are made, and plants are the primary or first link in what is known as the food chain (which describes the feeding

relationships of all living things), cellulose is a very important substance (Raven and Johnson, 1996). In nature cellulose is usually associated with other polysaccharides such as xylan or lignin. Cellulose is a linear polysaccharide polymer with many glucose monosaccharide units (Raven and Johnson, 1996). The acetal linkage is beta which makes it different from starch. This peculiar difference in acetal linkages results in a major difference in digestibility in humans and other non-ruminants (Boxer, 1997). Non-ruminants and humans are unable to digest cellulose because the appropriate enzymes to break down the beta acetal linkages are lacking (Boxer, 1997).

Cellulose has been identified as the simplest of the polymers in the cellulosics, being composed of a continuous chain of D-glucose molecules linked in a β-1, 4 configuration (Brownm, 1993). These chains may contain more than 104 anhydroglucopyranose units giving a molecular mass of greater than 1.5Da (Brownm, 1993). Cellulose micelles are bunched together to form thread-like microfibrils. The individual cellulose polymers are hydrogen bonded between the ring oxygen of glucose molecule and the hydroxyl groups at position 3 (Brownm, 1993). The cellulosic fibrils are composed of highly ordered micelles possessing crystalline structure interspersed with disorderly areas of so-called amorphous cellulose (Brownm, 1993). Its density and complexity make it very resistant to hydrolysis without preliminary chemical or mechanical degradation or swelling (Thygesen *et al.*, 2003).

2.7.2 HEMICELLULOSE

Hemicellulose is a particularly heterogeneous polymer, in that it is composed variously of three hexoses, glucose, mannose and galactose and the two pentoses, xylose and arabinose together

with their uronic acids (Brownm, 1993). Hemicellulose is more soluble than cellulose and is frequently branched with degree of polymerization (DP) of 100 to 200 (Kuhad *et al.,* 1997; Leschine, 1995). Three well defined groups exist, namely; xylans that have a basic backbone of poly β-1, 4 xylan with additional side links to arabinose, glucuronic acid and galactans. The origin of lignocellulosic material defines the nature of the hemicelluloses (Brownm, 1993).

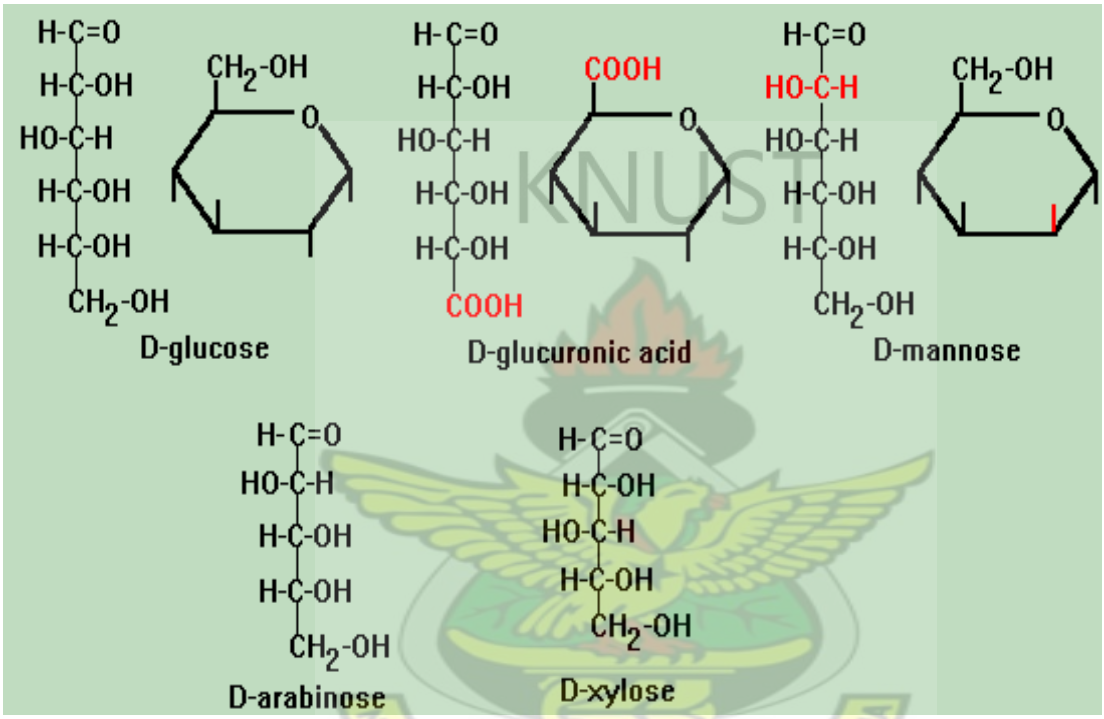


Fig 2.4 Some monomers of hemicellulose

Usually, all of the pentoses are present and there may even be small amounts of L- sugars. The hexoses as well as acids are formed by oxidation of sugars (Lebo *et al.,* 2001). Mannose and mannuronic acid tend to be present, and there can be galactose and galacturonic acid. Xylose is always the sugar present in the largest amount (Raven and Johnson, 1996). In contrast to cellulose that is crystalline, strong and resistant to hydrolysis, hemicellulose has a random, amorphous structure with little strength. It is easily hydrolyzed by dilute acid or base (Lebo *et al.,* 2001)

2.7.3 LIGNIN

Lignin is a complex chemical compound most commonly derived from wood and an integral part of the cell walls of plants (Lebo *et al.,* 2001). It is a natural, complex, heterogeneous phenylpropanoid polymer comprising 25-30% of plant biomass (Arora *et al.*, 2002). The term was introduced in 1819 by de Candolle and is derived from the Latin word 'lignum' meaning wood (Boerjan *et al.,* 2003)

It is one of the most abundant organic polymers on earth, superseded only by cellulose, employing 30% non-fossil organic carbon and constituting from a quarter to a third of the dry mass of wood (Boerjan *et al.,* 2003). Lignin has several unusual properties as a biopolymer, not least being its heterogeneity in lacking a defined primary structure (Lebo *et al*., 2001). Lignin is three-dimensional polymer of phenylpropane unit (Lebo *et al*., 2001). It is a large, cross-linked, racemic macromolecule with molecular masses in excess of 10,000 atomic mass unit (Davin and Lewis, 2005). It is relatively hydrophobic and aromatic in nature (Hatakka, 2001). The degree of polymerization in nature is difficult to measure, since it is fragmented during extraction and the molecule consists of various types of substructures which appear to repeat in a haphazard manner (Kawai and Ohashi, 1993).

Different types of lignin have been described depending on the means of isolation (Davin and Lewis, 2005). The true formulation of lignin in the natural state can only be deduced from the

theoretical reconstruction of the various degradation products (Brownm, 1983). There are three monomers that make up almost all lignin found in nature. They are biosynthesized in plants via the shikimic acid pathway and their structures are shown in Fig 2.5

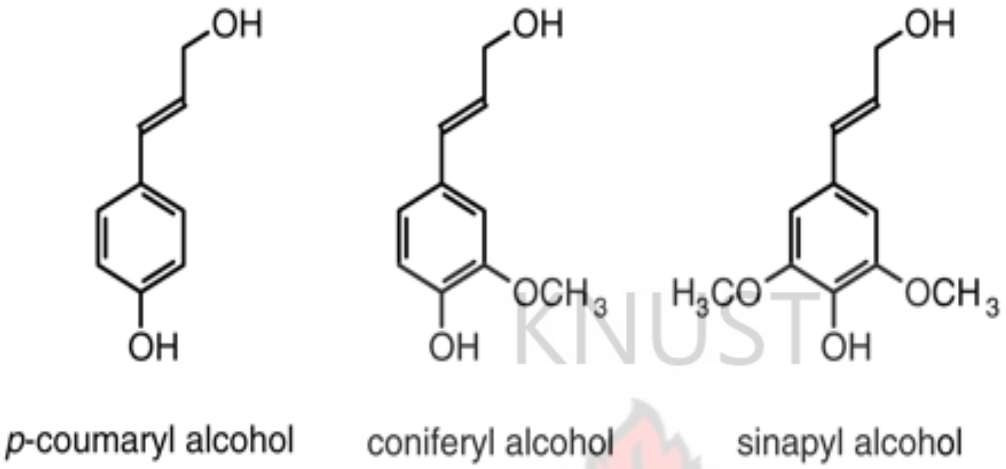


Fig 2.5 Structures and names of monomer units of lignin

2.8 BIODEGRADATION OF LIGNOCELLULOSE

Lignocellulose consisting of cellulose, hemicellulose and lignin (Betts *et al*., 1991) forms a complex substrate therefore making its degradation quite difficult. Its biodegradation, however, is not dependent on environmental factors alone, but also on the degradative capacity of the microbial population (Waldrop *et al*., 2000). The rate and extent of lignocellulose biodegradation is mostly dependent on the make-up of the microbial community (Tuor *et al*., 1995). The concerted actions of enzymes possessed by these microbial communities are required for complete hydrolysis and utilization of lignocellulosic materials (Waldrop *et al*., 2000). The lignocellulose biodegradation therefore essentially relies on the level of degradation of the cellulose and lignin components (Reid, 1989). The bioconversion process of lignocellulose into value-added bioproducts is illustrated below in Fig. 2.6.

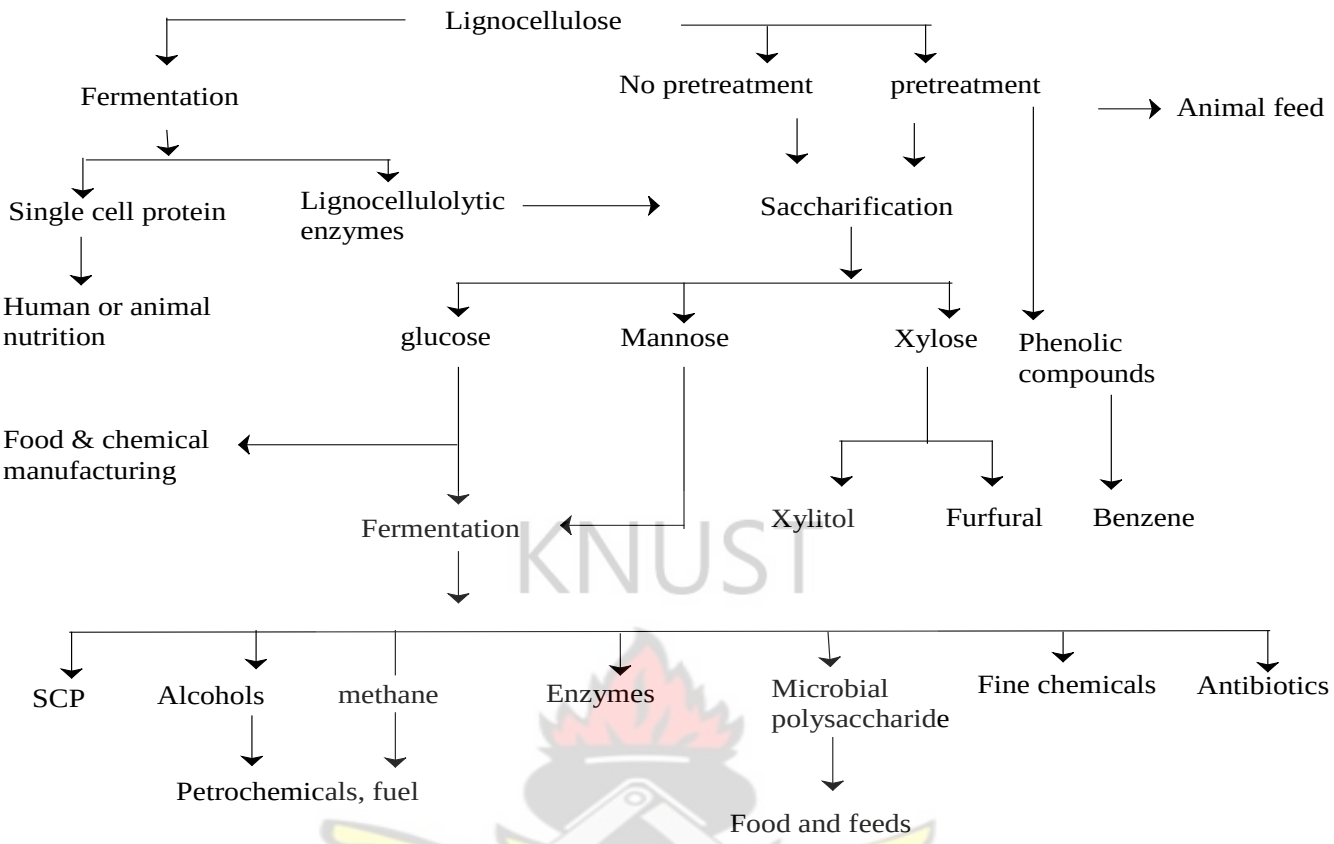


Fig 2.6 Generalized process stages in lignocellulose bioconversion into value-added bioproducts (Source: Reid, 1989**)**

2.9 LIGNOCELLULOSIC ENZYMES

2.9.1 CELLULASES

Cellulase refers to a class of enzymes produced chiefly by fungi, bacteria, and protozoans that catalyze the cellulolysis (or hydrolysis) of cellulose (Plate 2.7). However, there are also cellulases produced by other types of organisms such as plants and animals. Several different kinds of cellulases are known, which differ structurally and mechanistically (Chapin *et al.*, 2002)

