EFFECT OF CONTROLLED FERMENTATION USING *ASPERGILLUS NIGER* AND *TRICHODERMA HARZANIUM* ON NUTRIENT COMPOSITION OF PRE-TREATED BENGAL INDIGO (*INDIGOFERA ARRECTA*) SEEDS

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CHAPTER 1

INTRODUCTION

The genus *Indigofera* Linn. is a large genus of about 700 species of flowering plants belonging to the sub-family Papilionoideae in the family Fabaceae / Leguminosae. They occur throughout the tropical and subtropical regions of the world. Burkill (1995) recognized 60 species while Soladoye and Lewis (2003) recorded 60 species in Nigeria with over 60% abundance in the Northern region of the country with 27 species distributed across the South Western area of the country. *Indigofera* in Greek means indigo dye which is famous for the natural blue colors obtained from the leaflets and branches of this herb. The most important of the species are *Indigofera arrecta* and *Indigofera tinctoria.*

Indigofera spp. display excellent adaptation to a range of environments, and possess diverse morphological and agronomic attributes, significant to their use as forage and cover crops (Hassen *et al*., 2006). Some of these species, *Indigofera tinctoria* and *Indigofera suffruticosa* are used to produced indigo dyes while some have medicinal values such as *Indigofera articulate* used for the treatment of toothache, *Indigofera oblongifolia*, *Indigofera suffruticosa* and *Indigofera aspalthoides* are used as anti–inflammatories for treatment of insect stings, snake bites and swellings (Shahjahan *et al*., 2005); and *Indigofera arrecta* extract is used to relieve ulcer pain.

The stem of *Indigofera tinctoria* is chewed to cure cough and decoction of leaves is used to cure chest pains, epilepsy, nervous disorders, asthma, bronchitis, fever and complaints of stomach, liver, kidney and spleen- especially in Cameroon (Takawira-Nyenya and Cardon, 2005). The twine paste cures dislocation. Also the warm leaves dismiss bruises (Ibe and Nwufo, 2005). Phytochemical investigation of *Indigofera* species shows that they are rich in organic and fatty acids, flavonoids such as carotenoids and coumarins (Yinusa *et al.,* 2007).

Indigofera arrecta is readily found in abundance in the locality of Samaru- Zaria, Kaduna State and the young leaves are eaten as vegetable. In the northern part of the country among the Hausa, it is called "Ba-ba", in the south-west among the Yoruba, it is known as "Elu-aja" and in eastern part of the country among the Igbos, it is known as "Uri". *Indigofera arrecta* seeds contain several anti-nutritional factors which limit their consumption and affect the digestibility and bioavailability of nutrients. Seeds are well known rich sources of minerals but the bioavailability of these minerals is usually low due to the presence of antinutrients and enzyme inhibitors (Valencia *et al*., 1999). These antinutrients and enzyme inhibitors interfere with absorption of nutrients from foodstuff thus affecting their metabolism. The seed of *Indigofera arrecta* is known for its hardness which is due to the presence of a hard seed coat which is the seed's primary defense against adverse environmental conditions. Seed hardness is the effect of two components: hardness of the seed coat

and hardness of the seed interior. Hardness is related to seed coat (testa) impermeability and also to cotyledon impermeability. In the testa it may involve lignins as well as tannins, whereas in cotyledons it may be primarily lignification as cotyledons have low concentrations of phenolic compounds. Some anti-nutritional factors such as trypsin and cysteine inhibitors and lectins are heat-labile compounds and their negative effects are, therefore, markedly reduced by cooking (Boufassa,*et al*., 1986; Akinyele, 1989), while tannins and phytic acid are heat-stable compounds that retain negative effects on mineral and protein bioavailability after cooking (Ogun *et al*., 1989).

All necessary experimental steps employed in producing fermentable substrates are referred to in a single term as pretreatment. Pretreatment strategies have generally been categorized into biological, physical and chemical processes, or a combination of these approaches.Pretreatments change the structure of cell walls and polymers by disrupting intermolecular forces holding them together, allowing greater access by enzymes and water. Phenolic compounds in the seed coat contribute to seed hardness and inhibition of microorganism growth hence the need for pretreatment in order to increase the accessibility to enzyme hydrolysis or fermentation.

The presence of lignin also impedes enzymatic hydrolysis, as enzymes bind onto the surface of lignin and hence do not act on the cellulose chains (Palonen *et al*., 2004). Biodegradability can be enhanced by pretreatment of lignocellulolytic materials, including acid or alkali treatment, ammoniaand urea, physical grinding and milling, fungal degradation and steam explosion, and combined alkali and heat treatment (Galbe and Zacchi, 2007). Gharpuray *et al.*(1983) examined several of these pretreatment options individually and in combination, and found that those treatments which enhanced specific surface area were most effective at increasing enzymatic hydrolysis.

Fermentation has always been an important part of human lives with exclusive benefits as food. It can produce vital nutrients or eliminate antinutrients. Several experiments have demonstrated that fermentation of legumes enhances their nutritive value (Zamora *et al*., 1979; Akpapunam and Achinwehu, 1985), reduces some anti-nutritional endogenous compounds such as phytic acid (Kozlowska *et al*., 1996), and exerts beneficial effects on protein digestibility and biological value of legumes. *Trichoderma* spp. and *Aspergillus* spp. are good secretors of lignocellulolytic enzymes(Archer and Peberdy, 1997). They are producers of tannase (Dapiya *et al*., 2010) and phytase (Aseri *et al*., 2009, Newkirk *et al*., 2001) which are two enzymes capable of degrading tannin and phytic acid respectively. The incubation of substrate with *Trichoderma harzanium* and *Aspergillus niger*markedly reduce the phytate and

tannin in *Jatropha curcas* seeds(Balewu*et al*.,2011). Efforts have been focused on how to combine pretreatment and solid state fermentation to maximize productivity and to make the overall process economically efficient.

This study is aimed at evaluating the effect of pretreatment on nutrient composition of *Indigofera arrecta* seeds using controlled fermentation by *Aspergillus niger*and *Trichoderma harzanium* in mono and co-culture.

1.1 Statement of Research Problem

As the world population continues to increase, food security on a global scale must become an even greater priority. Along with population growth, economic growth and the rise of the middle class in developing countries are expected to further increase demand for meat, milk, and eggs (FAO, 2009).The consumption of conventional feedstuffs like soybean, maize and sorghum by human being is undermining their availability to animals.The price of the conventional source of protein in livestock ration has risen due to competition between man and livestock thus, there is a big challenge of matching available feedstuff supplies with animal needs.Hence, the world is becoming increasingly aware of the looming food insecurity, the possibility of raising animal on unconventional but easily sourced and available feedstuffs in the tropics and subtropics deserves more attention (Belewu *et al*., 2009).

The primary constraint to livestock production in developing countries is the scarcity and fluctuating quantity and quality of the feed supply.The seed of *Indigofera arrecta* is an underutilized resource that has received little attention. The plant is readily and abundantly found in farms and wasteland among grasses and is destroyed alongside with grasses during the clearing of the farm. Its usage has been limited probably due to the hardness of the seed and antinutritional content. Hence the need for suitable pretreatment and treatment conditions to enhance its nutritive value. The growth of fungi in natural substrates is usually slow and this limitation must be overcomed by suitable mechanical and chemical pretreatment of the raw substrate (Cen and Xia, 1999). The solid state fermentation of *Indigofera arrecta* with *Trichoderma harzanium* and *Aspergillus niger*may be a promising method of enhancing the usage of *Indigofera arrecta* thereby making available renewable feedstuff.

1.2 Justification

In Nigeria, the unbearable scarcity of animal feed motivated nutritionists to look for alternative sources of protein for livestock animals from lesser known seeds like *Indigofera arrecta.*

The calcium concentration of *Indigofera* species is relatively high ranging from 0.5 to 2.0%, where normal ranges of 0.20 to over 0.30% would satisfy animal

requirement. Crude protein content of *Indigofera* spp is sufficiently high for consideration of these species as potential protein supplements in low protein quality diets. The amount of the nutritionally essential amino acids present in defatted seed meals of *Indigofera* species is comparable to that present in legumes (Van Etten *et al.,* 1967). The quantity of non-essential amino acids, glycine, aspartic acid, and serine in these *Indigofera* species is more compared to the amount found in legumes (Roger *et al*., 1973). However, they contain several anti-nutritional factors which limit their consumption and affect the digestibility and bioavailability of its nutrients (Bressani, 1993). Antinutritional and toxic factors are considered to defend seeds against environmental vagaries and thus help to protect them. These factors though good for the plant, cause deleterious effect or are even toxic to animals and man. Therefore, fermentation being one of the oldest and most economical method of food production and preservation can be used in an attempt to reduce or eliminate those anti-nutritional factors and hence render the wild *Indigofera arrecta*seed useful.

The main aim of pretreatment is to increase surface area, to decrystallize cellulose, and to remove hemicellulose and lignin. Pretreatment has to overcome the resistance of the plant cell walls to deconstruction in order to separate the cellulose from lignin and hemicelluloses to make it more accessible to microbes or enzyme hydrolysis (Brodeur *et al*., 2011). Therefore, choosing the appropriate pretreatment for *Indigofera arrecta* seeds is a compromise between minimizing the degradation of the

hemicellulose and cellulose components while maximizing the ease of fermentation of the substrate using mono and co-culture of *Trichoderma harzanium* and *Aspergillus niger*.

1.3 Null Hypothesis

Mono and co-culture fermentation do not eliminate the antinutritional factors and do not exert beneficial effects on the proximate composition and mineral content of pretreated *Indigofera arrecta* seeds.

1.4 Aim

The aim of this study is to evaluate the effect of controlled fermentation using *Aspergillus niger*and *Trichoderma harzanium* on nutrient composition of pretreated *Indigofera arrecta* seeds.

1.5 Specific Objectives

i. To evaluate the effect of pretreatment on the lignin, cellulose and hemicellulose content of pretreated *Indigofera arrecta* seeds.

ii. To evaluate the effect of pretreatment on the proximate, antinutritional and mineral composition of *Indigofera arrecta* seeds.

iii. To determine the proximate, antinutritional and mineralcomposition of pre-treated *Indigofera arrecta* seeds after fermentation with mono and co-culture of *Aspergillus niger*and *Trichoderma harzanium*. .

iv. To determine the amino acid composition of pre-treated *Indigofera arrecta* seeds after fermentation with mono and co-culture of *Aspergillus niger*and *Trichoderma harzanium*.

v. To determine the maximum phytase and tannase activity of pretreated *Indigofera arrecta* seeds after fermentation with mono and co-culture of *Aspergillus niger*and *Trichoderma harzanium*.

CHAPTER 2

LITERATURE REVIEW

2.1 Seed Structure of Legumes

The legume seed has two distinct parts, the seed coat and cotyledon. The hilum, micropyle, hypocotyls (embryonic stem), epicotyl (embryonic stem tip) and the radicle (embryonic root) are the minor parts of the seed. Both the hilum and micropyle are found on the surface of the seed coat. Also, the hilum is a conspicuous feature, and it represents the scar left on the seed when it separates from the funicle. The micropyle is a canal in the seed coat of the nucellus through which the pollen tube usually passes during fertilization.

2.1.1 Seed Coat

The seed coat is the outer protective covering of the cotyledon and is also referred to as the testa. The seed coat develops from the integument of the ovule and has three distinctive cells palisade, subepidermal, and parenchyma cells. Additionally, these cells are compactly arranged enhancing the function as a barrier to the external environment. The seed coat has been identified to be rich in mineral nutrient, such as calcium, magnesium, iron, zinc, potassium, and copper, as well as phytate, tannins, and phenolics (Adebooye and Singh, 2007). Adebooye and Singh (2007) investigated the minerals, tannins, phytate, amino acids, and fatty acids profile of whole and decorticated cowpeas. They observed a significant loss of the minerals,

tannins, phytate, and fatty acids (linolenic and arachidonic acids) when the seed coat was removed, but reported no loss in the amino acids of the decorticated (removal of seed coat) seeds. Pyrinyawiwatkul *et al*. (1996) agreed to the slight difference between the amino acid profile of the seed coat and the cotyledon. Also, Adebooye and Singh (2007) documented the complete absence of arachidonic fatty acids when the cowpea seeds were decorticated. Therefore, the researchers concluded that decortication leads to losses of minerals in cowpeas.

The seed coat is one of the main factors which determine germination capacity, vigor and longevity of seeds. It has a protective function during imbibition, avoiding cell rupture and loss of intracellular substances (Duke and Kakefuda, 1981), and also protects the embryonic axis (Carvalho and Nakagawa, 2000). It is derived from the integuments of the ovule where the primine gives rise to the testa and the secundine gives rise to the tegmen. By means of a cross section of the testa of a soybean seed, three layers may be distinguished, the epidermis, the hypodermis and the inner parenchyma(Swanson *et al*., 1985). This last layer, composed of the spongy parenchyma, is present in the entire testa of the seed, except for the hilar region. It has from 6 to 8 cell layers, tangential to the surface of the testa, formed by thin walls and absent protoplasm, with the outermost part of this parenchyma being formed by large, elongated cells, while the innermost part by smaller and significantly branched cells (Esau, 1977).