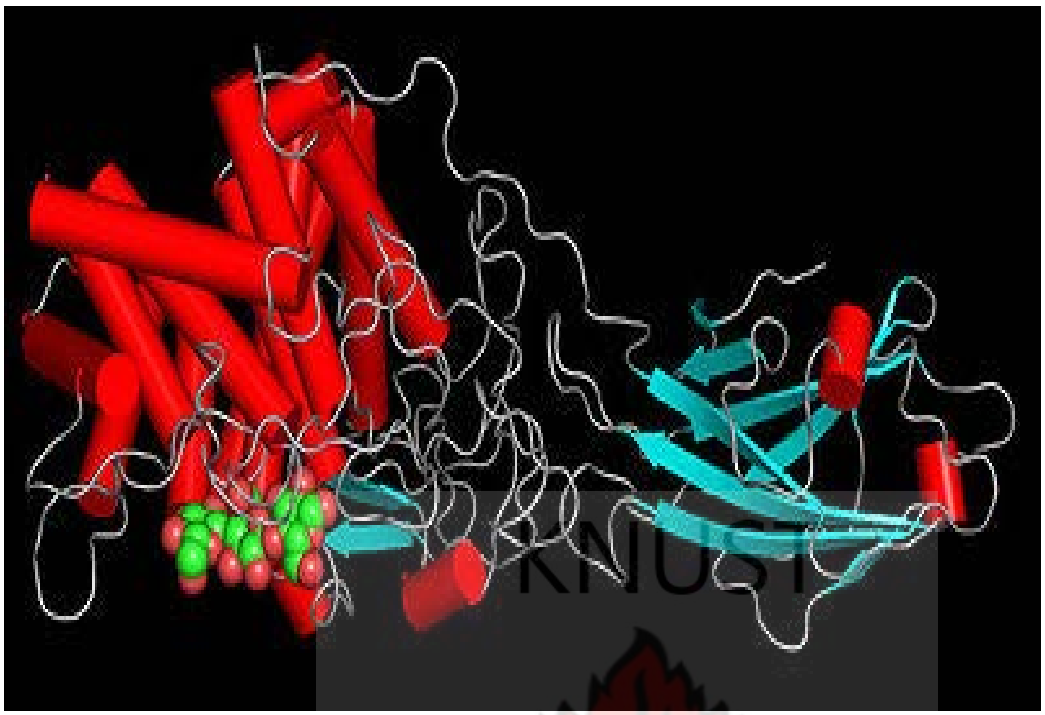


Plate 2.7 Model of Cellulase enzyme

Source: Chapin *et al.*, (2002)

There are five general types of cellulases based on the type of reaction catalyzed.

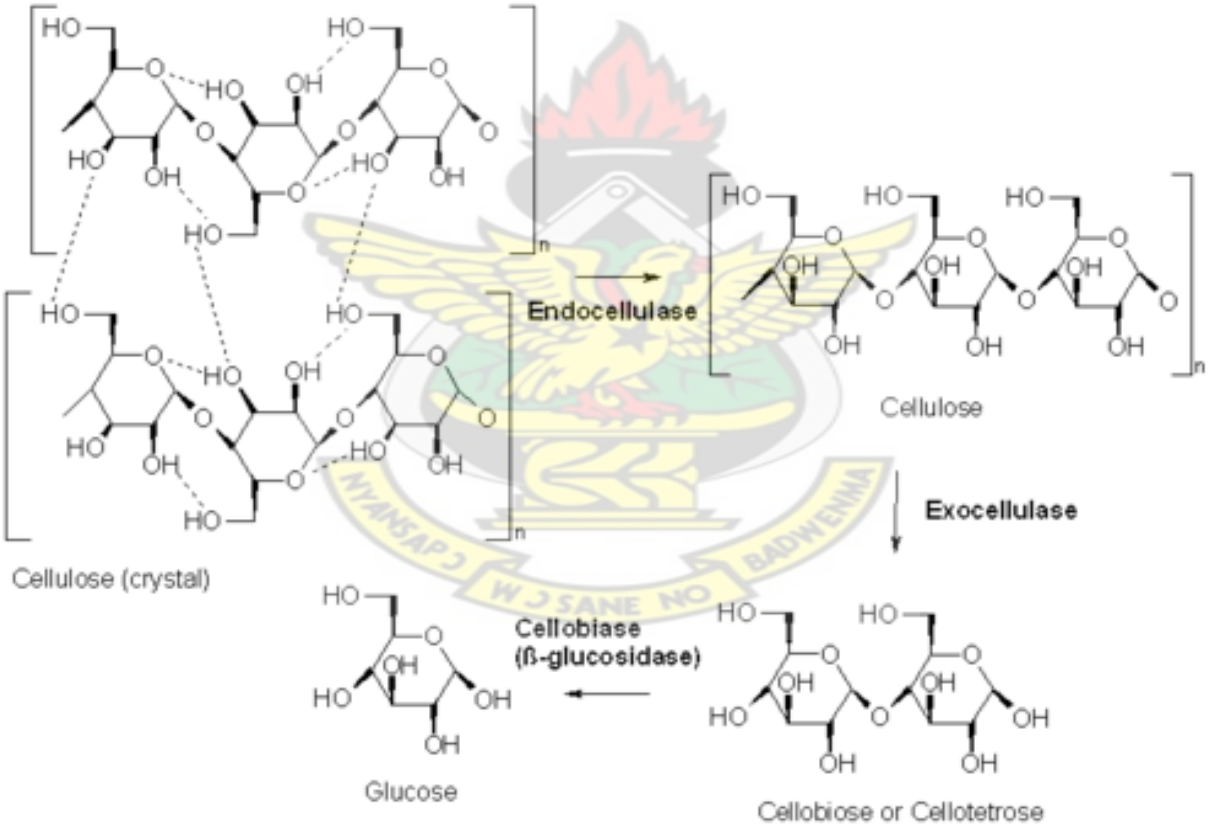
1. Endo-cellulase breaks internal bonds to disrupt the crystalline structure of cellulose and expose individual cellulose polysaccharide chains
2. Exo-cellulase cleaves 2-4 units from the ends of the exposed chains produced by endo-cellulase, resulting in disaccharides such as cellobiose (Deobald and Crawford, 1997)

The last three reactions explain the mechanism of cellulolysis as shown in Fig. 2.8

3. The breakdown of the non-covalent interactions present in the crystalline structure of cellulose by endo-cellulases

4. Hydrolysis of the individual cellulose fibers to break it into smaller sugars by exo-cellulases
5. Hydrolysis of disaccharides and tetrasaccharides into glucose by beta-glucosidase (Deobald and Crawford, 1997)

There are two main types of exo-cellulases or cellobohydrolases (CBH) – one type working progressively from the reducing end, and one type working progressively from the non-reducing end of cellulose (Tomme *et al*., 1995)



Source: Deobald and Crawford (1997)

Fig 2.8 Mechanism of cellulolysis

2.9.2 LIGNASES

Identifying lignin degrading microorganisms has been hampered because of the lack of reliable assays, but significant progress has been made through the use of a ^{14}C-labelled lignin assay (Freer and Detroy, 1982). Fungi breakdown lignin aerobically through the use of a family of extracellular enzymes collectively termed “lignases”. Two families of lignolytic enzymes are widely considered to play a key role in the enzymatic degradation: phenol oxidase (laccase) and peroxidases (lignin peroxidase (LiP) and manganese peroxidase (MnP) (Krause *et al*., 2003; Malherbe and Cloete, 2003)

Other enzymes whose roles have not been fully elucidated include H_2O_2-producing enzymes: glyoxal oxidase (Kersten and Kirk, 1987), glucose oxidase (Kelley and Reddy, 1986), veratryl alcohol oxidases (Bourbonnais and Paice, 1988), methanol oxidase (Nishida and Eriksson, 1987) and oxido-reductase (Bao and Renganathan, 1991).

2.10 THE IMPORTANCE OF BIOPROCESSING COCOA BEAN SHELLS

Though cocoa bean shell contains high nitrogen and other essential nutrients, its utilization as animal feed material (especially for monogastrics) is very limited due to the high fibrous nature and the high levels of anti-nutritional factors, particularly theobromine (Gohl, 1981). Considering the rate of processing of cocoa into its products and by-products, there is currently a very alarming threat to the environment since there is no proper disposal method of the cocoa bean shells after deshelling the beans, especially in developing countries such as Ghana.

Animals such as pigs and poultry, collectively known as monogastrics have difficulty in digesting high fibre containing diets unlike ruminants which have rumen microorganisms aiding fibre digestibility to some extent (Church, 1976)). The inability of monogastrics to utilize diets containing high fibre adversely affect their physiological development such as poor growth performance, poor laying capacity and low feed efficiency (Olubamiwa *et al*., 2000). This poor output eventually affects low rate of animal production.

There is therefore the need to address an alternative use of cocoa bean shells by improving the nutritive value thus enhancing the fibre digestibility and minimizing the levels of anti-nutritional factors so as to qualify the feedstuff for its maximum use in animal feed. One of the most recent and efficacious methods that has proven to enhance the fibre digestibility is by fermentation with *Pleurotus* spp (Alemawor *et al*., 2009). Degradation of theobromine found in cocoa products and by-products has also been achieved by fermentation of cocoa by-products with *Aspergillus niger* (Adamafio *et al*., 2011)

2.11 *PLEUROTUS SPP* AS BIOCATALYSTS

Pleurotus spp. is commonly known as giant mushroom. It is called shimeji or hiratake by Asians. The Pleurotus genus gathers several species, such as *P. ostreatus*, *P. pulmonaris*, *P. sajor caju*, *P. cornucopiae* and *P. ostreatoroseus*. *Pleurotus* is spread all round the world in its natural habitat, mainly in forest environments (Bononi *et al*., 1999). These fungi are also provided with enzymes that degrade lignin present in vegetables, this being the reason why they are known as wood white rot fungi (Abreu *et al*., 2007). Their taste is very pleasant and they are among the mushrooms with highest production in several regions of the world (Ibekwe *et al*., 2008).

Several kinds of residues may be used for *Pleurotus* spp. cultivation such as wheat straw, corn, cotton, coconut, crushed sugarcane and sawdust. In favourable environments (temperature, relative humidity, luminosity) they produce lignocellulase enzymes, mainly laccase (LAC) and Mn-peroxidase (MnP), which convert these lignocellulosic residues into food. However, the addition of supplements to these substrates, such as wheat bran, rice and soy is recommended, in order to obtain a satisfactory development (Bononi *et al*., 1999; Eira, 2004; Bernardi *et al*., 2008). Fast and efficient development and low production cost in the most varied agro-industrial residues make *Pleurotus* spp. a very profitable cultivation target (Adu- Amankwah, 2006))

2.12 *IN VITRO* DIGESTIBILITY STUDIES

The nutritive quality of any feedstuff is evaluated by the availability of its nutrients to the animal in question for good growth performance (Tatli and Cerci, 2006). However, nutritional management may be influenced by several factors such as the physiological and hormonal conditions of the animal (Cone *et al*., 1996). Monogastric animals such as poultry and pigs do not have any microorganisms in the GIT unlike ruminants therefore always need high energy diets containing readily available simple sugars (Chang *et al*., 1998) to complement their energy requirements. The bioavailability of a feedstuff can therefore be investigated outside the animal by the application of *in vitro* digestibility studies (Tilley and Terry, 1963) which employs enzymatic action on feed material in a test tube to evaluate the utilization of the feed by the animal. The only drawback with *in vitro* digestibility is that these methods cannot mimic the complex and dynamic conditions in the digestive tract of live animals where exogenous and endogenous secretions and nutrients are mixed and where there are also interactions in

concentration, inhibition, microbial, neutral and hormonal effects though relatively simple, inexpensive, rapid and high level of precision achieved (Tilley and Terry, 1963).

CHAPTER THREE

3.0 MATERIALS AND METHODS

3.1 MATERIALS

Substrate

The dried cocoa bean shells of unspecified variety were obtained from the Cocoa Research Station at Mpintin in the Western region of Ghana.

Drying equipment

Solar dryers were used for drying the samples which are located at the Department of Biochemistry and Biotechnology Annex of Kwame Nkrumah University of Science and Technology (KNUST)

Pasteurization unit

A bucket-like autoclave of regulated temperature and pressure was used for the pasteurization of the substrates obtained from the Plant Pathology Laboratory of the Department of Soil and Crop Sciences, KNUST.

Aluminium trays and foils

Aluminium trays (dimensions 108cm x 65cm), plastic bowls and foils used for the fermentation process were obtained from Central Market, Kumasi.

Fungi for fermentation

Oyster mushroom (*Pleurotus ostreatus*) spawn for the first stage fermentation was obtained from Robart Farms, a mushroom production farm at Kenyasi in Kumasi.

Identification and isolation of *Aspergillus niger* for the second fermentation was undertaken at the Pathology Laboratory of the Faculty of Agriculture, K.N.U.S.T, Kumasi.

Plate 3.1 : Dried cocoa bean shells as substrate

3.2 PRELIMINARY STUDY

Preliminary experiment was conducted to ascertain the order of the two-stage fermentation as far as the two fungi mentioned earlier are concerned i.e. fermentation of the cocoa bean shells with *Pleurotus ostreatus* followed by *Aspergillus niger* or vice versa.

Procedure

Some amount of the cocoa bean shells (CBS) (unmeasured) was milled into 2mm particle size and composted for four days. The composted CBS used as substrate for fermentation was supplemented with 4% Calcium Carbonate (Thulasi *et al*., 2010) due to the low pH obtained. The substrate was placed in plastic bowls and each inoculated with *P. ostreatus* spawn and *A. niger* spore suspension separately in triplicate. The inoculated substrates were then incubated at room temperature for one week.

After the one week period of incubation, growths were observed in both fungi inoculated substrates. The *P. ostreatus* fermented and the *A. niger* fermented CBS were sun-dried for five days and then pasteurized. The pasteurized *P. ostreatus* fermented CBS was then inoculated with *A. niger* spore suspension as the pasteurized *A. niger* fermented CBS was also inoculated with *P. ostreatus* spawn and incubated at room temperature for one week. This was done in triplicate.

After the one week period of incubation, it was observed that *P. ostreatus* was unable to grow on the *A. niger* fermented CBS under the same conditions unlike the *A. niger* which was able to grow on the *P. ostreatus* fermented CBS.