The intermediate layer of the testa, the hypodermis, is formed of cells in hourglass form, orpillar cells, or even osteosclereid cells. It consists of a uniform cell layer through the entire testa, except for the hilar region. The cell wall of its sclerenchyma cells is not uniform, with the presence of large intercellular spaces (Corner, 1951). The epidermis, outside of the testa, remains uniseriate and gives rise to the palisade layer, characteristic of leguminosae seeds. This layer consists of macrosclereids (Malpighi cells) with wall of unequal thickness, having a cuticle present over their outermost wall. Its cells are elongated and arranged perpendicular to the surface of the testa, with thick cell walls (Esau, 1976). In soybean seeds, the thickness of the four testa layers altogether, including the cuticle, starting from the surface, may vary from 70 to 100 micrometers, there being variation among cultivars. Nevertheless, this characteristic is a constant with each cultivar and is controlled genetically (Caviness and Simpson, 1974). The presence or lack of pores and their quantity, shape and size on the surface of the testa is also controlled genetically. The pores seem to be related to water absorption, such that in hard seeds they are either absent or they exist in small quantity (Calero *et al*., 1981).

2.1.2 Cotyledon

The main food storage organ of legumes seeds is the cotyledon, and it accounts for about 85% of the mass of the seed. It is an organized structure, composed of starch granules embedded in a protein matrix, with protein bodies surrounded by a

lipoprotein membrane (Liu *et al.,* 1992; Aguilera and Stanley 1985). Most legumes are dicotyledenic with a cell wall rich in cellulose (25.9-30.9%), hemicellulose (15.9-23.7%), lignin (0.4-0.6%), and pectin (28.5-41.2%). The middle lamella is found between cells cementing individual cells of the cotyledon, and mostly contains pectic substances, which dictate the physical strength of the tissue (Aguilera and Stanley, 1985). Reyes-Moreno and Paredes-Lopez (1993) described pectic substance as a group of closely associated polysaccharides from the primary cell walls and intercellular regions of higher plants. The major component of the pectic substance is D-galacturonic, which is composed of methyl esters, pectin, the de-esterified pectic acid and its salts; pectate, and certain neutral polysaccharides lacking the galacturonan backbone (Banerjee and Chauhan, 1981). Changes in pectic substances following unfavorable storage condition, which may be due to divalent cations released from phytate that bridge with pectin, have been associated with the development of hard to cook (HTC) defect in legumes (Mattson 1946; Hentges *et al*., 1991; Liu *et al*., 1993).

2.2 Hard-Shell Defect

Some legumes do not imbibe water when soaked for a long time. Generally, this occurs when seeds are stored in high temperatures and low relative humidity conditions (Hentges *et al*., 1991; Reyes-Moreno and Paredes-Lopez 1993; Coelho *et al.,* 2007). This phenomenon is referred to as the hard-shell defect (HSD). Structural

alterations in the seed coat, as a result of high storage temperature, cause the HSD condition in legumes (Coelho *et al.*,2007). This defect renders the affected seeds not suitable for consumption.

Indigofera arrecta seeds, like those of many leguminous species, have hard and impermeable coats. Many leguminous seeds have wide dormancy durations induced by the appearance of a hard water proof coat. These seeds exhibit seed coat-imposed dormancy which may be due to impermeability of testa to water (Ballard, 1973; Roleston, 1978; Cavanagh., 1980). In the case of soybeans, differences in the lignin content among seed coat have been observed by various authors (Tavares *et al*., 1987; Carbonell *et al*., 1992; Alvarez, 1994; Carbonell and Krzyzanowski, 1995; Panobianco, 1997; Menezes, 2008).

The term lignin is used to designate a group of substances with similar chemical units indicated as polymers derived from “p-coumaryl”, “conyferyl” e “sinapyl" alcohols (Lewis and Yamamoto, 1990). Impermeable to water, lignin is also very resistant to pressure and not very elastic and it is the most abundant plant polymer after cellulose, being found in greater quantity in the cell wall, around 60% to 90% (Egg-Mendonça, 2001), and its deposition occurs during the formation of the cell wall. The growth and development of the cell wall may be divided into two phases: growth of the primary wall, a phase in which the cell increases in size, and growth of

the secondary wall, a phase in which deposition of lignin polymers occurs to the extent that the cell wall becomes progressively thicker as of the internal edge of the primary wall, in the direction of the center of the cell. The inclusion of lignin on the cell wall originates in the middle lamella, going in the direction of the interior of the secondary wall. According to Jung and Allen (1995), the effect of this lignin deposition pattern makes the middle lamella/primary cell wall region more intensely lignified. This lignin deposition is important not only to lend rigidity and resistance to plant tissue, such as stem and leaves, but especially for the seed coat of soybean seeds, it has been correlated with resistance to mechanical damage (Alvarez, 1994; Panobianco *et al*., 1999), providing mechanical resistance to the tissue and protection against infestations by microorganisms to the cell wall (Tavares *et al*., 1987).

Hard or impermeable seeds, according to Woodstock (1988), may be the result of compacted organization of cellulose microfibriles on the cell wall. This, for its part, may be impregnated with waterproof substances, such as lignin, waxes, suberins or tannin. They are abundantly composed of cellulose and hemicellulose polysaccharides, and of phenylpropanoid polymers such as lignin (McDougall *et al*., 1996).

2.3 Less Digestible Components of Cell Wall.

Lignocellulose is the primary building block of plant cell walls. It is the fibrous material that forms the structural frame work of the plant cell wall. Lignocellulosics are composed of heterogeneous complex of carbohydrate polymers (cellulose, hemicelluloses and lignin). Lignocellulosics are resistant to degradation and offer hydrolytic stability and structural robustness, which is mainly due to cross linking between polysaccharides (cellulose and hemicellulose) and lignin via ester and ether linkages. Cellulose and hemicellulose are densely packed by layers of lignin that offer protection against enzymatic hydrolysis. Lignocellulose-rich feedstock needs to be decomposed by pretreatment to improve its digestibility.

The main chemical component of feeds that determine the rate of digestion is the neutral detergent fibre (NDF) which is a measure of the cell wall content. Cell wall carbohydrates can be quantified by determination of neutral detergent fibre (NDF), which includes cellulose, hemicellulose and lignin as the major components (Van Soest *et al*., 1991). There is a negative relationship between NDF content of feeds and rate at which they are digested. One consequence of those feeds that are equal in digestibility, but differ in NDF (cell content) content is that they have different intakes. Due to the variability of NDF in rumen degradation and itsinfluence

onanimal performance, the knowledge of NDF digestibility in forage is critical for effective ruminant feeding (Oba and Allen, 1999).

Apart from the anti-nutrients, the crude fibre content also plays a vital role in poor digestibility of plant ingredients (Hastings, 1964; Atack *et al*., 1979; Jackson *et al*., 1982; De Silva *et al*., 1990; Bairagi *et al*., 2002, 2004; Ramachandran *et al*., 2005). The results of several feeding trials indicated that the tannin, phytic acid and crude fibre level of plant leaf meals and seed meals can be considerably reduced after being fermented with extracellular enzymes especially cellulase, amylase and protease producing bacterial strains in solid state fermentation process (Bairagi *et al*., 2002, 2004; Ramachandran *et al*., 2005).

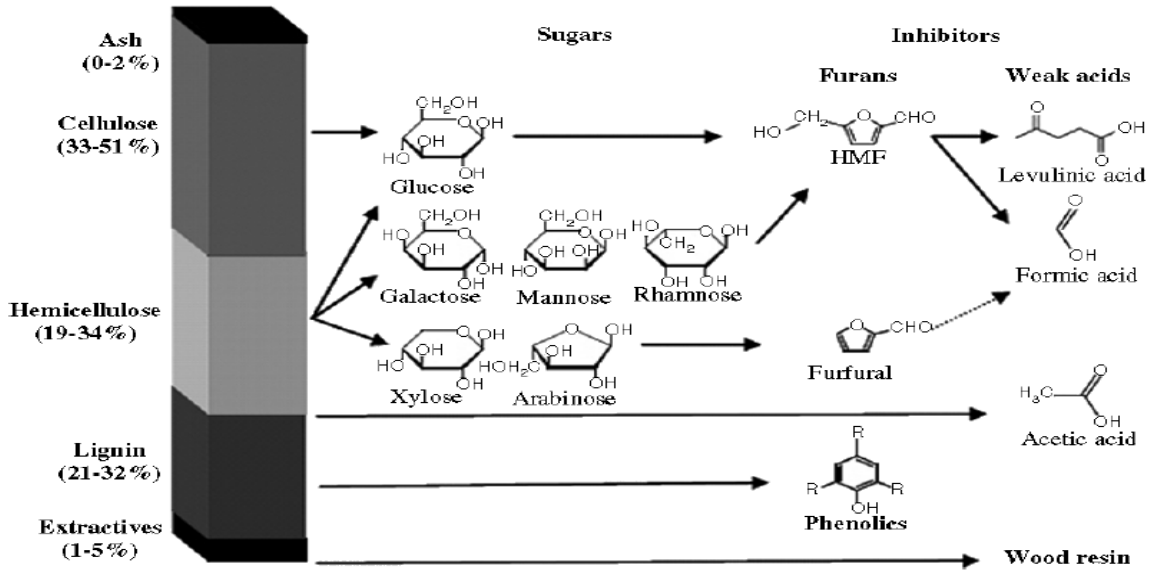


Fig 2.1: Average Composition of Lignocellulosic Biomass and Main Derived Hydrolysis Products (Almeida *et al*.,2007)

2.3.1 Cellulose

Cellulose is the main structural constituent in plant cell walls and is found in an organized fibrous structure. This linear polymer consists of D-glucose subunits linked to each other by β-(1, 4)-glycosidic bonds. Cellobiose is the repeat unit established through this linkage, and it constitutes cellulose chains. The long-chain cellulose polymers are linked together by hydrogen and van der Waals bonds, which cause the cellulose to be packed into microfibrils. Hemicelluloses and lignin cover the microfibrils. Fermentable D-glucose can be produced from cellulose through the action of either acid or enzymes breaking the β - (1, 4) - glycosidic linkages. Cellulose in biomass is present in both crystalline and amorphous forms. Crystalline cellulose comprises the major proportion of cellulose, whereas a small percentage of unorganized cellulose chains form amorphous cellulose. Cellulose is more susceptible to enzymatic degradation in its amorphous form (Beguin and Aubert., 1994).

2.3.2 Hemicellulose

Hemicellulose is the second most abundant polymer (20–50% of lignocellulose biomass) and differs from cellulose in that it is not chemically homogeneous. Hemicelluloses are branched, heterogenous polymers of pentoses (xylose, arabinose), hexoses (mannose, glucose, galactose) and acetylated sugars. They have

lower molecular weight compared to cellulose and branches with short lateral chains that are easily hydrolyzed (Fengel and Wegener, 1984).

Among the key components of lignocellulosics, hemicelluloses are the most thermo-chemically sensitive (Hendricks and Zeeman, 2009; Levan *et al*., 1990). Hemicelluloses within plant cell walls are thought to 'coat' cellulose-fibrils and it has been proposed that at least 50% of hemicellulose should be removed to significantly increase cellulose digestibility. Nevertheless, severity parameters must be carefully optimized to avoid the formation of hemicellulose degradation products such as furfurals and hydroxymethyl furfurals which have been reported to inhibit the fermentation process (Palmqvist and Hahn-Hägerdal, 2000).

2.3.3 Lignin

Lignin is the third most abundant polymer in nature. It is present in plant cell walls and confers a rigid, impermeable, resistance to microbial attack and oxidative stress. Lignin is an amorphous heteropolymer network of phenyl propane units (p-coumaryl, coniferyl and sinapyl alcohol) held together by different linkages (Hendricks and Zeeman, 2009). Lignin is generally accepted as the 'glue' that binds the different components of lignocellulosic biomass together, thus making it insoluble in water. Because of its close association with cellulose microfibrils, lignin has been identified as a major deterrent to enzymatic and microbial hydrolysis of lignocellulosic biomass (Avgerinos and Wang, 1983). Chang and Holtzapple, (2000)

showed that biomass digestibility is enhanced with increasing lignin removal. In addition to being a physical barrier, the detrimental effects of lignin include:

i) Nonspecific adsorption of hydrolytic enzymes to "sticky" lignin;

ii) Interference with, and non-productive binding of cellulolytic enzymes to lignin-carbohydrates complexes; and

iii) Toxicity of lignin derivatives to microorganisms.

2.4 Pretreatment of Lignocellulosic Materials

Lignocellulosic biomass contains three major components (cellulose, hemicellulose and lignin), which made the plant body rigid and very hard. Among these three components, lignin is the most important part which makes the plants more hardened. The digestibility of plant material in the rumen is related to the proportion and lignification of plant cell wall. Presence of lignin and hemicellulose makes the access of enzymes to cellulose fibers difficult. Therefore, the removal of lignin and hemicellulose as well as the increase of porosity during the pretreatment process increases the hydrolysis rate significantly (Dwivedi *et al*., 2009). The purpose of pretreatment is to disrupt the highly-coordinated cellulose, hemicellulose and lignin interactions in the cell wall, and make the polysaccharide chains more accessible to the enzymes. Pretreatment also removes lignin and hemicellulose, reduces cellulose crystallinity, and increases the porosity of the materials (Mosier *et al*., 2005).

The presence of lignin in lignocelluloses leads to a protective barrier that prevents plant cell destruction by fungi and bacteria for bioconversion. For the conversion, the cellulose and hemicellulose must be broken down into their corresponding monomers (sugars), so that microorganisms can utilize them. Unlike readily bio degradable substances (substrates) such as starch and sucrose that can be easily bio-degraded into the monosaccharide, for the utilization of lignocellulosic materials in a bioconversion process, pretreatment is necessary. Therefore, the first step is the integration of chemical and physical treatment systems for dissolving hemicellulose and improving accessibility of the cellulose for hydrolytic enzymes (Godliving, 2009).

Lignin is the prime factor influencing the digestibility of plant cell wall material. As concentrations of lignin increase, digestibility, intake and animal performance usually decreases. Lignin limits cell wall (fibre) digestion by providing a physical barrier to microbial attack and the concentration of both fibre and lignin increases as plants mature (Van Soest, 1978). Different feedstock contain different amount of lignin that must be removed via pretreatment to enhance biomass digestibility. The lignin is believed to melt during pretreatment and coalesces upon cooling such that its properties are altered; it can subsequently be precipitated (Brownell and Saddler, 1987; Converse 1993). Delignification (extraction of lignin by chemicals) causes biomass swelling, disruption of lignin structure, increases in internal surface area, and increased accessibility of cellulolytic enzymes to cellulose fibers. Although not

all pretreatments result in substantial delignification, the structure of lignin may be altered without extraction due to changes in the chemical properties of the lignin. The pretreated biomass becomes more digestible than the raw biomass even though it may have approximately the same lignin content as non-pretreated biomass.

The presence of lignin and hemicellulose makes the accessibility of enzymes and acids to cellulose more difficult, thus reducing the efficiency of the hydrolysis process. Pretreatment is required to alter the size and structure of the biomass, as well as its chemical composition, so that the hydrolysis can be achieved rapidly and with greater yields.

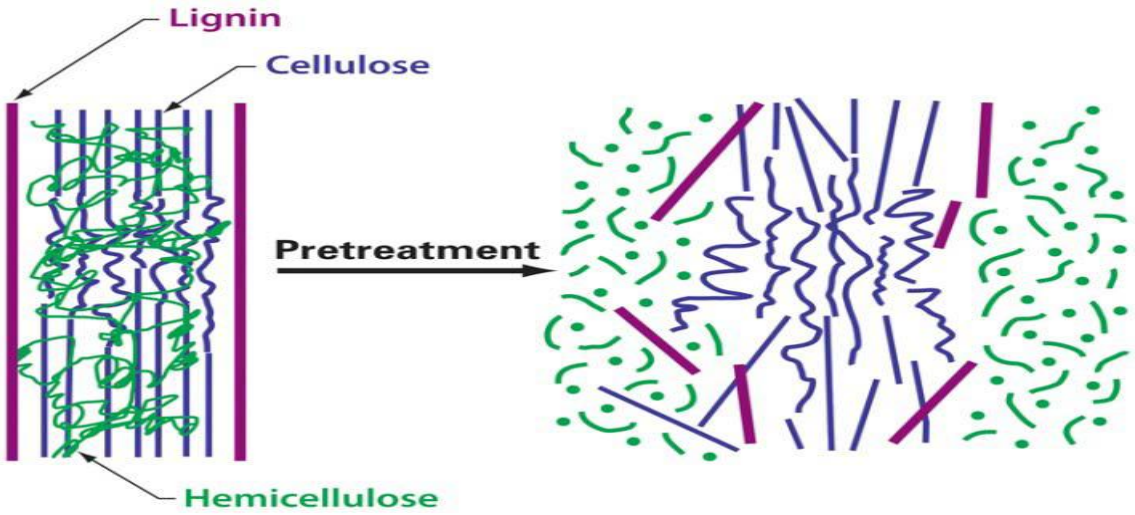


Fig 2.2: Schematic Goals of Pretreatment on Lignocellulosic Material (modified from Mosier *et al*., 2005).

2.5 Goals of Pretreatment

A lot of beneficial effects of pretreatment of lignocellulosic materials have been known for a long time. The goal of the pretreatment process is to remove lignin and hemicellulose, reduce the crystallinity of cellulose, and increase the porosity of the lignocellulosic materials. Each pretreatment has its own effect(s) on the cellulose, hemicellulose and lignin, the three main components of lignocellulosic biomass (Hendriks and Zeeman, 2009**).**

Ideally, pretreatment methods should meet following requirements:

I. low cost,
II. Ability to reduce or remove the maximum amount of extractives and inhibitors including lignin,
III. Retain a majority of the polysaccharides (avoid the degradation or loss of carbohydrate),
IV. Consume little energy,
V. Consume few chemicals and
VI. Produce no pollution.

2.6 Assessment of Pretreatment

In theory, the ideal pretreatment process produces a disrupted, hydrated substrate that is easily hydrolyzed but avoids the formation of sugar degradation products and fermentation inhibitors. Assessment of biomass pretreatment processes depends on a parameter called the "severity factor", which is defined as the combined effect of

temperature, acidity, and duration of pretreatment. Studies on biomass pretreatment have used the severity factor for comparing pretreatment results even though it does not provide an accurate measure of the severity. In other words it is used for rough estimates (Galbe and Zacchi, 2007). Nevertheless, severity parameters must be carefully optimized to avoid the formation of hemicellulose degradation products such as furfurals and hydroxymethyl furfurals which have been reported to inhibit the fermentation process (Palmqvist and Hahn-Hägerdal, 2000). Pre-treatment assessment is conducted by;

I. Analyzing the sugars (both monomers and oligomers) released in the liquid and the carbohydrate content of the water insoluble solids (WIS) after pretreatment. This gives the total amount of recoverable carbohydrates.
II. Enzymatic hydrolysis of either the washed or unwashed WIS;
III. Fermentation of the pretreated material to assess its fermentability directly or target the concentration and inhibition of fermenting microorganisms with respect to growth.
IV. Fermentation of either the slurry or washed water insoluble solids (WIS) for biofuels production;
V. Assessment of additional biotechnological potential of the pretreated fractions such as value added products.

2.7 Types of Pretreatment

Pretreatment methods can be roughly divided into different categories: physical (milling and grinding), physicochemical (steam pretreatment/autohydrolysis, hydrothermolysis, and wet oxidation), chemical (alkali, dilute acid, oxidizing agents, and organic solvents), biological, electrical, or a combination of these.

2.7.1 Mechanical Communition

Coarse size reduction, chipping, shredding, grinding, milling are amongst the different mechanical size reduction methods that have been used to enhance the digestibility of lignocellulosic biomass (Palmowski and Muller, 1999). These treatments increase the available specific surface area, and reduce both the degree of polymerization (DP) and cellulose crystallinity (Sun and Cheng, 2002). Smaller substrate particle would provide large surface area for microbial metabolism, which is a desirable factor in enzyme production. However, too small substrate particle will lead to substrate agglomeration, which may interfere with microbial respiration/aeration, and result in poor cellular growth (Pandey *et al*., 2000).

Osma *et al. (*2007) reported that particle size, porosity and chemical composition of the substrates play crucial role in higher enzyme production. It has been shown that further reduction of biomass particle size below 40 mesh (0.400 mm) has little effect on the rates and yields of biomass hydrolysis (Chang *et al*., 1997). The objective of chipping is to reduce heat and mass transfer limitations. Grinding and milling are more effective at reducing the particle size and cellulose crystallinity than chipping

probably as a result of the shear forces generated during milling. The type and duration of milling as well as the kind of biomass determine the increase in specific surface area, final DP and the net reduction in cellulose crystallinity. They result to improved digestibility of cellulose and hemicellulose to glucan and xylan, respectively; they further enhance enzymatic digestibility with lower enzyme loads. Mechanical pretreatment also result to substantial lignin depolymerization via the cleavage of uncondensed-aryl ether linkages (Ineou *et al*., 2008).

2.7.2 Steam Explosion

Steam explosion a common method used in the pretreatment of lignocellulosic materials. In this method, substrate is treated with high-pressure saturated steam, and then the pressure is suddenly reduced, which makes the substrate undergo an explosive decompression. The ideal steam-pretreatment condition for a given feedstock should yield water insoluble fraction (WIF), which is highly susceptible to enzymatic hydrolysis, composed of mainly of cellulose and lignin, which also yields a highly fermentable water soluble fraction (WSF) composed of mainly partially hydrolyzed hemicellulose and other soluble process derived aromatic compounds (Boussaid *et al*., 1999; Robinson *et al*., 2002).

2.7.3 Pretreatment with Alkali

The primary mechanism of alkaline hydrolysis is the saponification of intermolecular ester bonds crosslinking xylan hemicellulose and other components, in order to increase the porosity of lignocellulosic materials (Tarkow and Feist, 1969). Sodium hydroxide, lime, and ammonia are the common alkali reagents applied in pretreatment of cellulosic biomass. Sodium hydroxide is the strongest base among the three, giving the highest degree of lignin removal (Wang *et al*., 2010; Xu *et al*., 2010). Alkaline pretreatment disrupts the lignin structure and breaks the linkage between lignin and the other carbohydrate fractions in lignocellulosic biomass, thus making the carbohydrates in the heteromatrix more accessible. The reactivity of remaining polysaccharides increases as the lignin is removed. Acetyl and other uronic acid substitutions on hemicellulose that lessen the accessibility of enzymes to cellulose surface are also removed by alkali pretreatments (Chang and Holtzapple, 2000; Mosier *et al*., 2005; Galbe and Zacchi, 2007). However, most of the alkali is consumed.

Pretreatment with alkali removes lignin and part of the hemicellulose, thus increasing the accessibility of enzymes to cellulose in later phases of hydrolysis. All of the cellulose and most of the hemicellulose is left in an insoluble polymeric form. This is due to better fermentation efficiency, because formation of inhibitory byproducts is avoided. Downside of the method is a slightly lower sugar conversion rate. Pretreatment with alkali is best used for biomass with high lignin content (Hamelinck *et al*., 2005; Mosier *et al*., 2005;Gupta, 2008).

Alkaline thermochemical pretreatment process is a well known method that can be used to dissolve complex substrates before biological treatment. Compared with acid or oxidative reagents, alkali treatment appears to be the most effective method in breaking the ester bonds between lignin. Pretreatment with alkaline solutions increases the digestibility of cellulose and favors the solubilization of lignin (Alvira, 2010). For example, pretreatment with NaOH solutions leads to swelling and the increasing of internal surface of cellulose (Alvira *et al*., 2010). Although alkaline pretreatments show great efficiency as regards the lignin solubilization, they are less efficient concerning the solubilization of cellulose and hemicelluloses (Girio*et al*., 2010).

2.7.4 Pretreatment with Acid

It has been proven to be very effective in disrupting the hemicellulose interaction with lignocellulose. The removal of hemicellulose increases the accessibility of the enzyme to the cellulose, subsequently increasing the efficiency of enzymatic hydrolysis. Furthermore, it could yield close to the theoretical yield for carbohydrates when combined with the hydrolysis of hemicellulose (xylose, 70% to 90% yield) (Liu and Wyman, 2004). Although combined sugar yield is high using acid for biomass pretreatment, there are several drawbacks. First, the necessity of neutralization before fermentation generates gypsum; second, this pretreatment process involves high capital investment, large chemical consumption and acid

recovery costs (Laxman andLachke,2009). Most important of all, the relatively large amount of extractives and lignin left in the solid portion of the pretreated biomass and the byproducts from this process consequently impede fermentation reaction. Dilute acid pretreatment breaks down the cell wall structure and exposes the cellulose for increased enzyme accessibility (Mosier *et al*., 2005). Approximately 70% of the lignin, 5% of the hemicellulose, and 95% of the cellulose remains after dilute acid pretreatment (Kim *et al*., 2003). The main objective of the acid pretreatment is the solubilization of the hemicellulosic fraction of the biomass, in order to increase the accessibility of the enzymes in the enzymatic hydrolysis reaction (Alvira *et al*., 2010).