In view of the outcome of the preliminary experiment conducted, *P. ostreatus* fermentation followed by *A. niger* was selected for the two-stage fermentation.

EXPERIMENT ONE

3.3 FERMENTATION OF COCOA BEAN SHELLS WITH *Pleurotus ostreatus*

3.3.1 SUBSTRATE PREPARATION

3.3.1.1 Size reduction

Dried cocoa bean shells used as substrate for the fermentation was milled into a uniform particle size of 2 mm using a hammer mill.

3.3.2 Composting

During composting, the substrate was spread on black plastic sheet and water sprinkled and turned inside out until 60-65% moisture content was attained which was achieved by taking a handful of the wetted substrate and squeezing it, ensuring no water droplets drip out between the fingers, an improvised method of moisture determination. The substrate was then heaped to a height of 1.5m (Alemawor *et al*., 2009) and covered with the plastic sheet to protect it from direct effect of sun and rain. It was left for four days (Alemawor *et al*., 2009) with intermittent turning over every two days to ensure equal distribution of water and heat throughout. This also ensured uniform degradation and replenishing of oxygen. The temperature observed after the first four days was 44^{o}C. The temperature and the pH at the end of the composting were 56^{o}C and 5.74 respectively. The composted substrate was then supplemented with 4% Calcium Carbonate (Thulasi *et al*., 2010) (to control the pH of the material during fermentation). The significance of the composting process is basically to allow the residing microbes to degrade the substrate and make some nutrients available for initial fermentation.

3.3.3 Pasteurization

The composted substrate was wrapped in aluminium foils in 200g portions and arranged in a pan of the bucket-like autoclave suspended in water and tightly covered under pressure. Pasteurization was done at 121^{o}C at 15psi for 15minutes.

3.3.4 Inoculation

After the pasteurized substrates were cooled, the bottle containing the spawn was shaken to loosen the grains and then 2 grams of grains was introduced onto 200 grams of the substrates in each tray. A control experiment was set where triplicate substrates were not inoculated with the *P. ostreatus* spawn. This was done not only to ascertain the effectiveness of the fermentation by the fungus but also to check the sterility of the fermentation process.

3.3.5 Incubation

Plastic trays with the inoculated substrates were kept in an incubation room undisturbed to allow the growth of the mycelia through the substrates. Experiments were done in triplicate by week up to 8 weeks having a total of twenty-four inoculated substrates.

At the end of each week, the fermentation process was terminated by drying the triplicate substrates in the solar dryer for five days. The dried substrates were then milled to homogeneity and kept in the solar dryer for chemical analysis.

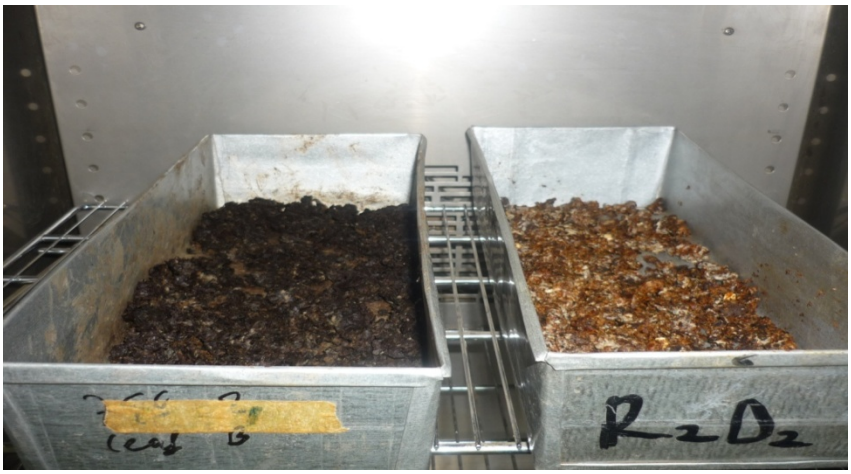


Plate 3.2 Drying of substrates in solar dryer

3.3.6 Chemical Analysis

The chemical composition of the treated substrates was determined by proximate analysis which included crude protein, crude fibre, ether extract (crude fat), ash and moisture (AOAC, 2002) and by Van Soest Method of Analysis (Van Soest, 1995) for the determination of the fibre components of the treated substrates namely; Acid detergent fibre (ADF), Neutral detergent fibre (NDF) and Acid detergent lignin (ADL). The mineral elements, Calcium and Potassium were determined using Flame Photometry and the Phosphorus using the spectrophotometric Vanandium Phosphomolybdate method . The anti-nutritional factor (theobromine) was determined using gravimetry method by Holmes (AOAC, 2002).

Acquisition of dried cocoa bean shells

↓

Size reduction

↓

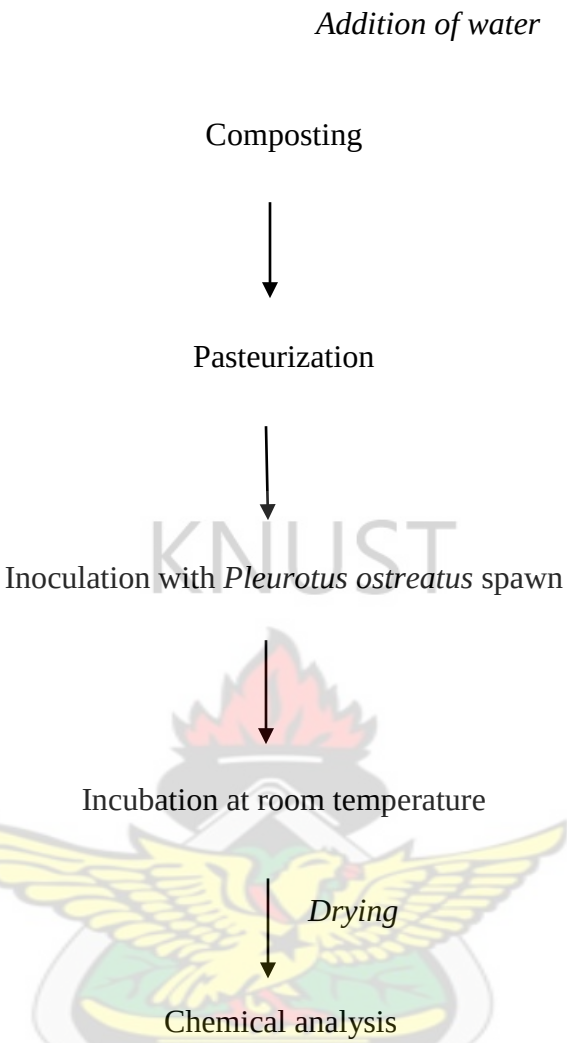


Fig. 3.1 Flow diagram for the bioprocess

3.3.6.1 Determination of Moisture

Two (2.0) grams each of raw, uninoculated and inoculated samples were weighed into already cleaned and weighed crucibles in duplicate. The crucibles were placed in a (Wagtech)

thermostatically controlled oven at 110 ^{o}C for 24 hours. The crucibles were then removed and placed in a dessicator to cool and reweighed. The procedure was repeated until a constant weight was obtained. Moisture content was calculated by difference and expressed as a percentage of the initial weight of the samples.

3.3.6.2 Determination of Ash

Two (2.0) grams each of raw, uninoculated and inoculated samples were weighed into already cleaned and weighed crucibles in duplicate. The crucibles were placed in a furnace (Vecstar) preheated at 600^{o}C for 2 hours. The crucibles were removed, cooled and reweighed. The masses of the crucibles and their contents were found by subtraction. The ash content was calculated by difference and expressed as a percentage of the initial weight of the sample.

3.3.6.3 Determination of Calcium and Potassium using Flame Photometry

Reagents and Materials

8N Hydrochloric acid

Distilled or deionized water

Volumetric flasks (100ml)

Funnels

Whatman No. 40 filter paper

Water bath

Porcelain crucibles

Muffle furnace (500- 600^{o}C)

Procedure

One (1.0) gram of finely ground sample was weighed into a porcelain crucible and placed in a muffle furnace and heated at 500^{o}C for 4 hours. After 4 hours, the inorganic content was removed and allowed to cool. The content was then moistened with 2 ml distilled water after which 5.0 ml of 8N HCl (Mix 2 parts of concentrated HCl with 1 part of water) was slowly and carefully added. The solution in the crucible was covered and placed on steam water bath for 20 minutes after which it was filtered through Whatman No. 40 filter paper trapping the filtrate into a 100ml volumetric flask. The crucible was well washed with distilled water through the filter paper and the same to the filter paper several times. The volumetric flask was made up to the 100 ml mark and shaken vigorously to ensure complete mixing and stoppered. The solution was then used in the determination of Ca and K using the Flame Photometer instrument. Appropriate standard solutions of the elements were prepared and used to calibrate the instrument in each element determination.

3.3.6.4 Determination of Total Phosphorus using Spectrophotometric Vanadium Phosphomolybdate method

Reagents and Materials

Ammonium Molybdate – Ammonium Vanadate in HNO_3 (Vanadomolybdate)

Standard Phosphorus Solution: 50ppm P

Digestion block/Heating mantle

Digestion flask

Spectrophotometer

Beakers – various sizes

Flasks – various sizes

Procedure

After the samples have been ashed and digested with 8N HCl (Refer to the initial steps for the determination of Ca and K), 10ml of the digest was measured into a 50ml volumetric flask and 10ml of Vanadomolybdate reagent added. The contents were made up to volume with distilled water, shaken vigorously and kept for 30 minutes. A development of yellow colour was obtained which was read at 430nm on a spectrophotometer. The percentage transmittance was recorded from which the absorbance was determined hence the P content from the standard curve was calculated.

3.3.6.5 Determination of Crude protein

3.3.6.5.1 Digestion

Two (2.0) grams of raw, uninoculated (control) and treated samples were put into digestion flasks and a half tablet of Selenium-based catalyst added. Twenty-five (25.0) millilitres of

concentrated Sulphuric acid was added to each of the flasks and shaken thoroughly to ensure complete soaking of the samples. The flasks were then placed on a digestion burner and heated slowly with intermittent shaken until a clear greenish solution was obtained (usually one hour period). The digested samples (digests) were allowed to cool and transferred into a 100ml volumetric flask and made up to the mark with distilled water.

3.3.6.5.2 Distillation

Kjeldahl nitrogen distillation apparatus (Tecator™ Kjeltec System) used was flushed out using distilled water for about five minutes. Twenty–five (25) milliliters of 5% boric acid was measured into a 250ml conical flasks and two drops of mixed indicator added. Each of the 100ml diluted digests was transferred into a Kjeldahl distillation tube and then 50ml of 40% NaOH added. The apparatus was switched into operation whereby steam was generated and boiled the mixture in the tube. This process liberated gaseous ammonia which was collected into the conical flask until a 100ml of the distillate was obtained. The colour change observed in the conical flask was pink to bluish green. A blank test was conducted without the test samples.

3.3.6.5.3 Titration

The distillates were titrated with 0.1N HCl solution. The end-point was noted during the titration which was from the bluish green colour to colourless. The volume of HCl used (titre values) was then recorded after colour changes to pink by a drop.

3.3.6.6 Determination of Crude fibre

Crude fibre is the non-soluble or indigestible portion of the carbohydrate of a feed material and is normally determined on defatted samples or samples with negligible amounts of fat content. Since the cocoa bean shell was obtained by analysis to have negligible fat content, crude fibre measurement was not preceded by crude fat determination. Two (2.0) grams of each of the samples was weighed into a 750 ml Erlenmeyer flask and few chips added and 200 ml of 1.25% sulphuric acid was added to each of the samples. The flask was then set on a hot plate and connected to a condenser. The content was timed at the onset of boiling. At the end of the thirty minutes, the flask was removed and the contents filtered through a linen cloth in a funnel. Boiling water was used to wash the content till the acid was totally removed. The distillate was discarded and the residue was placed back into the Erlenmeyer flask containing the chips using a spatula and 200 ml of 1.25% NaOH was added and flask reconnected to the condenser. The content was left to boil for thirty minutes after which the flask was disconnected and the content filtered using linen cloth. The content was washed with boiling water till the base was completely removed and then finally washed with ethanol to break any lumps. The residue was transferred into a porcelain crucible and dried in an oven for 2 hours at 130^{o}C. The weight of the dried residue now composed of the crude fibre and minerals was recorded and then placed in the furnace for thirty minutes to burn off the organic material (crude fibre) leaving the inorganic (minerals). The content after ashing was allowed to cool after which the weight was taken. The crude fibre was obtained by a subtraction of the weight of the ash from the weight of the dried residue before ashing.

3.3.6.7 Nitrogen- free Extracts (NFEs)

Nitrogen-free extracts (NFEs) represents the soluble carbohydrate of a feed, such as starch and sugars. This is determined simply by subtracting the average of each of the other components (percent crude protein, crude fat, crude fibre, moisture and ash) from 100 (Crampton *et al*., 1969).

3.3.6.8 Metabolizable Energy (M.E)

The calorie content or M.E of a feed is dependent on the amounts of crude protein, crude fat, and carbohydrate in the product. Carbohydrates are not measured directly, but can be estimated by calculating the "nitrogen-free extract" (or NFE) in the product. The M. E is calculated by multiplying each of the average percentages for the calorie-containing nutrients by the appropriate "modified Atwater" value. Protein and carbohydrate are assigned a value of 3.5. Fat is much more calorie dense, hence has a value of 8.5. The results of the three calculations are added. Then, to convert the answer to kcal/kg, the sum is multiplied by 10 (Crampton *et al*., 1969).

3.3.6.9 Detergent fibre analysis

Detergent fibre analysis was conceptualized to highlight the major constituents of the plant cell wall namely; cellulose, hemicellulose and lignin which are not accounted for by the crude fibre of the proximate analysis. Therefore the two main components of the detergent fibre analysis

proposed by Van Soest (1995) were acid detergent fibre (ADF) and neutral detergent fibre (NDF).