Pretreatment with dilute acid has been the most widely used method for pretreatment of the lignocellulosic material. This method uses cheap chemicals, mild operating conditions and is simple to perform. Downside of the dilute acid pretreatment method is a low conversion rate and formation of byproducts that are inhibitory for the following fermentation process. Besides sulfuric acid, nitric acid also has shown good results in cellulose-to-sugars conversion yields, but nitric acids higher price makes it less cost effective.

2.7.5 Biological Pretreatment

Biological pretreatments have been mostly been associated with the action of fungi capable of producing enzymes that can degrade lignin, hemicellulose, and polyphenols. White-rot and brown-rot fungi both belong to the basidiomycetes, whereas soft-rot fungi are ascomycetes, and their activity is usually related to high or low moisture content of wood (Blanchette, 1995). White- and soft-rot fungi have been reported to degrade lignocellulose material, with white-rot being the most effective at biological pretreatment of biomass (Lee 1997; Sun and Cheng, 2002). Brown-rot fungi primarily attack cellulose, whereas white and soft-rot attack both lignin and cellulose via the production of enzymes such as lignin peroxidases, polyphenol oxidases, manganese-dependent peroxidases, and laccases that degrade the lignin. The idea of using white- rot fungi to improve the digestibility of lignocellulose waste for ruminants was first developed in 1902 by Falck (Cohen, 2002) who suggested the use of fungi for the improvement of lignocellulosic wastes. Since then, a considerable amount of work has been conducted on the upgrading of lignocellulosic matter to feed fungi using white-rot. A wide range of lignocellulosic biomass have been pretreated with white-rot fungi and used as ruminant feed. Hatakka *et al.* (1993, 1994) reported the selective delignification of wood and wheat straw by selected white-rot fungi such as; *Phanerochaete chrysosporium, Phlebia radiata, Dichmitus squalens, Rigidosporuslignosus,* and *Jungua separabilima.* Lignin depolymerization by these fungi takes weeks to achieve significant result but can be very selective and efficient (Hatakka 1994; Hatakka *et al*., 1993). Biological

pretreatment of lignocellulose could improve the nutritional value (Okano *et al*., 2009) and *invitro* digestibility (Zadrazil and Puniya, 1995), increase bioavailability of nutrients and decrease antinutritional factors (Mandebvu *et al.,* 1999).The rate of biological pretreatment however, is too slow for industrial purposes. The residence time of 10–14 days, the requirement of careful growth conditions, and the large amount of space to perform biological pretreatments are the disadvantages that make this method of pretreatment less attractive on an industrial scale. The fact that some of the carbohydrate fraction is consumed by the microorganism could also be a disadvantage.

Ustilago maydis

Filamentous fungi are among the most potent degraders of lignocellulosic biomass as they produce a high number and a broad variety of enzymes that have different and complementary catalytic activities (Lundell *et al*., 2010). The degradation of lignocellulose by filamentous fungi has been studied in a range of basidiomycetes and ascomycetes. Among wood-decaying basidiomycetes, the white-rot fungi *Phanerochaete chrysosporium* is known to secrete a wide range of enzymes such as lignin peroxidases and glycosyl hydrolases (GHs) (Ravalason *et al*., 2008).

Recently, there has been growing interest in the potential of plant-pathogenic fungi to optimize the enzymatic pretreatment processes for the optimal hydrolysis of lignocellulosic biomass (Gibson *et al*., 2011). A recent large-scale screening using

156 ascomycetes revealed that the plant pathogens were more active than the non-pathogens on several lignocellulosic substrates (King *et al*., 2011). *U. maydis* is a well characterized basidiomycete fungus that parasitizes maize. It belongs to the smut fungi, which share a biotrophic mode of growth, i.e., they grow on the host plant without killing it. Post-genomic approach revealed the unexpected potential of *U.maydis*, which contained unique enzymatic equipment that significantly supplemented the *T.reesei* enzymatic cocktail. (Marie Couturier *et al*., 2012). The putative *U. maydis* oxido-reductases could play a crucial role in improving the hydrolysis of plant cell walls.



Figure 2.3: Morphology of fungi (*U. maydis)* on PDA medium

2.7.6 Combined Pretreatment

These treatments are a combination of physical, chemical, biological and enzymatic treatments used in order to get the best of all treatments hence minimizing the disadvantages of individual treatments.

2.8 Effects of Fermentation

One of the processing methods that have been shown to significantly reduce or eliminate the level of antinutrients and also improve the nutritional quality of plant materials is solid fermentation using fungi (Bressani, 1973; Gloria *et al*., 1995; Jacqueline *et al*., 1996; Obiozoba, 1998; Muhammad *et al*., 2000; Muhammad, 2007; Adeyemi, 2008). Fermentation enhances the quantity, quality and bioavailability of nutrients, mineral elements, vitamins, essential amino acids and protein by improving protein and fibre digestibility. Fermentation gradually changes the characteristics of the food by the action of enzymes, produced by some bacteria, moulds and yeasts (Bray *et al*., 1999; Adeyemi, 2008*).*

Several simple processing methods, such as decortication, soaking, cooking, germination, enzymes and fermentation, have been used to reduce the amount of tannins, phenols and phytates (Hassan and El-Tinay,1995; Mahgoub and Elhag, 1998; Youssef, 1998;Elmaki *et al.*,1999; Matuschek *et* a*l.*,2001; Urbano *et al*., 2007; Porres *et al.*,2008). Many studies have demonstrated that fermentation processes are

an excellent way of diminishing antinutritional factors like tannin and phytate (Towo *et al*., 2006; Correia *et al*., 2005).

2.9 Production of Enzymes by Solid State Fermentation

Solid state fermentation (SSF) systems have generated much interest lately, because they offer some economical and practical advantages including higher product concentration, improved product recovery, very simple cultivation facilities, reduced wastewater output, lower capital investment, and lower plant operation cost (Latifian *et al.*, 2007).

Solid state fermentation is a suitable process for enzyme production using filamentous fungi since, SSF is well adapted to the metabolism of the fungus and it gives the natural habitat for fungal growth (Singhania *et al*., 2009).

In studies where the enzyme concentration obtained from submerged fermentation (SmF),Submerged liquid fermentation(SLF) and solid state fermentation (SSF) have been compared, it has been observed that in SSF the titles are higher and also, the produced enzymes are stable to wider ranges of temperature and pH. This has been evidenced for enzymes such as the amylase (Ramesh and Lonsane, 1991), pectinase (Sólis-Pereíra *et al.,* 1993; Maldonado *et al.,* 1998), tannase (Lekhaand Lonsane, 1994) and protease (George *et al*., 1997). In these studies it has demonstrated that SSF presents attractive advantages for the production of microbial enzymes. More

recent studies in SSF involve the production of enzymes with potential utilization in the food industry. Such enzymes are a) tannin acyl hydrolase, b) protopectinase, c) caffeinase, d) mannanase, e) phytase, f) xylanase and g) protease. Extracellular enzymes are recovered from the fermentation medium by centrifugation, filtration, fractional precipitation, chromatographic separation, electrophoresis, membrane separation, freeze drying or a combination of these methods (Skinner, 1975). Intracellular enzymes are extracted by disruption of cells in a homogeniser or mill. Recovery is more difficult and the yield is lower than for extracellular enzymes, because some enzymes are retained within the cell mass. If required, the specific activity of the enzyme is increased by precipitation using acetone, alcohols or ammonium sulphate or by ultrafiltration.

2.9.1 Tannase

Tannins are water soluble polyphenolic compounds which are widespread in plants and found in bark, galls, leaves, fruits and wood. Tannins are the fourth most abundant plant constituents after cellulose, hemicelluloses and lignin (Rana and Bhat, 2005). Tannase (E.C. 3.1.1.20) also known as tannin acyl hydrolase, is the enzyme which catalyses decomposition of hydrolysable tannins especially gallo-tannins to glucose and gallic acid (Van de Lagemaat and Pyle, 2005). Gallic acid, the product of tannin hydrolysis finds application in many fields including dye-making, pharmaceutical, leather and chemical industries (Hota *et al*., 2007). These

compounds are mostly soluble (except those with high molecular weights), and are capable of forming stable complexes with proteins and other polymers such as cellulose, hemicellulose, pectin and with minerals (Haslam,1996).Tannin acyl hydrolases, commonly referred to as tannases (E.C. 3. 1. 1. 20), are inducible enzymes produced by fungi, yeast and bacteria. Tannases have recently been characterized by their activity upon phenolic complexes. They are able to hydrolyze the ester bond (galloyl ester of gallic acid) of substrates such as tannic acid, epicatechin gallate, epigallocatechin gallate, and chlorogenic acid.

Tannins are known to occur in the vacuoles of plant cells (Forsyth, 1964; Jung and Fahey, 1983). All tannins are polyphenolic compounds with a high molecular weight (MW 500-3,000) (Haslam, 1981). Tannins can be divided into condensed and hydrolysable tannins. Condensed tannins (proanthocyanidins) are the most widely distributed in vascular plants and are made up by condensation of hydroxyflavans, leucoanthocyanidin (flavan-3, 4-diol) and catechin (flavan-3-ol) (Cope and Burns, 1974). Hydrolysable tannins are restricted to angiosperm dicotyledons and usually contain glucose as a central core (Swain, 1979). Generally if ingested in excessive quantities, tannins inhibit the absorption of minerals such as iron which may, if prolonged, lead to anemia (Brune *et al*., 1989). This is because tannins are metal ion chelators, and tannin-chelated metal ions are not bioavailable. Many plants employ tannins to deter animals. Animals that consume excessive amounts of these plants fall ill or die. The lethal dose is said to be around 6% of the animal's body weight.

Tannins are known to affect the grazing behaviour and consequently depress the food intake in sheep (Cope and Burns, 1974). Feed utilization appears to be negatively correlated with tannin content due to depression in rumen microbial activity (Donnely and Anthony, 1970; McLeod, 1974; Walker, 1975; Terrill *et al.,* 1989).

2.9.2 Phytase

Phytase is an enzyme in which interest has increased remarkably in the past 20 years not only because of its wide range of applications in animal and human nutrition, but also, in response to heightened concerns over phosphorus pollution in the environment (Lei and Porres, 2003).

The main storage form of phosphorus in many seeds is phytate (myo-inositol hexakisphosphate). Phytate is the major inhibitor of mineral absorption from plant foods, and decreasing the content of phytic acid in meals of plant origin greatly improves the absorption of plant minerals (Kayode *et al.,* 2007). The nutritional importance of phytic acid lies in its ability to chelate several mineral elements especially calcium, magnesium, iron and zinc, thereby reducing availability in the intestinal track (Hertramf and Piedad-Pascual, 2000). Phosphate from phytic acid is poorly available, costly and non- renewable mineral therefore phosphate is commonly added to pork, poultry and fish diets to supplement the phosphorus to

meet nutritional requirement (Urbano *et al.*, 2007). In cereals, legumes and Brassicae, phytate was collected in the seeds during maturation and accounts for 50–80% of total phosphorus (Hong *et al.*, 2004). One of the main factors limiting the nutritional values of cereals and legumes in humans and animals is the interaction of phytic acid with essential dietary minerals (Mg, Ca, Zn, etc.), protein, or vitamins, which makes it an antinutritional factor. Phytate in plants are usually chelated with cations, protein and (or) starches and this chelated form called phytin constitutes between 1 and 3% by weight of many of the cereals and oilseeds used in animal feeds (Cheryan, 1980). As a result, reducing the phytate content in plant materials to increase their nutritional values is advisable. This can help explain the increased interest in phytate-degrading enzymes (Becerra *et al.*, 1996; Gargova *et al.*, 2003). In order to increase the bioavailability of minerals in phytate containing seeds there is one alternative, which is phytic acid removal from the food products. Many processes have been displayed to remove phytic acid from seeds, but the best results are achieved by the use of enzymatic hydrolysis (Greiner *et al.*, 2006). Phytase is either absent or present at a very low level in the gastrointestinal tract of monogastric animals (Selle and Ravindran, 2007). Dietary phytate is not digested in the intestine and consequently accumulates in faecal materials. Phytase is produced by bacteria, fungi and yeasts. Among them, strains of *Aspergillus niger*produce large amounts of extracellular phytase (Chelius and Wodzinski, 1994) and are more acid tolerant than bacteria and yeasts (Kim *et al*., 1998). Microbial phytases are of interest for

biotechnological applications, especially for the phytate reduction in foods and feedstuffs.