3.3.6.7.1 Acid Detergent Fibre (ADF) determination

Reagents

Sulphuric acid (98% purity)

Cetyl trimethyl ammonium bromide (CTAB)

Acetone

Preparation of Acid detergent solution: 27.84 ml of sulphuric acid was transferred into a 1000 ml volumetric flask and brought to the mark with distilled water and 20 grams of CTAB was added to form a clear solution by heating.

Procedure

One (1.0) gram of air dried sample was transferred into Berzelius beaker. A hundred (100) ml of the acid detergent solution was then added and heated to boil for 5 to 10 minutes and then allowed to boil for 60 minutes. The content was washed with hot water two to three times after which it was then washed with acetone until no further colour was removed and filtered into a pre-weighed crucible. The residue was put in an oven at 100^{o}C for 5 hours after which it was cooled in a dessicator and weight recorded.

3.3.6.7.2 Neutral detergent fibre NDF) determination

Reagents

Sodium lauryl sulfate (Sodium dodecyl sulphate)

Disodium dihydrogen ethylene diamine tetraacetic acid (EDTA- disodium salt)

Sodium borate decahydrate (Borax)

Disodium hydrogen phosphate anhydrous (Na_2HPO_4)

2- Ethoxy ethanol (ethylene glycol monoethyl ether)

Acetone

Sodium sulphite

Decahydronaphthalene

Preparation of Neutral detergent solution: 18.61 grams of EDTA and 6.81 grams of sodium borate decahydrate were weighed and transferred into a beaker. The content was then dissolved in 200 ml of distilled water by heating and 200 ml of a solution containing 30 grams of sodium lauryl sulphate and 10 ml of 2-ethoxy ethanol added. A hundred (100) ml of a solution containing 4.5 grams of disodium hydrogen sulphate was then added and the solution made up to one litre volume with distilled water and pH adjusted to 7.0.

Procedure

One (1.0) gram of the air dried sample was transferred into a refluxing flask and 100 ml of cold neutral detergent solution added. Two (2.0) ml of decahydronaphthalene and 0.5 grams of sodium sulphite were added and heated to boil and refluxed for 60 minutes. After the 60 minutes, the content was washed with hot water two to three times and finally washed with acetone two

times. The residue was transferred into a pre-weighed crucible and dried in an oven at 100^{o}C for five hours. The crucible was then cooled in a dessicator and weight recorded.

3.3.6.7.3 Acid detergent lignin (ADL) determination

Reagents

72% H_2SO_4 standardized to specific gravity of 1.634 at 20^{o}C

All other reagents are the same as those for ADF

Procedure

The ADF residue was placed in a 50 ml beaker and the contents covered with 72% H_2SO_4. The content was stirred with a glass rod intermittently to a smooth paste and allowed to stand for 3 hours. After the 3 hours, the content was filtered and washed with hot water through Gooch crucible (perforated) until free of acid and the glass rod rinsed and removed. The residue remaining in the crucible was dried in oven at 130 degrees Celsius for 2 hours. It was then cooled in a dessicator, weighed and weight recorded. It was put in muffle furnace at 600 degrees Celsius to ash for 2 hours after which it was cooled and weight recorded. The ADL was obtained by subtracting the weight of the ash from the weight of the dried residue.

EXPERIMENT TWO

3.4 FERMENTATION OF *Pleurotus ostreatus* FERMENTED COCOA BEAN SHELLS WITH *Aspergillus niger*

3.4.1 Isolation and Identification of *Aspergillus niger*

3.4.1.1 Preparation of Potato Dextrose Agar (PDA)

Materials

Potato – 200g

Dextrose – 20g

Agar – 20g

Tap water – 1L

Procedure

Two hundred (200) grams of already cleaned and peeled potatoes cut into 12 mm cubes was weighed into a beaker and then rinsed several times with water. The washed potato cubes were soaked in 1L of water and then boiled until soft (1 hour). The softened potato cubes were mashed and squeezed through a fine sieve. To the sieved potato liquid, 20 grams of agar was added and boiled till dissolved. Twenty (20) grams of dextrose was then added and stirred to dissolve. The mixture was transferred into a one litre conical flask and made up to the mark with water. The mixture was then sterilized at 15psi for 20 minutes after which it was allowed to cool and stored.

3.4.1.2 Isolation of *Aspergillus niger* from cocoa bean shells

A portion of the sterilized Potato Dextrose Agar (PDA) was poured into already sterilized Petri dishes in duplicate. The PDA containing Petri dishes were inoculated with the shells in a Laminar Flow and incubated at room temperature for seven days.

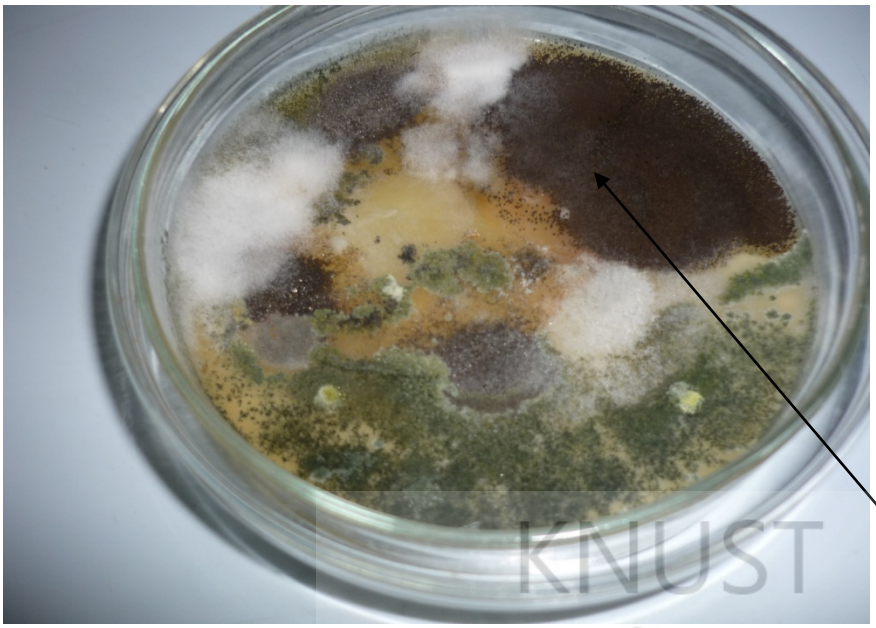


Plate 3.3 Colonies of different microorganisms cultured from cocoa bean shells

The identification of the *Aspergillus niger* was microscopically based on the morphological and physiological characteristics of the species. *A. niger* is usually identified by the black sporulation.

To obtain a pure culture of the *A. niger*, an already sterilized needle was used to pick some spores of the *A. niger* and inoculated into a PDA culture medium in triplicate.

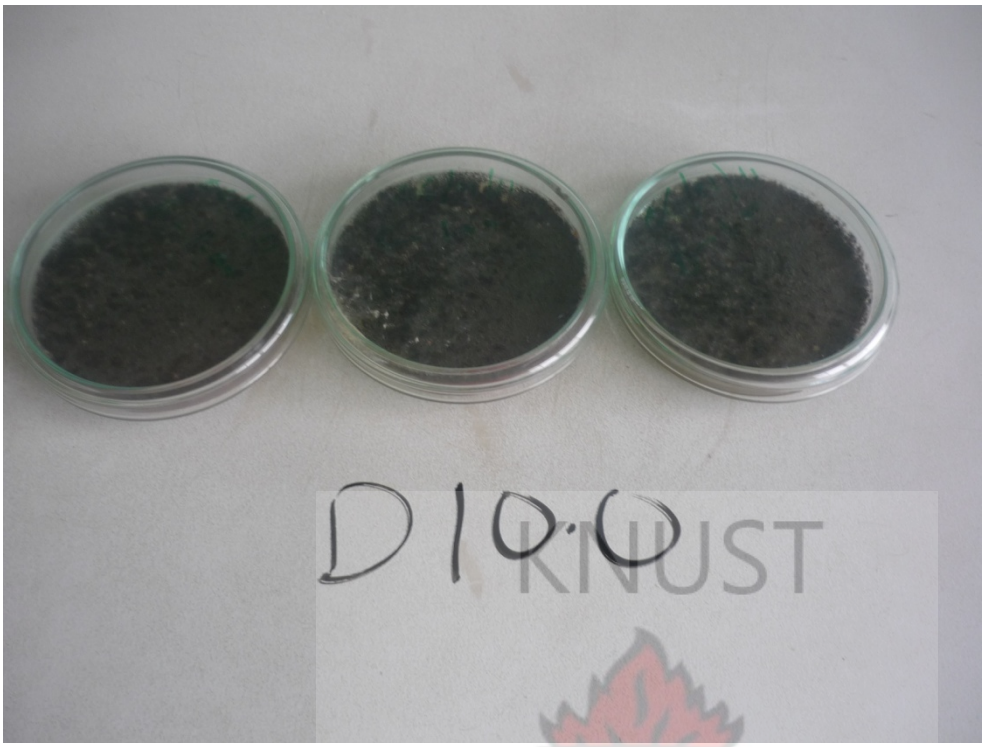


Plate 3.4 Pure cultures of *Aspergillus niger* incubated for six (6) days

3.4.2 Preparation of sub-cultures for fermentation

A 2 mm cork borer was used to pick spores of the *A. niger* from each of the pure cultures which were inoculated into already sterilized Petri dishes with PDA in seven replicates giving a total of 21 Petri dishes. The Petri dishes were then incubated at room temperature for six days.

3.4.3 Preparation of *A. niger* spore suspension

At the end of six days of incubation, spores of the mould were harvested by flooding each of the 6-day old cultures with 20 ml of sterilized distilled water and dislodging the spores by scraping. The spore suspensions were then decanted and pooled into a 500 ml Erlenmeyer flask to the mark.

Plate 3.5 Spore suspension of *Aspergillus niger*

3.4.4 Determination of Spore concentration

One (1) ml of spore suspension was taken from the stock into a 100 ml measuring cylinder and made to the mark with sterile distilled water. The diluted suspension was shaken and a portion pipetted 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 five regions of the haemacytometer and the average taken. The average value was then multiplied by 10^4 number of cells/ml and a dilution factor of 10. The spore concentration was obtained to be 2.5 x 10^6 cells/ml.

3.4.5 Substrate for *A. niger* fermentation

Dried and grounded *Pleurotus ostreatus* fermented cocoa bean shells (POFCBS) of week one to week eight were bulked into one sample which was used as substrate for the *Aspergillus niger* fermentation based on previous findings that *Pleurotus ostreatus* fermentation is incapable of biodegrading alkaloids in cocoa by-products (Alemawor *et al.,* 2009)

3.4.6 Pasteurization of Substrate

The POFCBS used as substrate for *A. niger* fermentation and the non-*Pleurotus ostreatus* fermented cocoa bean shells (NPOFCBS) were moistened with sterile distilled water, wrapped in aluminium foil and placed in a bucket-like autoclave at 121^{o}C for 15 minutes.

3.4.7 Inoculation of Substrate with *Aspergillus niger* spore suspension

Forty (40) grams of sterile cocoa bean shell powder (substrate) was inoculated with 80ml of the spore suspension of spore concentration of 2.2 x 10^{6}cell/ml (Krishnan *et al.*, 1954). A control experiment was set up having non-*Pleurotus ostreatus* fermented cocoa bean shells (NPOFCBS) and *Pleurotus ostreatus* fermented cocoa bean shells (POFCBS) without *Aspergillus niger* with POFCBS as the main control.

3.4.8 Incubation of inoculated substrates

Substrates inoculated with the *A. niger* spore suspension were kept in an incubation room at room temperature for seven days. After each day, the fermentation process was terminated by drying the substrates in an oven at 60^{o}C for two days to inactivate the *A. niger*. The dried substrates were then prepared for theobromine determination.

Plate 3.6 Incubation of inoculated substrates

3.5 Determination of Alkaloids in cocoa products and by-products

Alkaloids also called methylxanthines are compounds mostly found in cocoa products and by-products. They comprise theobromine, theophylline and caffeine which may be present in the cocoa products and by-products in varied amounts. Several methods have been described experimentally in the determination of alkaloids in cocoa products and by-products based on selective separation and extraction using chlorinated solvents and employing gravimetry, spectrophotometry, volumetry and chromatography (AOAC, 2002). The gravimetric method of Holmes (AOAC, 2002) was used for the determination of theobromine in the *Aspergillus niger* fermented cocoa bean shells which consists of extraction of the material with chloroform and formation of a complex by the reaction of theobromine with silver nitrate releasing nitric acid which is quantified by titrating against a standard alkali.

3.5.1 Determination of Theobromine in the *Aspergillus niger* fermented cocoa bean shells (ANFCBS)

Reagents

Chloroform

0.10N Sulphuric Acid

0.10N Sodium Hydroxide

0.10N Silver Nitrate

Phenol red indicator

Procedure

Due to the negligible amount of fat in the raw, control and fermented cocoa shells, the materials were directly used without refluxing with chloroform. A 0.5 gram of each of the samples was weighed and dissolved in 100ml of water containing 1.5ml of 0.10N Sulphuric acid by heating almost to boiling and the temperature maintained for 5 minutes. The content was then cooled to 40^{o}C and then 1.5ml of phenol red indicator solution added. A measured volume of approximately 1.5ml of Sodium hydroxide was added until the solution turned red. The colour was then brought back to yellow by the careful addition of 0.10N Sulphuric acid. A measured volume of 40ml 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;

1ml of 0.10N sodium hydroxide ≡ 18.01mg of theobromine

3.6 Experimental Design

The Complete Randomized Design (CRD) was used to sample the substrates for determining;

(i) The effect of *P. ostreatus* fermentation on the proximate composition, mineral contents, and the individual fibre components of the cocoa bean shells

(ii) The effect of *A. niger* fermentation on the theobromine content of the cocoa bean shells (CBS).