2.10 Biology of *Aspergillus niger*

Aspergillus niger is a member of the genus Aspergillus which includes a set of fungi that are generally considered asexual, although perfect forms (forms that reproduce sexually) have been found. Aspergilli are ubiquitous in nature. They are geographically widely distributed, and have been observed in a broad range of habitats because they can colonize a wide variety of substrates. *A. niger* is commonly found as a saprophyte growing on dead leaves, stored grain, compost piles, and other decaying vegetation. The spores are widespread, and are often associated with organic materials and soil.

The history of safe use for *A. niger* comes primarily from its use in the food industry for the production of many enzymes such as a-amylase, amyloglucosidase, cellulases, lactase, invertase, pectinases, and acid proteases (Bennett, 1985; Ward, 1989). In addition, the annual production of citric acid by fermentation is now approximately 350,000 tons, using either *A. niger* or Candida yeast as the producing organisms. Citric acid fermentation using *A. niger* is carried out commercially in both surface culture and in submerged processes (Berry *et al*., 1977; Kubicek and Rohr, 1986; Ward, 1989).

A. niger has some uses as the organism itself, in addition to its products of fermentation. For example, due to its ease of visualization and resistance to several anti-fungal agents, *A. niger* is used to test the efficacy of preservative treatments (Jong and Gantt, 1987). In addition, *A. niger* has been shown to be exquisitely sensitive to micronutrient deficiencies prompting the use of *A. niger* strains for soil testing (Raper and Fennell, 1965). There is also interest in using this fungus to perform certain enzymatic reactions that are very difficult to accomplish by strictly chemical means, such as specific additions to steroids and other complex rings (Jong and Gantt, 1987).

2.10.1 Identification and Taxonomy

As is the case of many fungi, the taxonomy of *Aspergillus* is primarily based on morphological features, rather than the physiological, biochemical features and genetic characteristics often used to classify bacteria. The genus *Aspergillus* is usually defined as asexual saprophytic fungi that produce large black or brown conidia by phialides that are arranged in a globose head radiating from a vesicle or spherical conidiophore.

This definition leads to inclusion of a complex assortment of organisms within the taxon. This is illustrated by the 132 species arranged in 18 groups by Raper and Fennell (1965) due to overlapping morphological or physiological characteristics. *Aspergillus niger* is both a species and a group within the genus *Aspergillus*.

2.10.2 Definition of the *Aspergillus niger* Group

Raper and Fennell (1965) designated 15 species as comprising the *Aspergillus niger* group, which includes all of the aspergilla with black conidia. The current concept of retention of the *A. niger* group is based on black conidia which seems dominant (Kusters-van Someren *et al*., 1990).

2.10.3 *A. niger* Species

While morphology provides a reasonable means of classification and assignment within the *A. niger* group, it is not a reliable means for identifying a given isolate from the field. The major distinction currently separating *A. niger*from the other species of *Aspergillus* is the production of carbon black or very dark brown spores from biseriate phialides (Raper and Fennell, 1965). On Czapek dox agar, colonies of *A. niger*consist of a compact white or yellow basal felt covered by a dense layer of dark-brown to black conidial heads (Ellis, 2006). Other features include the smooth and generally colorless conidiophores and spores that are #5 μm, globose, and have conspicuous ridges or spines not arranged in rows. *A. niger*isolates grow slowly on Czapek agar (Raper and Fennell, 1965). These physical characters such as spore color and rate of growth on a defined media are subject to change, especially under extended pure culture or selection and mutation.

Though *A. niger* is relatively stable to spontaneous mutation compared to other aspergilli, variation in morphology may still be a problem with some strains (Raper and Fennell, 1965). Thus this species may be misidentified with other *Aspergillus* spp.

2.10.4 Toxin Production by *A. niger*

Aspergillus niger can produce a variety of fungal metabolites, termed mycotoxins, depending upon growth conditions and the strain of the organisms. The mycotoxins include oxalic acid crystals, kojic acid, and cyclic pentapeptides called malformins. The mycotoxins range from moderately to highly toxic in terms of acute toxicity.

A. niger produces oxalic acid and kojic acid abundantly. These two products have only a slight acute toxicity. Oxalic acid has an intraperitoneal LD of 150 mg/kg in rats and kojic acid has an intraperitoneal LD of 250 mg/kg in mice (Ueno and Ueno, 1978).

Malformins produced by *A. niger* are more potent toxins, at least by the intraperitoneal route of administration. Malformin A has an intraperitoneal LD as low as 3.1 mg/kg in mice (Kobbe *et al*., 1977.) Pathologic signs accompanying fatality included dilatation with hemorrhage of the gastrointestinal tract and changes in the liver and kidney. Death occurred within four days. In contrast, oral doses up to 50 mg/kg failed to cause evidence of acute toxicity (Yoshizawa, 1975.)

The production of malformins is related to the composition of the growth substrate and usually occurs in stationary phase cultures. While not always true, mycotoxins are generally produced on solid substrates with high carbon/nitrogen solid content (Ciegler and Kurtzman, 1970; Anderegg *et al*., 1976). For example, malformins are produced when *A. niger* is grown on onion bulbs (Curtis et al., 1974) and on fermenting grains (Kobbe et al., 1977). A strain of *A. niger* recovered from mold-damaged rice produced malformin A. A survey to define the number of strains in nature that are malformin producers appears not to have been made.

2.11 Environmental Hazards

I. Hazards to Animals

Livestock ingesting *A. niger* contaminated feed have been shown to be adversely affected by mycotoxins. Calcium depletion and other physiological abnormalities including death can result from ingestion of *A. niger* colonized feed due to the fungal production of oxalic acid or malformins (Austwick, 1965). Chicks and mice were killed after being fed with moldy soybeans and mice died after eating contaminated wheat containing isolates of *A. niger* (Semeniuk, *et al*., 1971). The cause of death was assumed to be toxicosis, but pathogenicity was observed in some cases.

II. Hazards to Plants

A. niger has been isolated from 37 genera of plants (Farr *et al*., 1989). Often these reports involve co-isolation with other perhalps more destructive microorganisms or isolation from a stored plant product. There are reports of *A. niger* being a plant pathogen in peanuts (Jackson, 1962). Apparently, *A. niger* can induce a crown rot of peanuts due to *A. niger* -infected seed under specific hot, humid growth conditions. The mycotoxins described above, namely oxalic acid, malformin A, and malformin C, have been shown to cause significant growth effects such as root curling and top deformation in plants (Anderegg *et al*., 1976).

*A. niger*can cause the rotting of numerous fruits, vegetables, and other food products, thus causing substantial economic losses due to spoilage. For example, black rot of onions associated with *A. niger* is responsible for serious losses of onion bulbs in the field and in storage. There are also reports of *A. niger*-induced spoilage of mangos (Prakash and Raoof, 1989), grapes (Sharma and Vir, 1986), and tomatoes (Sinha and Saxena, 1987).

2.12 Biology of *Trichoderma* spp.

The genus *Trichoderma* is a soil-born fungus. They are saprobic on decaying wood and vegetable matter. Species of *Trichoderma* are frequently dominant components of the soil microflora in widely varying habitats. This may indicate that *Trichoderma* spp. could be aggressively competitive nature. In early period, Rifai and

Webster(1966) attempted to establish taxonomy approach to *Trichoderma*. This fungus grows easily in media such as potato dextrose agar (PDA), Malt agar (MA), Blakeslee's agar (BLA) and Czapek Dox agar (CDA) (Bissett, 1991a,b,c). This advantage of *Trichoderma* is built and has attracted numerous researchers worldwide. Until in the present, *Trichoderma* spp. are well known fungi as a biological control agent (BCA).

2.12.1 Morphological Characteristic of Genus

Rifai (1969) and Bissett (1991a,b) reported about the morphological characteristics of *Trichoderma*, used to characterize and differentiate species of *Trichoderma*. Characters useful for characterization and identification in other hyphomycete genera frequently are not as useful in differentiating *Trichoderma* species, usually because of the narrow range of variation of the simplified morphology in *Trichoderma*, or because descriptive terms to describe variation in color or pattern are not sufficiently precise to define differences between species. Identifications based on morphology characters remain the primary method for identification and verification of species in *Trichoderma*. Colony appearance, colony characters can be distinctive and characteristic of a species. But colony appearance is difficult to describe with sufficient precision for it to be very useful for identification. Most *Trichoderma* are fast growing fungi under optimum condition. The optimum temperature of most *Trichoderma* strains is between 25-30^{0}C.

Sporulate of *Trichoderma* gorw profusely on most media producing masses of powder, or in a few species slimy, green conidia. The green conidia are generally diagnostic of the genus but green conidia are also found in the unrelated genera *Penicillium* and *Aspergillus* as well as in the related genera *Clonostachys* and *Myrothecium*.

Conidiophores, the patterns of conidiophore branching and aggregation of conidiophores into fascicles and pustules are useful for identification of strains of *Trichoderma* to sections and species aggregates. *Trichoderma* species typically lack well defined conidiophores. Cells that produce conidia, phialides, arise at the ends of much branched hyphal systems. The branching can be typical and present a clear pattern or can be highly irregular. Typically in cultures that are more than one week old on weak media phialides can no longer be seen. Conidia produced from effused conidiophores, or from conidiophores aggregated into fascicles or pustules is a common characteristic of a species. Generally conidia are unicellular, typically ellipsoidal to oblong, with length/width ratio greater than 1.4, but some species have globose to subglobose or ovoidal conidia with length/width ratio 1.0-3. Conidia are always less than 5 µm long. Conidia of few species are warted but typically are smooth. For the color of conidia, typically green but may be white or yellow or gray, depending upon the species.

2.12.2 Morphological Classification

Trichoderma is classified in the asexual form of the Ascomycetes (Deuteromycetes) genus Hypocrea, a member of the Hypocreales. *Trichoderma* were classified into a lot of species, Rifai (1969) classified *Trichoderma* into nine species: *T. aureoviride, T. hamatum, T. harzianum, T. koningii, T. longibrachiatum, T. piluliferum, T. polysporum, T. pseudokoningii* and *T. viride*. Domsch *et al.* (1980) included 2 species in this genus; *T. reesei* and *T. saturnisporum*. Bissett (1984; 1991a,b,c) reported another 19 species of *Trichoderma* included *T. citrinoviride, T. atroviride, T. lactea, T. flavofuscum, T. crassum, T. croceum, T. fasciculatum, T. fertile, T. longipilis, T. minutisporum, T.spirale, T.oblongisporum, T. pubescens, T. strictipilis, T. strigosum, T. tomentosum, T. virens*, *Trichoderma* namorph of *Hypocrea gelatinosa* and *H. semiorbis*. Later, *T. atroviride* was replaced by *T. paceranosum* Rifai (1969) and Domsch *et al*. (1980) reported that morphology characteristics of phialides and conidia were generally based for morphological classified of *Trichoderma* into species.

2.12.3 General Physiology

Trichoderma are metabolically versatile yet are also eclectic. Danielson and Davey (1973a) reported nitrogen sources are generally well utilized from ammonium compounds to amino acids to protein, although nitrate assimilation is often poor and is species dependent. Carbon substrates are extremely diverse. Several *Trichoderma*

strains degrade hydrocarbons (Davies and Westlake, 1979; Ahmad and Baker, 1988), and this ability is perhaps reflected as they form major components of fungal population from soils polluted with oil (and Kjoller, 1976;Gudin and Chater, 1977; Pinholt *et al*., 1979).

It is manifest that *Trichoderma* are wondrously endowed in being able to attack diverse substrates including other fungi, their ability to compete for nutrients, and their production of antibiotics. They use these capabilities interactively to facilitate their development in particular habitats.

2.12.4 General Ecology

Trichoderma are comprised with a fast-growing group that appear to be extremely common in agricultural, prairie, forest, salt marsh and desert soil in all climatic zones (Danielson and Davey, 1973a; Domsch *et al*., 1980, Roiger *et al*., 1991; Wardle *et al*., 1993).

Characteristically, they are saprophytes, with the exception that they can attack other fungi. They could be detected by soil dilution plating. *Trichoderma* can constitute up to 3.1% of the total fungal propagules from a wide range of forest soils (Danielson and Davey, 1973). The competitive interaction between *Trichoderma* and other microbes are complex, encompassing competition for nutrients, action of lytic enzymes, besides antibiosis/symbiosis and fungistasis.

2.12.5*Trichoderma harzanium*

Fungal species of the *Trichoderma* genus are soil borne, green-spored ascomycetes, found all over the world (Schuster and Schmoll, 2010). *Trichoderma* spp. have been used as biological control agents against a wide range of phytopathogenic fungi for many years (Papavizas, 1985), *Trichoderma harzianum* being especially effective.*T. harzianium* is species aggregate, grouped on the basis of conidiophore branching patterns with short side branches short inflated phialides and smooth and small conidia (Rifai, 1969). These characteristics allow for the relatively easy identification of *Trichoderma* as a genus, but the species concepts are difficult to interpret and there is considerable confusion over the application of specific names (Rifai, 1969; Papavizas, 1985).