3.7 Data Analysis

Results were presented as mean standard values of three replicates each. A one-way analysis of variance (ANOVA) was carried out at $P < 0.05$ level of significance. Graphs were plotted using the GraphPad Prism (Version 5.0) software

CHAPTER FOUR

4.0 RESULTS AND DISCUSSIONS

4.1 Proximate composition of cocoa bean shells fermented with *Pleurotus ostreatus*

4.1.1 Moisture Content

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. Low moisture content is therefore required for a longer shelf life., there was a gradual increase of the moisture content from the

first week and then peaked at the sixth week at a moisture content of 18.17% after which it declined to 14.17% at the eighth week (Fig. 4.1). On the other hand, the control (uninoculated) samples had no significant difference ($p>0.05$) (Appendix 4A) in the moisture content throughout the fermentation period. The 5.88% increase in moisture content of the inoculated cocoa shells from the first week to the sixth week could be attributed to the mycelia growth of the mushroom. This observation agrees with Bano *et al* (1986), who stated that mycelia of mushrooms contain some amount of moisture. Sawyer (1994) also reported that water forms about 50% of the composition of mushrooms. The decline of the moisture content after the sixth week was as a result of the formation of fruiting bodies of the mushroom which utilizes nutrients as well as water for growth (Rypacek, 1966).

The moisture content of 14.17% obtained after the fermentation of the cocoa bean shells with *Pleurotus ostreatus* is slightly higher than the recommended level for feedstuffs which states a moisture level not more than 12% (Thiex and Richardson, 2003)

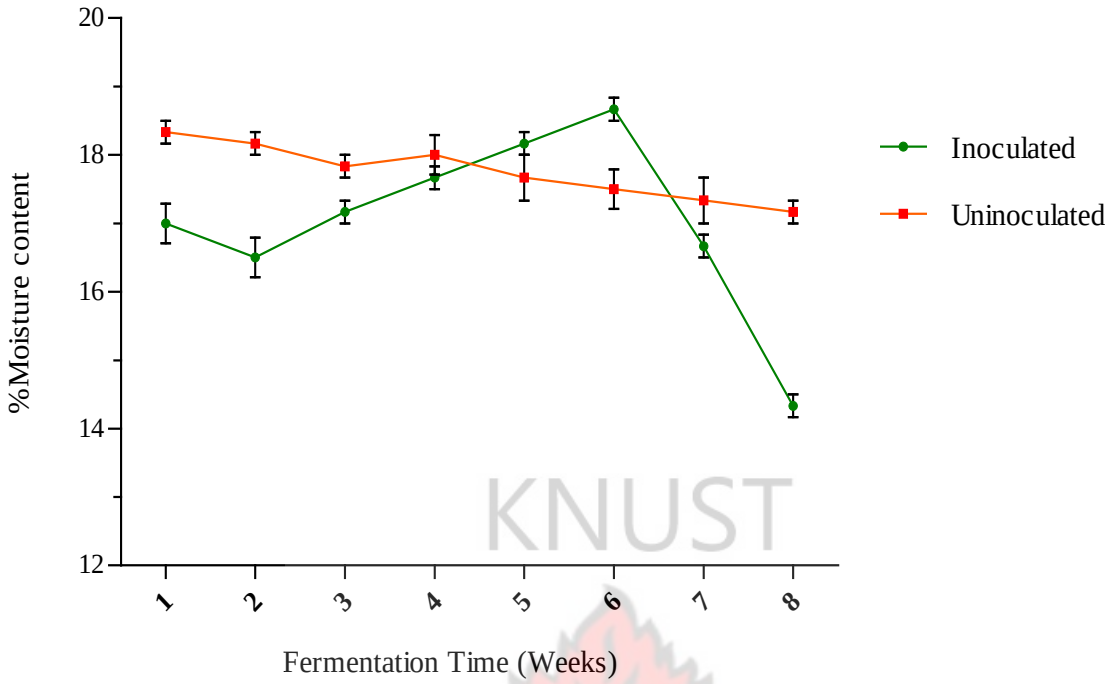


Fig 4.1 Effect of fermentation of cocoa bean shells with *Pleurotus ostreatus* on moisture content

4.1.2 Ash Content

The ash content of the inoculated samples had a gradual increase from the first week to the eighth week with values ranging from 11.18% to 18.25% respectively (Fig. 4.2). Significant differences ($p<0.05$) (Appendix 4B) existed in the inoculated cocoa bean shells unlike the control which had no significant difference in the ash content over the period of fermentation. The general increase in the ash content could be attributed to the enrichment of minerals by mycelia of the mushroom grown on the cocoa shells. According to Sawyer (1994), mushrooms have richer supply of minerals than many meat products and two times higher than in vegetables. Additionally, the fruiting bodies of mushrooms contain about 10% ash on dry matter basis (Bano *et al.*, 1986).

The fermentation of the cocoa shells with *P. ostreatus* generally impacted a positive effect on the total mineral content of the product compared to the control which though had a higher ash content at the first week realized no significant difference throughout the fermentation period. This result obtained actually confirms the assertion that the observed increase in the ash content of the inoculated cocoa shells was as a result of the mycelia growth on the cocoa shells. The 38.74% increase (from week one to week eight) in the ash content of the cocoa shells after the fermentation with the fungus makes the cocoa shells a very useful feed material for animals as minerals in diets of animals play several roles such as development of strong bones and also needed in metabolic processes preventing certain health-related problems associated with deficiency of minerals (Church, 1976).

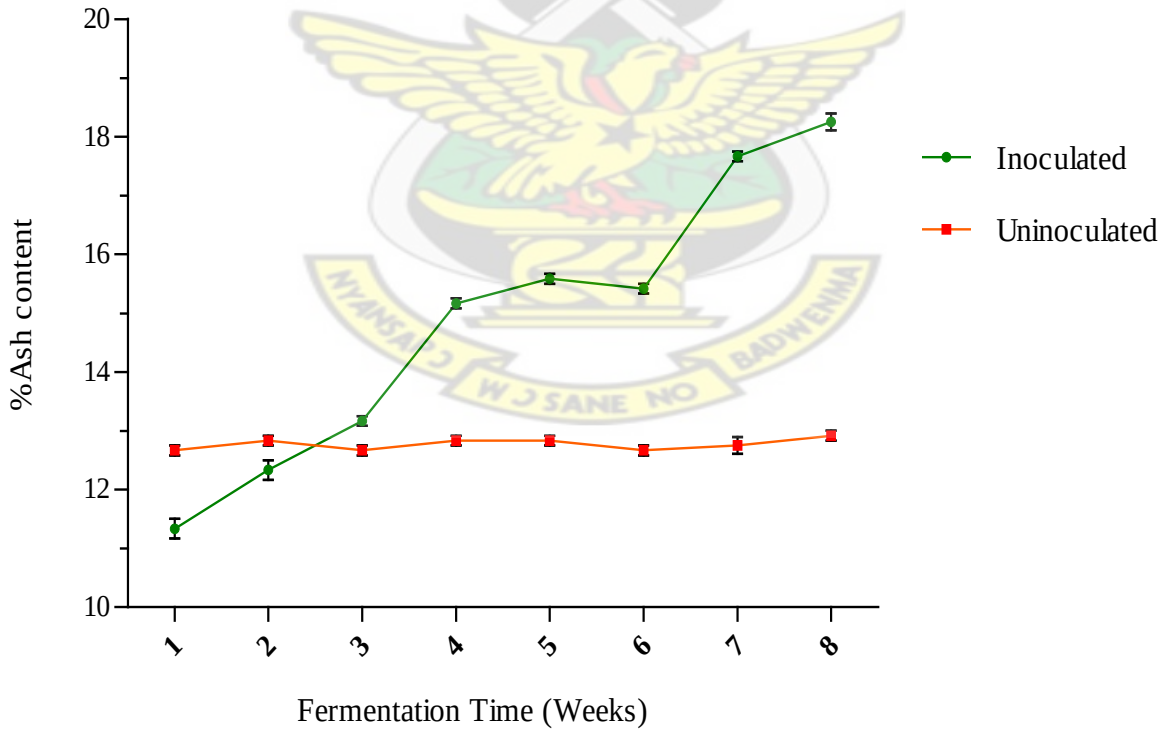


Fig 4.2 Effect of fermentation of cocoa bean shells with *Pleurotus ostreatus* on ash content

4.1.3 Crude protein

The result shown in Fig. 4.3 indicates an increase in the crude protein content of the cocoa bean shells fermented with *Pleurotus ostreatus* as compared to the control. The optimum level of the crude protein was attained at the sixth week of fermentation (26.17%) with the lowest level of 19.57% recorded in the first week of fermentation. The 25.22% increase in the crude protein level of the inoculated (treated) cocoa shells is in agreement with the report by Vijay *et al* (2007) that, *Pleurotus ostreatus* has the potential of converting cheap lignocellulosic materials into valuable protein materials at low cost. It was realized that there was an increasing trend of the crude protein content with fermentation time and then peaked by the sixth week (26.17%) after which there was a decline to 20.9% at week eight. The 20.14% decrease in the crude protein content after the sixth week of the inoculated cocoa shells could be attributed to the formation of fruiting bodies by the mushroom. This observation has been explained by Rypacek (1966) that, during the fruiting stage of the mushroom's life cycle, the mycelia utilize a lot of nutrients mainly proteins in forming the fruiting bodies which therefore accounted for the observed decrease after the sixth week of fermentation. Moreover, it was realized that, the crude protein of the *P. ostreatus* fermented cocoa bean shells had a lower value at week one compared to the control before rising in the succeeding weeks. This could be explained that, at the first week, the fungus had to use available nutrients e.g. proteins in growing (colonization) on the substrates hence the slight decrease in the crude protein at the initial stage of fermentation. However, the significant increase ($p<0.05$) (Appendix 4C) in the crude protein content of the cocoa shells inoculated with the mushroom compared to the control (uninoculated) throughout the fermentation period could possibly be attributed to the secretion of some extracellular enzymes such as cellulases, amylases and other lignin-degrading enzymes which are all proteins in nature in an attempt by the fungus to utilize available cellulose as source of carbon (Oboh *et al*., 2002).

Proteins are macromolecules that serve as energy source when metabolized and also have structural and mechanical functions such as actin and myosin in muscle and cytoskeleton formation (Schwarzer and Cole, 2005). Metabolism of proteins yields amino acids which enhances the growth and well-being of animals (Zagrovic *et al.*, 2002). Thus the increase in the protein content following fermentation with *Pleurotus ostreatus* will contribute positively to the utilization of the cocoa bean shells by animals as feed material.

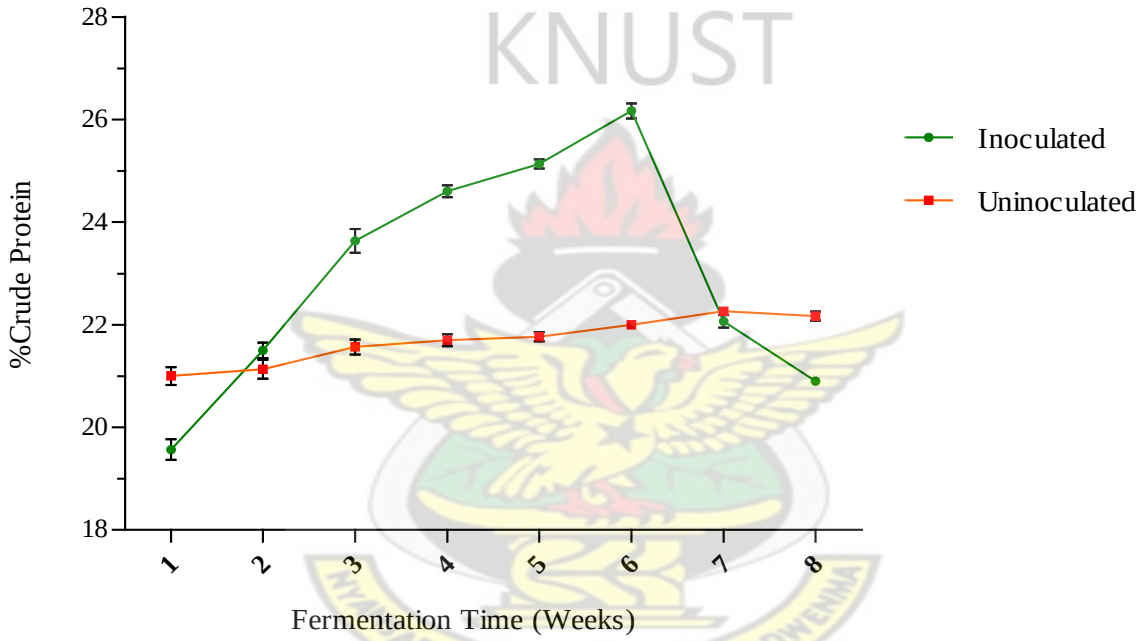


Fig 4.3 Effect of fermentation of cocoa bean shells with *Pleurotus ostreatus* on crude protein content

4.1.4 Crude Fibre

As fermentation period increases, there was a significant decline ($p<0.05$)(Appendix 4D) in the fibre content from 21.00% to 6.50% at the first and eighth week respectively (Fig. 4.4) when

cocoa bean shells was inoculated with *Pleurotus ostreatus*. The 69.05% decrease in fibre content was as a result of the fungus secreting enzymes responsible for the biodegradation of the fibre components compared to the control that had no significant change ($p>0.05$) (Appendix 4D) in the fibre content throughout the fermentation period. The enzymes secreted extracellularly by the fungus during the fermentation process were responsible for the decline in the fibre content by the disruption of cell wall (Chesson, 1993). The degradation of the complex carbohydrates into soluble components is the synergistic actions of several extracellular enzymes such as glucosidases which reduce oligosaccharides to their monomeric units (Aderemi and Nworju, 2007). It was further observed that, there was no significant decline in the fibre content after the sixth week of fermentation which confirms a similar observation by Alemawor *et al*., (2009) when cocoa pod husk was fermented with *P. ostreatus*.

The significant decrease in fibre content observed gives indication of reduced dietary fibre components such as cellulose, hemicelluloses and lignin which are not broken down by digestive enzymes in the gastro-intestinal tract (GIT) of monogastric animals (Hamzat *et al* 2006).

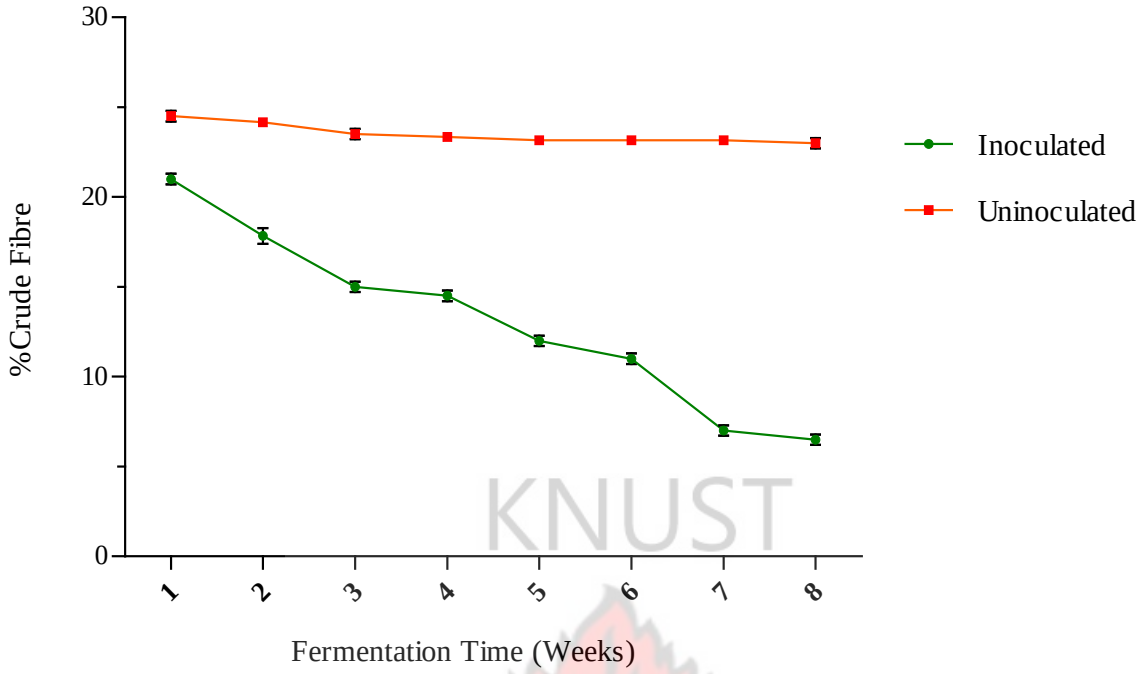


Fig 4.4 Effect of fermentation of cocoa bean shells with *Pleurotus ostreatus* on crude fibre content

4.2 Individual fibre components of cocoa bean shells fermented with *Pleurotus ostreatus*

4.2.1 Cellulose content

The decrease in the cellulose content of the cocoa shells (Fig. 4.5) was found to be significant ($p<0.05$) (Appendix 5A) unlike the control which showed no significant change ($p>0.05$) throughout the fermentation period. There was a 49.93% decrease in the cellulose content of the fermented cocoa shells at the sixth week, the optimum period of fermentation. The cellulose degradation was facilitated by the hydrolytic enzymes (cellulases) secreted by the fungus during the fermentation (Chesson, 1993). This observation confirms the result of Datta and Chakravarty (2001) that, the degradation of cellulose was due to the synergistic action of hydrolases collectively known as cellulases. Fungi are capable of producing a range of enzymes extracellularly and *P. ostreatus* is a source of cellulases, hemicellulases and laccases which help

in the degradation of complex carbohydrates into soluble sugars (Iyayi 2004). The availability of increased amount of soluble sugars due to the breakdown of cellulose would enhance digestibility of the cocoa shells hence making the non-ruminants utilize them maximally (Brimpong *et al*., 2009).

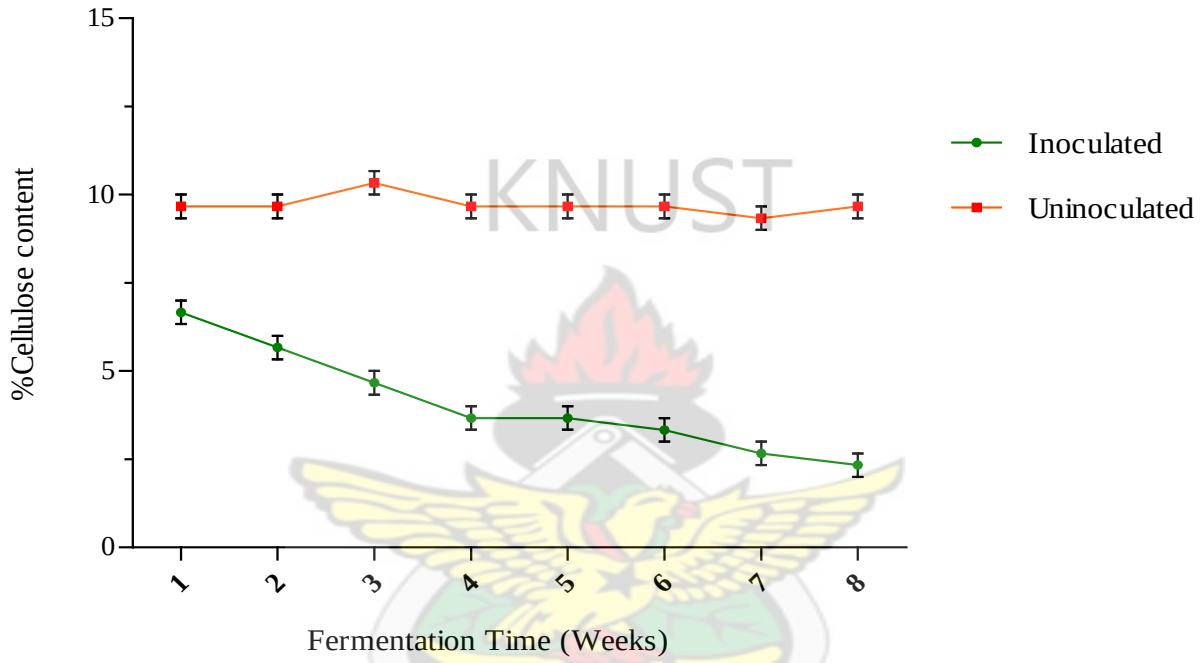


Fig 4.5 Effect of fermentation of cocoa bean shells with *Pleurotus ostreatus* on Cellulose content

4.2.2 Hemicellulose content

A general decline in the hemicellulose content was observed throughout the fermentation period (Fig. 4.6). The decrease in the hemicellulose content of the inoculated cocoa shells was found to be significant ($p<0.05$) (Appendix 5B) unlike the control which though had lower hemicellulose content at the initial stage had no significant decrease ($p>0.05$) (Appendix 5B) throughout the fermentation period. Though a decreasing trend of hemicellulose content was observed throughout the fermentation period, there was no significant decrease ($p>0.05$) observed after the

sixth week of fermentation which indicates that, the optimum duration for hemicellulose degradation of cocoa bean shells fermented with *Pleurotus ostreatus* is six weeks. Thus there was a 39.99% decrease in hemicellulose content by the optimum fermentation period (6th week). This result strongly supports that of Brimpong *et al* (2009) who also observed a 41% decrease in hemicellulose after complete colonization of corn cobs by the mycelia of the oyster mushroom with no further reduction thereafter.

The significant reduction ($p<0.05$) in the hemicellulose content of the cocoa bean shells fermented with *Pleurotus ostreatus* may enhance the digestibility of the shells when used in animal diets since hemicellulose like other fibre components, is hardly digested by animals, particularly non-ruminants.

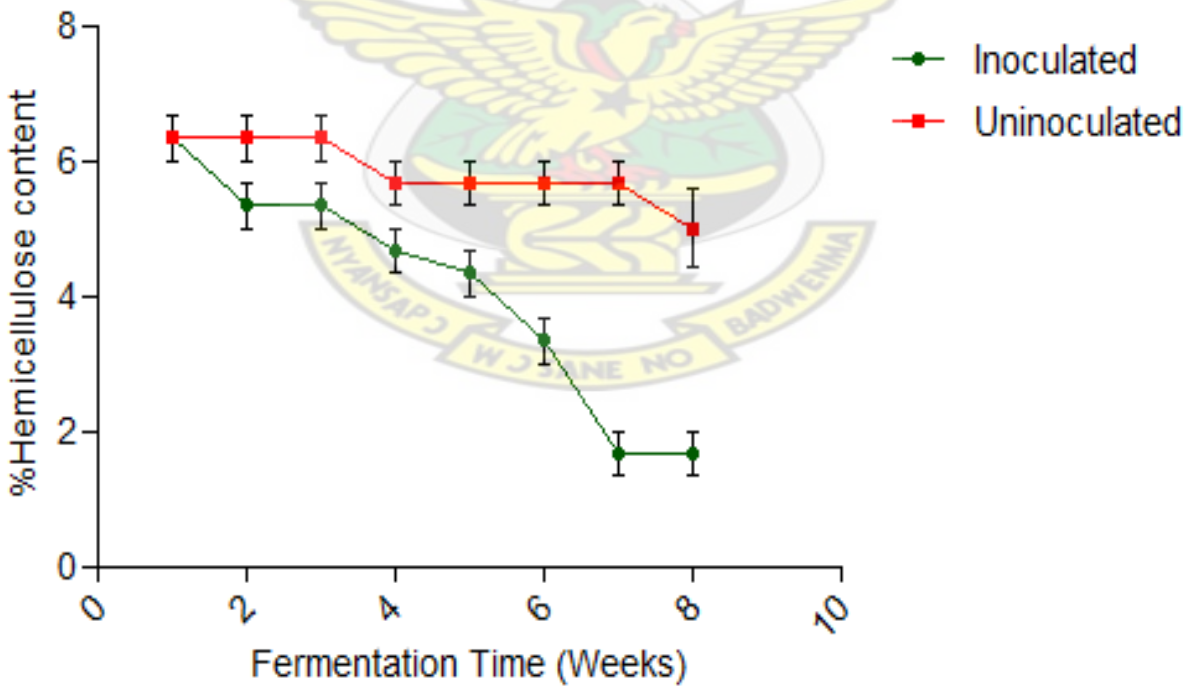


Fig 4.6 Effect of fermentation of cocoa bean shells with *Pleurotus ostreatus* on hemicellulose content

4.2.3 Lignin content

The lignin content of the cocoa shells had no significant decrease within the first week of fermentation but was significantly reduced ($p<0.05$) (Appendix 5C) after the 6th week recording 36.81% of degradation at the end of the fermentation period (Fig. 4.7). The decrease in lignin content of the cocoa shells was found to be significant ($p<0.05$) when compared to the control which had no significant change ($p>0.05$) (Appendix 5C) over the period of fermentation. An investigation conducted by Brimpong *et al* (2009) on the growth of oyster mushroom on corncobs reported a decrease of lignin content by 42.3% after complete colonization beyond which there was no further reduction. It could be observed that the availability of the cellulose for degradation was dependent on the extent of lignin degradation as stated by Datta and Chakravarty (2001). The degradation of lignin was as a result of the production of lignin-degrading extracellular enzymes such as lignin peroxidases (laccases) that oxidize both the aromatic rings and the aliphatic side chains to produce compounds more absorbable by the fungi (Cho *et al*., 1999). The significant decrease in the lignin content would make the cocoa shells a much more useful feed material for monogastric animals since lignin is one of the components contributing to the high fibre of the shells which makes it less useful as animal feed material (Hamzat *et al.*, 2006).

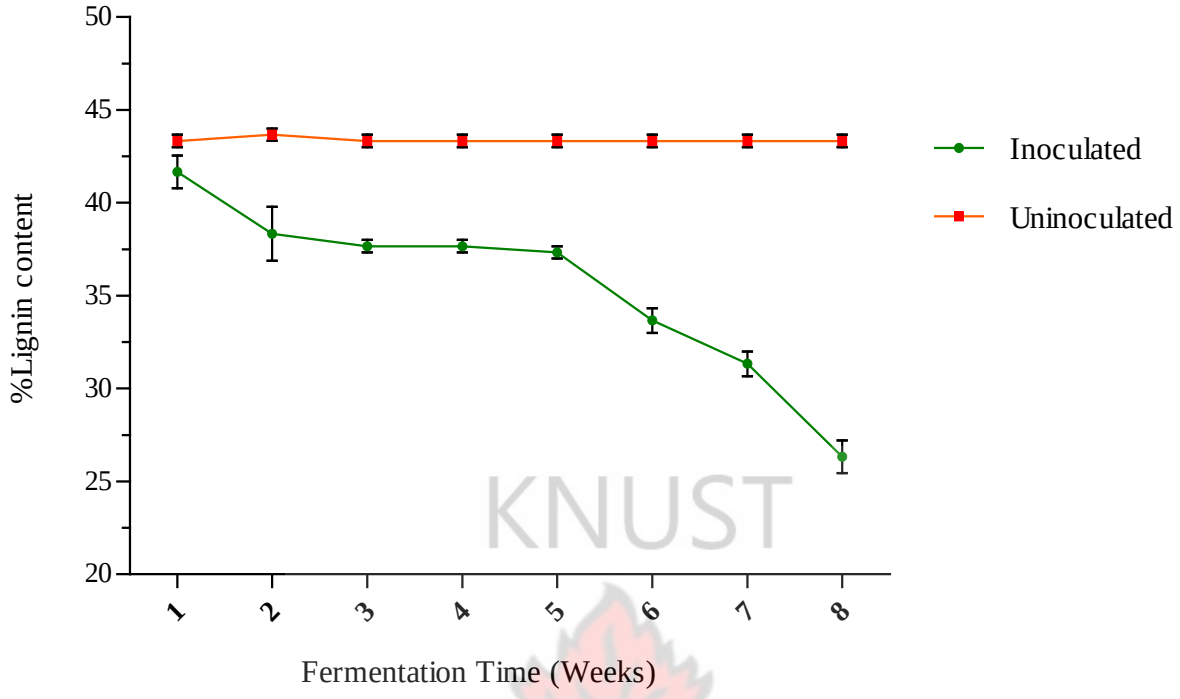


Fig 4.7 Effect of fermentation of cocoa bean shells with *Pleurotus ostreatus* on lignin content

4.2.4 *In vitro* digestibility studies

Although the *in vitro* digestibility test could not be carried out on the fermented product i.e. the *Pleurotus ostreatus* fermented cocoa bean shells to ascertain its digestibility due to technical difficulties, other reports on *Pleurotus ostreatus* fermentation of other agro- wastes indicated improved *in vitro* digestibility of products such as cocoa pod husks, cassava peels, rice straws etc. (Alemawor *et al.*, 2009). It could therefore be speculated that the *P. ostreatus* fermented cocoa bean shells would be equally digestible all conditions being equal.