2.12.6 Mixed Culture Fermentation

Mixed culture fermentation is that in which the inoculum consists of two or more organisms (Gutierrez-Correa *et al*., 1999).The mixed culturing of fungi may lead to better substrate utilization, increased productivity, and it also can strengthen and accelerate the bioconversion process on encouraging compatibility results reported in solid-state bioconversions than for liquid state fermentations (Alam *et al*., 2003; Molla *et al*., 2001). Strain compatibility is a critical factor in mixed culturing, and the most important observation was that synergistic interactions between compatible

partners may overcome nutritional limitations in poor agricultural residues (Gutierrez-Correa *et al*., 1999). The purpose of compatible mixed culturing is to let fungi work together mutually and degrade substrates faster. Many studies showed that evaluation of fungal possible compatible interactions was first studied in two agar media: potato dextrose agar (PDA) and malt extract agar (MEA) (Skidmore and Dickinson, 1976; Webber and Hedger, 1986).

Trichoderma reesei LM-UC4, the parent strain and its hyper cellulolytic mutant LM-UC4E1 were co-cultured with *Aspergillus niger*ATCC 10864 in solid substrate fermentation by Gutierrez-Correa *et al*., (1999). Bagasse was *Phanerochaete chrysosporium* supplemented with either soymeal or ammonium sulfate and urea, and the mutant strain was more responsive to mixed culturing than the parent strain when *A. niger*was the cooperating partner. Mixed culture fermentations are widely used in biotechnology for many processes including the production of antibiotics, enzymes, fermented food, composting, dairy fermentation, bioconversion of apple distillery and domestic wastewater sludge (Alam *et al*., 2001).

Table 2.1:Modes of Interaction between Filamentous fungi.

Interaction	Definition
1. Mutual intermingling	The growth where both fungi grow into one another without anymacroscopic sign of interconnections.
2. Partial intermingling	The growth where the fungus being observed is growing into the opposed fungus either above/below or touching each other without making any inhibition zone.
3. Invasion/replacement	One mycelium grows into the other and begins to consume another one, it may finally replace it.
4. Inhibition at touching point	The fungi approached each other until almost in contact and a narrow demarcation line, 1 to 2 mm, between the two colonies was clearly visible.
5. Inhibition at distance	Inhibition at a distance of >2 mm

CHAPTER 3

MATERIALS AND METHODS

3.1 Materials

3.1.1 Collection and Preparation of Samples

Indigofera arrecta fruits were collected from Zaria metropolis with identification done at the Herbarium of the Department of Biological Sciences, Ahmadu Bello University, Zaria, Nigeria. The fruits were sun-dried for 3-4 days, threshed and winnowed. Dust and other foreign materials were removed to obtain clean seeds. The whole seeds were then stored in plastic containers for subsequent analyses.

3.1.2 Isolation of *Aspergillus niger*

Isolation of *Aspergillus niger*from soil samples was performed by the soil dilution method (Johnson *et al*., 1959). Ten grams of soil sample was added to 90 ml distilled water on a shaker for one hour. Then, 10 ml aliquot from this suspension was transferred aseptically to 90 ml distilled water. This dilution process was repeated to get dilutions up to 10^{-5}. From the dilution suspension, one milliliter of aliquot from soil sample was spread on petri dishes containing Potato Dextrose Agar (PDA).The identification of *Aspergillus niger* was made by the simple microscopic method of

Ellis (2006). Pure isolates of *Aspergillus niger* were sub cultured on slants and stored at 4^0C.

3.1.3 Isolation of *Trichoderma harzanium*

Rice stems and leaves were cut into 5 mm pieces and added to 150 mL distilled water on a shaker for 30 minutes. Then, 10 mL aliquot from this suspension was transferred aseptically to 90 mL distilled water. This dilution process was repeated to get dilutions up to $10^{-5.}$ From the dilution suspension, one millilitre of aliquot from phyllosphere sample was spread on RB-S-F selective medium (Davet and Rouxel, 2000) in Petri dishes. The dishes were incubated at 26±1°C in the dark for 4 days. Individual isolates were identified at the species level using morphological keys and fungal species descriptions (Bisset, 1991a,b., Gams and Bisset, 1998; Zafari *et al.*, 2004). Pure isolates of *Trichoderma harzanium* were subcultured on slants and stored at 4^0C.

3.1.4 Identification of *Ustilago maydis*

Ustilago maydis was obtained from the Department of Microbiology, Ahmadu Bello University, Zaria and identified according to the method of Ellis (2006). The fungi

were authenticated microscopically and morphologically. Pure isolates of *Ustilago maydis* were sub cultured on slants and stored at 4^0C.

3.1.5 Chemicals

Chemicals and reagents used for the study were of analytical grade.

3.2 Methods

3.2.1 Pretreatments

The following pretreatments were carried out on the seeds of *Indigofera arrecta* seeds

- Mechanical Pre-treatment- One kilogram of the sample was dried in an oven at 55^oC for 24hr. After drying, the sample was ground in a bench mill and sieved to obtain a particlesize of 0.05- 0.250 mm fraction which was stored in clean polythene bags until required.
- Steam Pre-treatment- Two hundred grams of the ground sample was mixed with 1L ofdistilled water and autoclaved for 90min at 121^0C. The residues was washedthoroughly with distilled water and dried in an oven to a constant weight at 60^0C (Saddler *et al.*, 1993).

- Acid Pre-treatment- Two hundred grams of the sample was mixed with 1L of 1M HCl for 1 hour after which the mixture was neutralized with 1M NaOH (Chen *et al.*, 2002). The residues was then washed with distilled water and dried to constant weight at 60^0C in the oven.
- Alkaline Pre-treatment- Two hundred grams of the sample was mixed with 1L of 0.25 M NaOH solutions for 1 hour after which the mixture was neutralized with 0.1M HCl. The residue was washed with distilled water and dried to constant weight at 60^0C in the oven (Ali *et al*., 1991).
- Combined (Steam and Alkaline) Pre-treatment - Two hundred grams of the sample was mixed with 1L of 2%NaOH solution and autoclaved for 90min at 121^0C. The residue was washed with distilled water and dried to constant weight at 60^0C in an oven (Ali *et al*., 1991).
- Biological pre-treatment - Forty grams of the samplewas moistened with 200ml of distilledwater to obtain 70% w/w of moisture in 1L Erlenmeyer conical flask. Themixture was autoclaved for 30min at 121^0C. After cooling, it was inoculated withsporessuspension of *Ustilago maydis*and incubated at 28^0C for 21days.

3.2.2 Determination of Lignin, Cellulose and Hemicellulose Content

The procedure for Neutral- Detergent Fibre (NDF), Acid- Detergent Fibre (ADF), and Acid- Detergent Lignin (ADL) analysis was used according to the method reported by Goering and Van Soest. (1970).

- NDF—Neutral Detergent Fibre (total lignocellulosic content)

NDF fibre is the residue remaining after digestion in a detergent solution. The fibre residues are predominantly hemicellulose, cellulose and lignin. The NDF content is expressed as a dry mass basis fraction in percentage.

Procedure

One gram of air-dried sample was weighed into a conical flask. Reagents were added in the following order: 100mL room temperature neutral-detergent solution, 2mL decahydronaphthalene, and 0.50g sodium sulfite. The mixture was then heated to boiling and refluxed for 60 minutes. The residue in the crucible was washed with 50mL of hot water (90^{o}C) and then the liquid was filtered. The washing procedure was repeated 3 times after which the sample was washed and filtered dry twice with acetone in same manner. The crucible was then dried at 100^{o}C for 8 hours in the drying oven and cooled in a desiccator and then weighed. The yield of recovered neutral-detergent fibre was reported as the lignocellulosic content. The dried crucible was ashed for 3 hours at 500-550^{o}C in the muffle furnace, cooled in a desiccator and weighed. The ash content was reported as the ash insoluble in neutral-detergent.

Calculations

The neutral-detergent fibre fraction was computed as:

$$NDF = \frac{(W_0 - Wt) \times 100}{S}$$

Where W_0 was weight of dry crucible including fibre

Wt was weight of dry crucible

S was oven dried sample weight

- ADF- Acid Detergent Fibre (lignin and cellulose)

The acid-detergent fibre procedure provides a rapid method for lignocellulose determination. The fibre residues are primarily cellulose and lignin but can also include silica. The difference between neutral-detergent and acid-detergent fibre is an estimate of hemicellulose; however, this difference include some protein attached to the cell walls. The acid-detergent fibre was used as a preparatory step for lignin determination. The ADF content is used as a dry basis fraction in percentage.

Procedure

One gramme of air-dried sample was placed in a conical flask, to which 100mL of room temperature acid-detergent solution was added. Two milliliters of decalin was added in the mixture and the mixture was heated to constant boiling and refluxed for 60 minutes. After boiling, the mixture was transferred into a crucible and washed with 50 mL of hot water (90^oC) and the liquid was filtered. The washing procedure

was repeated 3 times and filtered dry with acetone in same manner until color removal was no longer apparent. The crucible was then dried at 100^{o}C for 8 hours in the drying oven and cooled in a desiccator and then weighed. The crucible was dried at 100^{o}C for 8 hours in the drying oven, cooled in a desiccator and weighed once completely cooled.

Calculations

The acid detergent fibre was computed as:

$$ADF = \frac{(W_0 - Wt) \times 100}{S}$$

Where W_0 was weight of dry crucible including fibre

Wt was tared weight of dry crucible

S was oven dried sample weight

- ADL- Acid Detergent Lignin

In the acid-detergent lignin procedure, the acid-detergent fibre (ADF) procedure is used as preparatory step. The detergent removes the protein and the other acid soluble material that would interfere with the lignin determination. The ADF residue consists of cellulose, lignin, cutin and acid-insoluble ash (mainly silica). Treatment with 72 % H_2SO_4 dissolves cellulose. Ashing of the residue will determine the crude lignin fraction including cutin. The ADL content is expressed as a dry matter basis fraction in percentage.

Procedure

The crucible containing the acid-detergent fibre was placed in a ceramic dish for support. About 50 mL cooled (15^{o}C) 72% H_2SO_4 was added to the residue and stirred with a glass rod to a smooth paste, breaking all lumps. Another 50 ml of 72% H_2SO_4 was added and stirred at hourly intervals and the excess acid drained away. After the acid-detergent fibre was treated with 72% H_2SO_4 for 3 hours, it was filtered to remove as much acid as possible, and washed with hot water (90^{o}C) until it was free from acid (pH of 7). The filter crucible was then placed in a drying oven at 100^{o}C for 2 hours and weighed after cooling in a desiccator overnight. The remaining residue was ashed in a muffle furnace at 500^{o}C for 2 hours. Then the crucible was cooled in a desiccator and weighed once completely cool.

Calculations

The acid-detergent lignin was determined from Equation:

$$ADL = \frac{S \times 100}{L}$$

Where L was loss upon ignition after 72% H_2SO_4 treatment

S was oven-dry sample weight

3.3 Fermentation Process

3.3.1 Selection of Simultaneous Tannin and Phytate Degrading *Aspergillus niger* and *Trichoderma harzanium* Isolate

Screening for simultaneous tannin and phytic acid degrading *Aspergillus niger* and *Trichoderma harzanium* isolates were carried out by simple agar plate method; an enrichment culture technique in minimum medium as adopted by Gustavo *et al*. (2001). The selection medium (g/l); $NaNO_3$-3.00, KH_2PO_4-1.00, M_gSO_4. $7H_2O$-0.50, F_eSO_4. $7H_2O$-0.01, agar- 30.0 was autoclaved at 121^oC for 15 min and supplemented with 1% tannic and phytic acid previously filter-sterilized through sterile Acrodisc 0.2 µm (Gelman Sciences) with pH- 4.0 adjusted using 100 mM NaOH. Strains of *Aspergillus niger* and *Trichoderma harzanium* isolated was then point inoculated and incubated at 30^oC for 72 h.

3.3.2 Preparation of Innoculum

The *Aspergillus niger* and *Trichoderma harzanium* spore inoculum were seperately prepared from the isolate that exhibit tannin and phytate utilization potential by adding 10 ml of sterile distilled water containing 0.2% Tween 80 (polyoxyethlene sorbitan monoolate) to a fully sporulated slant culture. The spores were dislodged by vigorous shaking and spore number estimated by direct microscopic enumeration using cell-counting haemocytometer. (Neubauer chamber; Merck, S.A., Madrid, Spain). The volume of spores suspension were adjusted to 2.75 x 10^7 spores/ml and the harvested *Aspergillus niger* and *Trichoderma harzanium* spores were used as

inoculums singly in the fermentation of the pretreated *Indigofera arrecta* seeds (Dapiya *et al*., 2010).