4.3 Changes in mineral content of cocoa bean shells fermented with *Pleurotus ostreatus*

4.3.1 Calcium

It was observed that there was an improvement in the calcium content of the cocoa shells fermented with the *Pleurotus ostreatus* spawn (Fig. 4.8). The calcium content of the raw cocoa shells was found to be 304.33 mg/kg whereas that of the control and inoculated cocoa shells recorded 309.33 mg/kg and 1092.11 mg/kg respectively, an increase of 71.7% when compared to the 6th week control (optimum period of fermentation). This increase in calcium of the cocoa shells is attributed to the mycelia growth of the mushroom and subsequent enrichment of the cocoa shells with the mineral as the oyster mushroom is known to contain appreciable amount of calcium (Sawyer, 1994). Mushrooms were also found to be rich sources of minerals and vitamins (Oghenekaro *et al*., 2009). Calcium, the most abundant mineral in the human body, has several important functions. More than 99% of total body calcium is stored in the bones and teeth where it functions to support their structure (Heaney *et al*., 2000). The remaining 1% is found throughout the body in blood, muscle, and the fluid between cells (Subar *et al*., 1998). Calcium is needed for muscle contraction, blood vessel contraction and expansion, the secretion of hormones and enzymes, and sending of messages through the nervous system (Heaney *et al*., 1991). The tremendous improvement in calcium content of the cocoa shells fermented with *P. ostreatus* has greatly enhanced the mineral content of the shells and its use as feeding material for animals which will help maintain the general health of the animal especially for growth (Heaney *et al*., 1991).

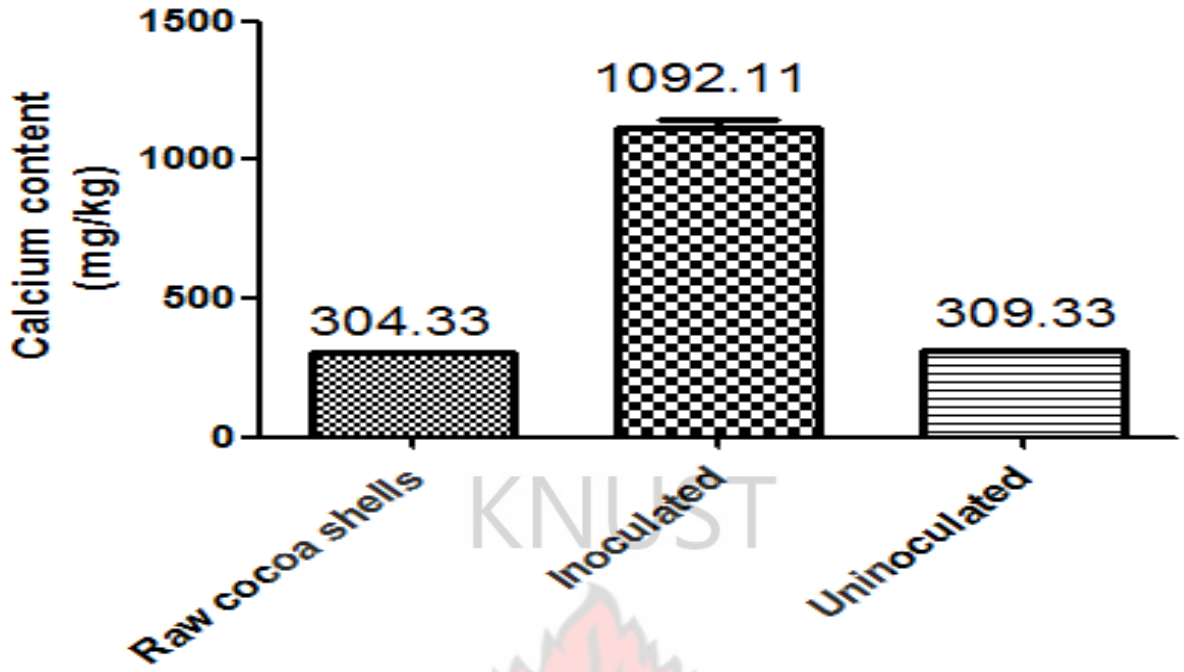


Fig 4.8 Changes in calcium content of cocoa bean shells fermented with *Pleurotus ostreatus* at 6th week of fermentation

4.3.2 Phosphorus

Of the three mineral elements analyzed in the cocoa shells, phosphorus had the least improvement by the bioprocess (Fig. 4.9). This result confirms earlier work done by Alemawor *et al* (2009) where phosphorus recorded a least value of the mineral elements after *P. ostreatus* fermentation of cassava peels over the same period. The respective values were 54.0 mg/kg, 52.0 mg/kg and 73.33 mg/kg for the raw, the uninoculated (control) and the inoculated cocoa shells, an increase of 29.09% at the 6th week of fermentation (Fig. 4.9). This increase is attributed to the

mycelia growth of the mushroom and subsequent enrichment of the cocoa shells with the mineral as *Pleurotus ostreatus* is also known to be rich in phosphorus and other elements needed by the body (Sawyer, 1994). Phosphorus is essential in the diet of animals and its deficiency contributes to the most widespread of all the mineral deficiencies affecting livestock (Chase, 1999). In the body, phosphorus constitutes about 80% of the animal's skeleton and also plays a major role in the formation of bones and teeth (Chase, 1999). In ion-exchange phosphorylation, phosphorus is needed for the transfer and utilization of energy and also complexes with Ca in a ratio between 2:1 and 1:1 for lactation in dairy animals (Chase, 1999). Thus the improvement of the phosphorus levels in the cocoa shells following the fermentation with *P. ostreatus* will contribute immensely to the general well-being of animals when used as feed material in their diets.

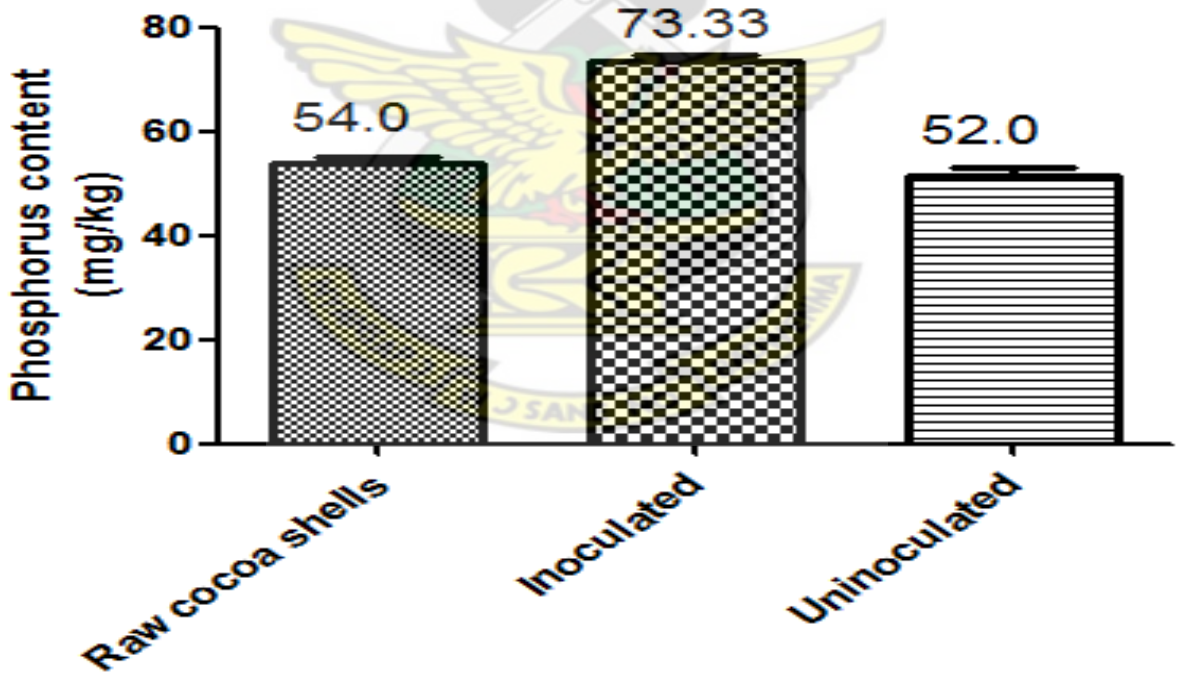


Fig 4.9 Changes in phosphorus content of cocoa bean shells fermented with *Pleurotus ostreatus* at 6th week of fermentation

4.3.3 Potassium

The potassium content of the treated cocoa shells was significantly improved ($p<0.05$) recording 766.33 mg/kg when compared with the control at the sixth week of fermentation (Fig. 4.10). The raw shells and the control recorded 381.67 mg/kg and 371.00 mg/kg of potassium content which was not significantly different ($p>0.05$) (Fig. 4.10). The 51.59% increase in potassium of the cocoa shells at the 6th week of fermentation is attributed to the mycelia growth of the mushroom and subsequent enrichment of the cocoa shells with the mineral as *Pleurotus ostreatus* is known to contain appreciable amount of the mineral element (Sawyer, 1994). Potassium is essential for the maintenance of a potential difference across cell membranes and therefore plays an important role in the generation of nerve and muscle action potentials (Skelly, 2002). The mineral element is therefore needed for the proper functioning of the nervous system, especially nerve impulse transmission. Potassium is also a primary constituent of primary cell metabolites such as nucleotide and co-enzymes (flavin phosphate, pyridoxal phosphate and thiamine phosphate) (Durand and Kawashima, 1980). It is essential for all energy transactions within animal cells, formation of adenosine di- and tri- phosphate (ADP and ATP) and guanine triphosphate (MacDonald *et al.*, 1981). Potassium therefore plays an important role in the growth of animals (Campell and Roberts, 1995) and therefore its enrichment in the cocoa shells would help meet potassium requirement when fed to animals.

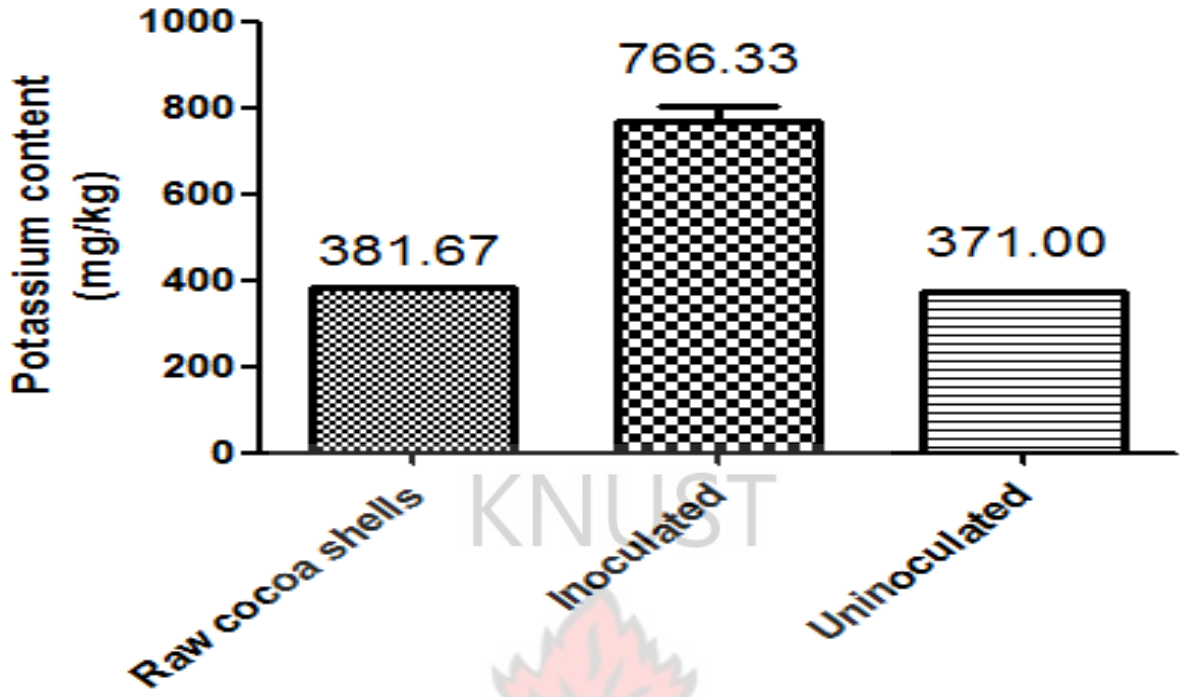


Fig 4.10 Changes in Potassium content of cocoa bean shells fermented with *Pleurotus ostreatus* at 6th week of fermentation

4.4 Changes in theobromine content of cocoa bean shells fermented with *Aspergillus niger*

The level of theobromine in the raw shells, though lower compared to literature (EFSA, 2008), was significantly decreased ($p<0.05$) (Appendix 7) when fermented with *Aspergillus niger* as compared to the raw shells and the uninoculated (controls) (Fig. 4.11). There was therefore a reduction of 72.89% theobromine content after the *A. niger* fermentation of the cocoa shells for seven days. This result confirms a study conducted by Adamafio *et al* (2011) who reported a 71.8% detheobromination in cocoa pod husk fermented with *Aspergillus niger* over the same period. It was further observed that, the theobromine levels of both the *Pleurotus ostreatus* fermented cocoa bean shells (POFCBS) and the non- *Pleurotus ostreatus* fermented cocoa bean

shells (NPOFCBS) serving as controls were not significantly changed ($p<0.05$) (Appendix 7) at the end of the seven days fermentation period. This result confirms the observation of Alemawor *et al* (2009) that, methylxanthines usually present in cocoa products and by-products are not affected by *Pleurotus ostreatus* fermentation. The detheobromination of the cocoa bean shells fermented with *A. niger* compared to the raw shells and the controls gives the assertion that, the degradation of theobromine by *A. niger* using theobromine as a sole carbon and energy source occurs via the demethylase route which involves 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 was also observed that, significant differences occurred in the theobromine levels of the raw shells (135.77 mg/g) and the controls (96.65 mg/g and 97.25 mg/g). This could be attributed to the heating methods used before the fermentation processes such as pasteurization since heating is reported to reduce the theobromine level in cocoa by-products (Menon, 1982).

Thus in order to reduce the theobromine content of cocoa bean shells for use in animal feed, *A. niger* fermentation will be a more environmentally friendly alternative compared to other methods reported to achieve the same purpose ((Menon, 1982).

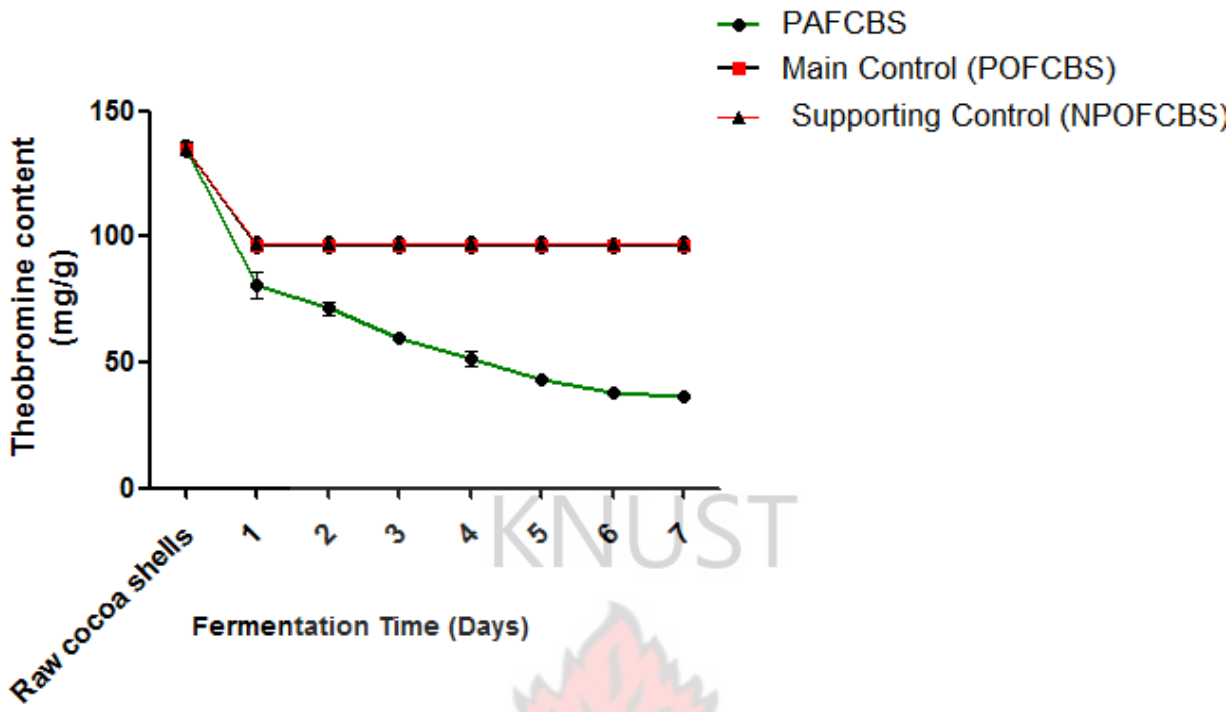


Fig. 4.11 Changes in theobromine content of cocoa bean shells fermented with *Aspergillus niger*

Key definitions: PAFCBS- *P. ostreatus and A. niger* fermented cocoa bean shells, POFCBS- *Pleurotus ostreatus* fermented cocoa bean shells, and NPOFCBS- Non-*Pleurotus ostreatus* fermented cocoa bean shells

4.5 Summary of the nutritional status of cocoa bean shells fermented with *P. ostreatus* and *A. niger*

The nutritional and anti-nutritional composition of the fermented cocoa bean shells were greatly enhanced as compared to the unfermented material (Table 4.1). The breakdown of complex carbohydrates in the CBS by the *P. ostreatus* fermentation was evident in the decrease in crude fibre content from 24.35% to 15.00% which was further reduced to 9.90% by the fermentation with *A. niger* (Table 4.1). This was as a result of the synergistic actions of the extracellular

enzymes such as cellulases, xylanases, glucanases, glucosidases and lignin-degrading enzymes secreted by the fungi by its growth on the surface of the cocoa bean shells (substrate) (Aderemi and Nworju, 2007, Ilyas *et al*., 2011). The reduction in crude fibre content of the fermented CBS with *P. ostreatus* and *A. niger* which reflected in the reduction of the individual fibre components (cellulose, hemicellulose and lignin) eventually resulted in a 33.64% increase (from 2,309.11KCal/Kg to 3,479.50KCal/Kg) in Metabolizable Energy (M.E) in the fermented product (Table 4.1).

Additionally, *A. niger* fermentation was able to greatly reduce the theobromine content of the CBS thus from 96.65mg/g to 36.62mg/g (Table 4.1), a theobromine reduction of 62.11%. A 28.81% reduction (from 135.77mg/g to 96.65mg/g) in theobromine content was also observed in both the non-*P. ostreatus* and *P. ostreatus* fermented CBS which meant that the reduction in theobromine content was not by the effect of the *P. ostreatus* fermentation. This could, however, be attributed to the heating processes applied before and after the *P. ostreatus* fermentation process since theobromine is greatly affected by heat as reported by Menon (1982).

TABLE 4.1: Nutritional and Anti-nutritional composition of fermented and unfermented cocoa bean shells

Nutrient	Unfermented cocoa bean shells (UCBS)	*Pleurotus ostreatus* fermented cocoa bean shells (POFCBS)	*Pleurotus ostreatus* and *Aspergillus niger* fermented cocoa bean shells (PAFCBS)
Crude Protein (%)	18.6	26.1	25.9
Crude Fibre (%)	**24.35**	**15.00**	**9.90**

Nitrogen-free Extracts (NFE)(Soluble Carbohydrates)	35.05	25.78	45.30
Metabolizable Energy (M.E.) (Kcal/Kg)	**2,309.11**	**2,163.83**	**3,479.50**
Ether Extract (%)	1.00	0.50	0.50
Ash content (%)	6.00	18.50	18.40
Moisture content (%)	15.00	14.00	-----
Acid detergent fibre (ADF) (%)	52.00	37.00	34.00
Neutral detergent Fibre (NDF) (%)	60.00	41.00	36.00
Cellulose (%)	11.00	3.00	1.00
Hemicellulose (%)	8.00	4.00	2.00
Lignin (%)	45.18	33.00	33.00
Theobromine content (mg/g)	**135.77**	**96.65**	**36.62**

4.6 CONCLUSION AND RECOMMENDATIONS

4.6.1 CONCLUSION

The optimum periods obtained for the *Pleurotus ostreatus* and *Aspergillus niger* fermentation processes were six weeks and five days respectively.

The selected order of the two-stage fermentation with the fungi i.e. *P. ostreatus* followed by *A. niger* significantly enhanced the nutritive value of the cocoa bean shells, particularly the decline

in the fibre and theobromine contents respectively unlike the vice versa. The *P. ostreatus* fermentation also increased the protein and minerals contents significantly.

These results obtained have therefore rendered the cocoa bean shells more useful as animal feed material particularly for monogastric animals.

4.6.2 RECOMMENDATIONS

Notwithstanding the suitability of the fungi treated cocoa bean shells as feed material for animals, it is recommended that:

(a) An assay should be conducted to ascertain the mycotoxicity level in the fungi treated cocoa bean shells since the strain of *Aspergillus niger* used in the study was not specified whether or not it produced Aflatoxin during the fermentation process.

(b) Secondly, an *in vitro* digestibility studies should be conducted to be able to ascertain the level of fibre digestibility of the fermented cocoa bean shells in terms of available total sugars.

(c) Finally, a large-scale fermentation of cocoa bean shells and its subsequent utilization in feeding trials of broiler or layer chicks should be investigated.

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APPENDICES

PHOTOGRAPH OF *PLEUROTUS OSTREATUS* FERMENTED COCOA BEAN SHELLS

APPENDIX 1

(A) FIRST WEEK

(B) SECOND WEEK

(C) THIRD WEEK

(D) FOURTH WEEK

(E) FIFTH WEEK

(F) SIXTH WEEK

(G) SEVENTH WEEK

(H) EIGTH WEEK

(I) CONTROL

APPENDIX 2A OYSTER MUSHROOM (*PLEUROTUS OSTREATUS*) SPAWN

APPENDIX 2B SIX (6) DAYS OLD *ASPERGILLUS NIGER* ISOLATED FROM COCOA BEAN SHELLS (C)

APPENDIX 3 FORMULAE USED IN THE CHEMICAL ANALYSIS

1. % Moisture = $\frac{\text{Loss in weight of sample} \times 100}{\text{Original weight of sample}}$

2. % Ash = $\frac{\text{Weight of ash} \times 100}{\text{Weight of sample}}$

3. % Crude Fibre = $\frac{\text{Weight of crude fibre obtained} \times 100}{\text{Weight of defatted residue used}}$

4. % Total Nitrogen (g/kg) = $\frac{(V_A - V_B) \times N_A \times 14.01}{W \times 10}$

V_A = Volume in ml of standard acid used in the titration of sample

V_B = Volume in ml of standard acid used in the titration of blank

N_A = Normality of the acid used

W = Weight of sample

% Crude Protein = % Total Nitrogen x 6.25 (Protein factor)

5. %NFE = 100 – [%E.E + %CP + %CF + %Ash + %Moisture]

Where NFE = Nitrogen-free extract

E.E = Ether extract

CP = Crude protein

CF = Crude fibre

6. M.E (Kcal/Kg) = [(3.5 x CP) + (8.5 x E.E) + (3.5 x NFE)] x 10

Where M.E = Metabolizable Energy

CP = Crude protein

E.E = Ether extract

NFE = Nitrogen-free extract

7. NDF = Cellulose + Hemicellulose + Lignin + Minerals
8. ADF = Cellulose + Lignin + Minerals
9. Hemicellulose = NDF – ADF
10. ADF = Lignin
11. Phosphorus (P)(µg) content in 1.0g of plant sample = C x df

C = concentration of P (µg/ml) as read from the standard curve

df = dilution factor, which is 50 x 10 = 500

APPENDICES 4 ANOVA TABLES FOR PROXIMATE COMPOSITION

APPENDIX 4A MOISTURE

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	32.61627	1	32.61627	23.65364	0.000173	4.493998
Within Groups	22.06258	14	1.378911			
Total	54.67885	15				

APPENDIX 4B ASH

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	12.08681	1	12.08681	7.334387	0.015509	4.493998
Within Groups	26.36742	14	1.647964			
Total	38.45423	15				

APPENDIX 4C CRUDE PROTEIN

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	1361.61	1	1361.61	242.0406	0.00	4.60011
Within Groups	78.7576	14	5.625543			
Total	1440.368	15				

APPENDIX 4D CRUDE FIBRE

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	448.7009	1	448.7009	24.52119	0.000144	4.493998
Within Groups	292.776	14	18.2985			
Total	741.477	15				

APPENDICES 5 ANOVA TABLES FOR FIBRE COMPONENTS

APPENDIX 5A CELLULOSE

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	67.43476	1	67.43476	9.476743	0.007198	4.493998
Within Groups	113.853	14	7.115815			
Total	181.2878	15				

APPENDIX 5B HEMICELLULOSE

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	13.78125	1	13.78125	11.45455	0.003782	4.493998
Within Groups	19.25	14	1.203125			
Total	33.03125	15				

APPENDIX 5C LIGNIN

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	552.1165	1	552.1165	16.21705	0.000975	4.493998
Within Groups	544.727	14	34.04544			
Total	1096.843	15				

APPENDICES 6 ANOVA TABLES FOR MINERAL COMPOSITION

APPENDIX 6A CALCIUM

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	41.6025	1	41.6025	5.539751	0.033727	4.60011
Within Groups	105.1374	14	7.509814			
Total	146.7399	15				

APPENDIX 6B PHOSPHORUS

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	58.71391	1	58.71391	19.54176	0.000581	4.60011
Within Groups	42.06349	14	3.004535			
Total	100.7774	15				

APPENDIX 6C POTASSIUM

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	8.500939	1	8.500939	6.287205	0.023323	4.60011
Within Groups	21.63362	14	1.352101			
Total	30.13456	15				

APPENDIX 7 THEOBROMINE

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	8941.536864	1	8941.537	60.15075	0.00000516	4.747225
Within Groups	1783.825371	12	148.6521			
Total	10725.36224	13				