3.3.3 Fermentation of Sample

Each flask containing 10g of *Indigofera arrecta*seeds pretreated using various methods was mixed with mineral salt solution(g/l; KH_2PO_4, 2.5; KNO_3, 5.0; $MgSO_4$. $7H_2O$, 1.0; Na_2SO_4, 1.0; $FeCl_2$, 0.02; $ZnSO_4$. $7H_2O$, 0.0015; $CuSO_4$. $5H_2O$, 0.003 and $MnSO_4$, 0.001) in distilled water in the ratio 1:2 autoclaved and inoculated with $2.75x\ 10^7$ per ml spores suspension from each microbe separately and in co-culture.

3.4 Enzyme Extraction

The enzyme from each flask was extracted with 0.2 M acetate buffer; pH 5.5 (50 ml for 10 g of substrate) (Manjit *et al.,* 2008). Then, these flasks were kept on the rotary shaker at 150 rev/min for one hour. The contents were squeezed through a wet muslin cloth. The enzyme extract was centrifuged at 10,000*g* for 20 min at 4°C and the clear supernatant was used as crude enzyme.

3.4.1 Enzyme Activity Assays

Phytase and tannase activities was evaluated according to Hwang *et al*. (2005) and Mondal *et al*. (2001), using sodium phytate and tannic acid as substrates, respectively.

- Tannase Activity

Tannase activity was estimated by colorimetric method (Mondal *et al.,* 2001). The reaction mixture contained 0.3 ml of substrate tannic acid (0.5% w/v in 0.2 M sodium acetate buffer, pH 5.5) and 0.1 ml of enzyme. This reaction mixture was incubated at 30^{o}C for one hour. The enzymatic reaction was terminated by addition of 3 ml of BSA (Bovine Serum Albumin) solution (1mg/ml) which also precipitated the residual tannic acid. A control was prepared side by side using heat denatured enzyme. The tubes were then centrifuged (5, 000 x g, 10 min) and the precipitate was dissolved in 3 ml SDS-triethanolamine (1% w/v, SDS in 5% v/v, Triethanolamine) solution. One ml of $FeCl_3$ reagent (0.01 M $FeCl_3$ in 0.01N HCl) was added to the tube and was kept for 15 min at room temperature for stabilization of the color. Absorbance was read at 530 nm against the blank (i.e. without tannic acid). The specific extinction co- efficient of tannic acid at 530 nm was found to be 0.577 (Mondal *et al.,* 2001). Using this co-efficient, one unit of tannase activity is defined as the amount of enzyme required to hydrolyze 1Mm of substrate (tannic acid) in 1 min under assay conditions.

- Phytase Activity

The phytase activity was determined by the incubation of 1 ml of the culture sample at 37^{o}C for 30 min in a 0.2M sodium acetate buffer (pH 5.5) containing 0.5% sodium phytate (dodecasodium salt of phytic acid [$C_6H_6O_{24}P_6Na_{12}$]; Sigma, St. Louis, Mo. USA). The reaction was terminated by addition of 1 ml of trichloroacetic acid (15% [w/w]). After the addition of 2 ml of a coloring reagent (3.66 g of $FeSO_4 \cdot 7H_2O$, 0.5 g of $(NH_4)_6Mo_7O_{24} \cdot 4H_2O$, and 1.6 ml of concentrated H_2SO_4 in 50 ml of distilled water), the chilled sample in ice water was followed by incubation for 10 min at 30^{o}C. The released phosphate was determined at 750 nm using a UV-VIS spectrophotometer (JASCO International Co. Ltd., Tokyo, Japan). A standard curve for sodium phosphate was made for each independent experiment. One unit was defined as 1m mole of phosphate produced per min.

3.5 Determination of Proximate Composition

3.5.1 Ash Content Determination

The term ash refers to the residue left after combustion of the oven dried sample and is a measure of the total mineral content. Determination of the ash content was done according to (AOAC, 1990). Three different crucibles were preheated in a muffle furnace at about 525^{o}C. Each crucible was cooled in a desiccator and weighed.

One gramme of each sample was weighed into different crucible. The crucible and their contents were transferred into a muffle furnace set at 525^{o}C and allowed to stay for 1 hour. The weights of crucible and content were taken and recorded.

The percentage ash was calculated using the expression below

$$\% \, ash = \frac{weight \, of \, ash \, (g) \times 100}{weight \, of \, dry \, sample}$$

3.5.2 Determination of Moisture

The method employed for the determination of moisture content in the sample based on the measurement of the loss in weight due to drying at a temperature of about 105^{o}C as described by, AOAC, 1980).

Two grammes of sample was weighed into a clean watch glass dried in an air circulated oven at 105^{o}C to constant weight and its contents was cooled in a desiccator and reweighed.

The percentage moisture was obtained using the expression

$$\% \, moisture = \frac{loss \, in \, weight \, on \, drying \, \times 100}{Initial \, sample \, weight \, (g)}$$

3.5.3 Determination of Crude Lipid Content

The lipid content was determined by the procedure described by AOAC (1990). A clean dry round bottom flask containing antibumping granules was used. Exactly 210cm^3 of petroleum ether (80^{o}C) was poured into the flask fitted with soxhlet extraction unit. The weighed sample was transferred into the thimble, which was already fixed into the soxhlet extraction unit. Cold water was put on to circulate. The heating mantle was switched on and heating rate adjusted until the solvent was refluxed at a steady rate. Extraction was carried out for eight hours.

The sample was removed and dried to constant weight in an oven and reweighed.

$$\%\ lipid\ w/w = \frac{weight\ of\ lipid\ extracted \times 100}{weight\ of\ dry\ sample}$$

Where the weight of lipid extracted was given to be the loss in weight of the sample after extraction and drying with oven.

3.5.4 Determination of Crude Fibre

Crude fibre was determined by the method of AOAC (1990). Two grams of the sample was placed in a round bottom flask. 100ml of 0.25M H_2SO_4 was added and

the mixture boiled under reflux for 30minutes. The insoluble matter was washed several times with hot water until it was acid free. Thereafter, it was transferred into a flask containing 100ml of hot 0.312M NaOH solution. The mixture was boiled again under reflux for 30 minutes and filtered under suction; the insoluble residue was washed with hot water until it is base free. It was dried to constant weight (C2) and incinerated in a furnace at 550^0C for 2 hours. It was put off and allowed to cool down. It removed cooled in a desiccators and weighed (C3). The crude fibre was calculated as loss in weighed on ashing.

Weight of the original sample = W

$$\%\ crude\ fibre = \frac{C2 - C3}{W} \times 100$$

3.5.5 Determination of Nitrogen Content and Crude Protein

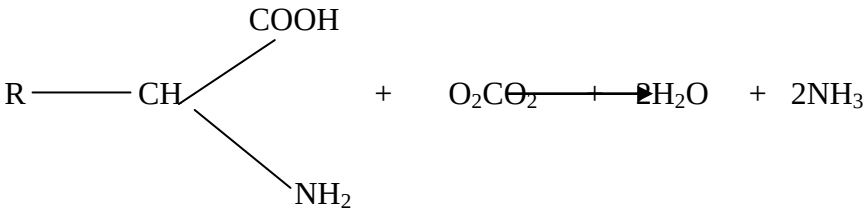
Principle: Nitrogen was used as an index of the protein termed crude protein as distinct from true protein.

The kjeldahl method of AOAC, (1990), was used.

Steps for Determination

A. Mineralization steps of organic substance in boiling sulphuric acid.

$$2H_2SO_4 \longrightarrow 2SO_2 + 2H_2O + O_2$$



$$2NH_3 + H_2SO_4 \longrightarrow (NH_4)_2SO_4$$

B. Distillation steps of ammonium sulphate after alkalization of the boric acid solution

$$(NH_4)_2SO_4 + NaOH \longrightarrow 2Na_2SO_4 + 2H_2O + 2NH_3$$

$$NH_3 + H_2O \longrightarrow NH_4OH$$

$$H_3BO_3 + NH_4OH \longrightarrow (NH_4)_3BO_3 + 3H_2O$$

C. Titration of ammonium with hydrochloric acid of standardized concentration

$(NH_4)_3BO_3 + HCl \longrightarrow 3NH_4Cl + H_3BO_3$

Procedure

Exactly 20g of the sample was weighed into 100ml Kjeldahl flask and a few antibumpimg granules were added. One gram of the mixed catalyst ($CuSO_4$ and K_2SO_4 in the ratio 8:1 respectively) and 15ml of concentrated sulphuric acid was added. The flask was placed on a kjeldahl digestion rack and heated until a clear solution was obtained. At the end of digestion the flask was cooled and the sample was quantitatively transferred to 100ml volumetric flask and made up to the mark with distilled water.

The digest (10ml) was pipetted into Marham semi micronitrogen steeland tube. Ten milliliters (10ml) of 40% NaOH solution was added cautiously. The sample was steam distilled liberating ammonia into a 100ml conical flask containing 10ml of 4% boric acid and a drop of methyl blue indicator until colour changed from pink to green. Thirty milliliters (30ml) volume of sample was collected. The content of the conical flask was titrated with 0.1M HCL. The end point was indicated by a colour change from green to pink and the volume (v) of the acid for each distillate was noted. Percentage nitrogen per sample was calculated using the expression below

$$\% \, Nitrogen = \frac{M \times v \times 14 \times 100 \times 100}{weight \; of \; sample \times 1000 \times 10}$$

Where M = Molarity of HCL

14 = atomic weight of Nitrogen 1000 = to convert to liter

100 = total volume of digest

100 = % conversion

10 = volume of digest taken

The crude protein was calculated as: % crude = 6.25× %N

6.25the conversion factor since it is assumed that protein contains 16% nitrogen.

3.5.6. Determination of Percentage Carbohydrate

The percentage carbohydrate was obtained by difference

Percentage carbohydrate = 100 – (% moisture + %protein + %fat + %ash +%crude fibre)

3.6 Determination of Mineral Content

The following minerals: magnesium, calcium, zinc, iron, potassium, and sodium were determined using atomic absorption spectrophotometry as described by AOAC, (1990).

Two grams of the sample was digested with 23ml of a mixture of HNO_3 (SG. 1.57) H_2SO_4 (SG. 1.84) and $HCIO_4$ (7:8:8 by volume). Then the cooled digest was diluted to 50ml with distilled water, filtered through a Whatman 45 filter paper and made up to 100ml with distilled H_2O in a glass volumetric flask. This final solution was for atomic absorption spectrophotometry.

3.7 Determination of Antinutritional Factors

3.7.1 Determination of Cyanide

The cyanide content was determined according to the method of AOAC (1984). Two grams of the sample was weighed into a flask and 100ml of distilled water added to it and allowed to hydrolyse for 1hr and the solution was steam distilled into 10ml of 2.5% NaOH solution. Twenty-five milliliters (25ml) of the distillate was pipetted into a conical flask, 2ml of 6M NH_4OH was added and 0.5ml of 10% KI solution was added, it was titrated with 0.02M $AgN0_3$ to a first turbid colour.

NB: 1ml of 0.02 M $AgN0_3$ = 1.08mg cyanide.

3.7.2 Determination of Tannins

i. Principle:

The tannin content was estimated spectrophotometrically by Folin-Denis method. The method is based on oxidation of the molecules containing a phenolic hydroxyl

group. The tannin and tannin- like compounds reduce phosphotungstomolybdic acid in alkaline solution to produce a highly coloured blue solution; the intensity of which is proportional to the amount of tannin and can be estimated against standard tannic acid solution at wavelength of 725 nm.

ii. Sample Preparation and Extraction of Tannins:

The sample was dried at 55°C and ground to pass through a sieve of 1mm diameter. Tannins extraction was done using 400mg ground sample in conical flask with 40 ml diethyl ether containing 1% acetic acid (V/V) and mixed to remove the pigment material. The supernatant was carefully discarded after 5 minute and 20 ml of 70% aqueous acetone was added and the flask wassealed with cotton plug covering with aluminum foil and kept in electrical shaker for 2 hours for extraction. Then it was filtered through Whatman filter paper No. 1 and sample was kept in refrigerator at 4°C until analysis.

iii. Standard Calibration Curve Preparation:

From the stock solution of tannic acid (0.5 mg/mI) using 0. 10, 20, 30, 40 and 50 µL in test tubes and volume was made to 1.0 ml. It gave a tannic acid concentration of 0, 5, 10, 15, 20 and 25µg respectively. Then 0.5 ml Folin reagent and 2.5 ml 20% sodium carbonate was added and the whole content was mixed properly and after 40 minutes, absorbance reading was taken at 725nm in spectrophotometer.

iv. Method:

Tannin was estimated according to the procedure of Makkar *et al*., (1998).

Fifty microlitres of tannins extract for each sample was taken in test tube and the volume made up to 1 .0 ml with distilled water. Then, 0.5 ml Folin Ciocalteu reagent was added and mixed properly. Then 2.5 ml 20% sodium carbonate solution added mixed and kept for 40 minutes at room temperature. Optical density was taken at 725 nm in spectrophotometer and concentration was estimated from the standard curve.

v. calculation:

$$\%\ \text{Tannin}\ (\text{mg}/100\text{g}) = \frac{\text{An} \times \text{C} \times \text{Df}}{\text{As} \times \text{W}} \times 100$$

Where: A_n = absorbance of test sample

A_s = absorbance of standard tannic acid

C = concentration of standard tannic acid (mg/ml)

Df= dilution factor =V_{ex}/ V_a

W =weight of test sample (mg)

V_{ex} =total volume of extract;

V_a = volume of extract analyzed

3.7.3 Determination of Phytic Acid

The phytic acid was determined using the procedure described by Lucas and Markakas (1975).

Two grammes of the sample was weighed into 250 mLs conical flask. 100 mL of 2% hydrochloric acid was used to soak each sample in the conical flask for 3 hrs and then filtered through a double layer of hardened filter papers. Fifty mililitres of each filtrate was placed in 250 mLs beaker and 107 mLs of distilled water added in each case to give proper acidity.

Ten mililitres of 0.3% ammonium thiocyanate solution was added into each solution as indicator and titrated with standard iron chloride solution, which contained 1.95mg iron per ml. The end point was brownish-yellow persisting for 5 min. The percentage phytic acid was calculated using the formula:

Calculation:

$$\%\ \text{phytic acid (mg/l00g)} = \frac{\text{Yx 1.19 x 100}}{\text{sample weight}}$$

Where: Y = titre value x 1 .95mg.

3.7.4 Determinition of Saponin

Saponins were determined by the gravimetric method of Brik *et al.*, (1963), as modified by Hudson and El-Defrawi (1979). Exactly 2g of dry ground sample was weighed into a thimble and transferred into the Soxhlet extraction chamber fitted with a condenser and a round flat bottomed flask. Some quantity of acetone enough to cause a reflux was poured into the flask for 3 hours by heating the flask on a hot plate and the solvent distilled off. This was the first extraction.

The methanol was enough to cause reflux. The saponin was then exhaustively extracted for three hours by heating the flask on a hot plate after which the final and initial weights of the saponin was noted.

$$\%\ Saponin = \frac{weigh\ of\ saponin}{weight\ of\ sample} \times 10$$

3.7.5 Determination of Trypsin Inhibitor Activity

This was done using the spectrophotometric method, described by Amtfield *et al*. (1985). A measured weight (10g) of the test sample was dispersed in 50ml of 0.5 M NaCl solution and stirred for 30min at room temperature. It was centrifuged and the supernatant filtered through Whatman No 42 filter paper. The filtrate was used for the assay. Standard trypsin was prepared and used to treat the substrate solution (N-a - benzoyl - Dl – arginine – p – anilide; BAPA). The extent of inhibition was used as a standard for measuring the trypsin inhibitory activity of the test sample extract. 2ml of the standard trypsin solution was added into a test tube containing 2ml of extract and 10ml of the substrate (BAPA). Standard trypsin solution (2ml) was added in another test tube containing only 10ml of the substrate. The latter served as the blank.

The content of the tubes were allowed to stand for 30min and then the absorbances of the solution measured spectrophotometrically at 410nm wavelength. One trypsin activity unit inhibited is given by an increase of 0.01 absorbance unit at 410nm.

$$Trypsinunitinhibited = \frac{Au}{As} \times 0.01 \times F$$

Where Au = Absorbance of test sample As = Absorbance of standard (uninhibited) sample

F = Experimental factor given as

$$\frac{Vf \times 1}{Va\ W}$$

Vf = Total volume of extract

Va = Volume of extract analysed

W = Weight of sample analysed

3.7.6 Determination of Lectins

The hemagglutinin content was determined using the spectrophotometric method of Onwuka (2005). One gramme of each sample was dispersed in a 10ml normal saline solution buffered at pH 6.4 with a 0.01M phosphate buffer solution. The solution was allowed to stand at room temperature for 30min and then centrifuge to obtain the extract.

To 0.1ml of the extract diluents in a test tube, 1ml of trypsinized rabbit blood was added. Control with the test tube containing only the blood cells was mounted and

both tubes were allowed to stand 4 hours at room temperature. Normal saline (1ml) was added to all the test tubes and allowed to stand for 10min after which the absorbance was read at 620nm.The test tube containing only the blood cells and normal saline served as blank.

The result is expressed as haemagglutinin units per milligram of the sample.

$$\text{Hemagglutinin unit/mg} = (b-a) \times F$$

Where b = absorbance of test sample solution

a = absorbance of the blank control

F = experimental factor given by:

$$F = (1/W \times V_f/V_a)\ D$$

Where W = weight of sample

V_f = total volume of extract

V_a = volume of extract used in the assay

D = dilution factor (if any)

The dilution factor D = 1ml to 10ml and 0.1ml out of the 10ml.

That is 10x 10/ 0.1 = 100ml.

3.7.7 Determination of Alkaloids

The gravimetric method of Horbone (1980) was adopted. Each sample (5g) was dispersed into 50ml of 10% acetic acid solution in ethanol. The mixture was shaken well and allowed to stand for 4 hours before filtering. The filtrate was evaporated to

one quarter (1/4) of its original volume. Drop wise conc. NH_4OH was added to precipitate the alkaloids. The precipitate was filtered off with a weighed filter paper. The precipitate was dried on the filter paper in the oven at 69^0 C for 30min and reweighed.

By weight difference, the weight of alkaloids determined and expressed by:

$$\%alkaloid = \frac{W2 - W1}{W} \; x \; \frac{100}{1}$$

Where W2 = weight of the precipitate before drying

W1 = weight of the precipitate after drying

W = weight of the sample

3.8 Determination of Amino Acid Profile

The amino acid profile in the sample was determined using methods described by Benitez (1989). The known sample was dried to constant weight, defatted, hydrolyzed, evaporated in a rotary evaporator and loaded into the Technicon sequential Multi-Sample Amino Acid Analyzer (TSM).

Defatting Sample:

The sample was defatted using chloroform/methanol mixture of ratio 2:1. The sample (4g) was put in extraction thimble and extracted for 15 hours in soxhlet extraction apparatus (AOAC, 2006).

Nitrogen Determination:

A small amount (200mg) of ground sample was weighed, wrapped in whatman filter paper (No.1) and put in the Kjeldhal digestion flask. Concentrated sulphuric acid (10ml) was added. Catalyst mixture (0.5g) containing sodium sulphate (Na_2SO_4), copper sulphate ($CuSO_4$) and selenium oxide (SeO_2) in the ratio of 10:5:1 was added into the flask to facilitate digestion. Four pieces of anti-bumping granules were added.

The flask was then put in Kjeldhal digestion apparatus for 3 hours until the liquid turned light green. The digested sample was cooled and diluted with distilled water to 100ml in standard volumetric flask. Aliquot (10m1) of the diluted solution with 10ml of 45% sodium hydroxide was put into the Markham distillation apparatus and distilled into 10ml of 2% boric acid containing 4 drops of bromocresol green/methyl red indicator until about 70ml of distillate was collected.

The distillate was then titrated with standardized 0.01 N hydrochloric acid to grey coloured Percentage Nitrogen = $\frac{(a-b) \times 0.01 \times 14 \times V \times 100}{W \times C}$

Where:

a. = Titre value of the digested sample

b. = Titre value of blank sample

v. = Volume after dilution (100ml)

W. = Weight of dried sample (mg)

C. = Aliquot of the sample used (10ml)

14. = Nitrogen constant in mg.

Hydrolysis of the sample

A known weight of the defeated sample was weighed into glass ampoule. Seven milliliter of 6NHCL was added and oxygen was expelled by passing nitrogen into the ampoule (this is to avoid possible oxidation of some amino acids during hydrolysis e.g methionine and cysteine). The glass ampoule was then sealed with Bunsen burner flame and put in an oven preset at $105^0C \pm 5^0C$ for 22 hours. The ampoule was allowed to cool before broken open at the tip and the content was filtered to remove the humin. It should be noted that tryptophan is destroyed by 6N HCL during hydrolysis.

The filtrate was then evaporated to dryness at 40^0C under vacuum in a rotary evaporator. The residue was dissolved with 5ml to acetate buffer (pH 2.0) and stored in plastic specimen bottles, which were kept in the freezer at 0^oC.

Loading of the hydrolysate into TSM analyzer

The amount loaded was between 5 to 10 microlitre. This was dispended into the cartridge of the analyzer. The TSM analyzer is designed to separate and analyze free acidic, neutral and basic amino acids of the hydrolysate. The period of an Analysis lasted for 76 minutes.

Method of Calculating Amino Acid Values from the Chromatogram Peaks.

An integrator attached to the Analyzer calculates the peak area proportional to the concentration of each of the amino acids. Alternatively, the net height of each peak produced by the chart recorder of TSM (each representing and Amino) was measured. The half-height of the peak on the chart was found and width of the peak on the half height was accurately measured and recorded. Approximately area of each peak was then obtained by multiplying the height with the width at half-height.

The norleucine equivalent (NE) for each amino acid in the standard mixture was calculated using the formula.

$$NE = \frac{\textit{Area of Norleucine Peak}}{\textit{Area of each amino acid}}$$

A constant S was calculated for each amino acid in the standard mixture:

Where S_{std} = NE_{std} x Molecular weight x μMAA_{std}

Finally, the amount of each amino acid present in the sample was calculated in g/16gN or g/100g protein using the following formula:

Concentration (g/100g protein) = NH x W@NH/2 x S_{std} x C

Where $C = \frac{\text{Dilution} \times 16}{\text{Sample Wt (g)} \times N\% \times 10 \times \text{Vol. loaded.}} \div NH \times W\ (nleu)$

Where: NH = Net height

W = Width @ half height

nleu = Norleucine

3.9Statistical Analysis

Mean and standard deviation (SD) of factors examined were calculated. The analytical technique used was factorial ANOVA (two way ANOVA). When there were statistically significant interaction effect, statistical comparison of cell means were explored using Duncan Multiple Range Test to separate the means.

CHAPTER 4

RESULTS

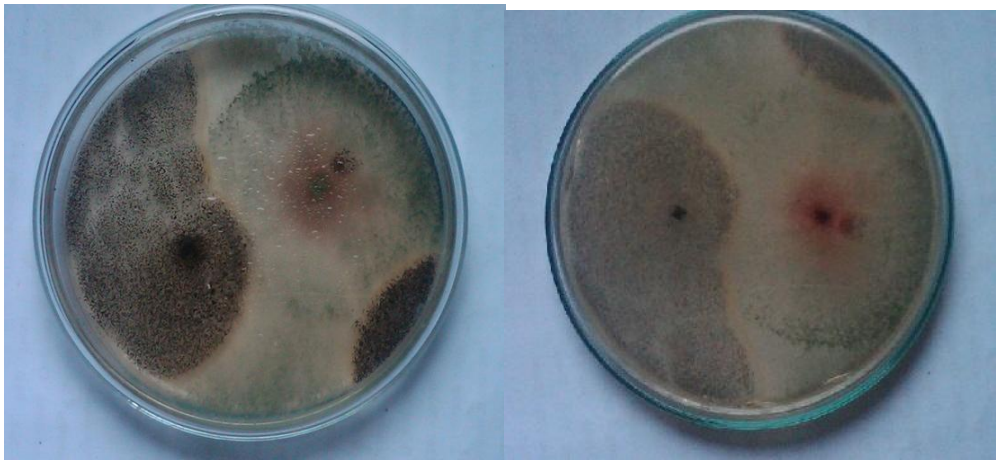
4.1 Screening and Compatibility Test of *Aspergillus niger*and *Trichoderma harzanium*.

Clear zones (colony) of the isolates of *Aspergillus niger* and *Trichoderma harzanium* as noted in Plate 1(A and B) respectively showed that the micro-organisms were capable of exhibiting simultaneous phytase and tannase activities on the agar plate supplemented with 1% tannic acid and sodium phytate.

Hyphal interactions and growth of *Aspergillus niger*and *Trichoderma harzanium* as shown in Plates 1C and the reverse in 1D were also studied to confirm the actual outcomes of the interactions.



A B



C D

Plate 1: Five- day old colony of A)mono-culture of *Aspergillus niger*, B) mono-culture of *Trichoderma harzanium*,C)Partial compatible mixed culture of *A. niger* and *T. harzanium* andD) reverse of mixed culture of both micro- organism onto PDA medium.

4.2 Lignin, Cellulose and Hemicellulose Content of Pretreated *Indigofera arrecta* Seeds

The changes in cellulose, lignin and hemicellulose content with respect to pretreatment of *Indigofera arrecta* seeds are shown in Table 4.1. There was a significant reduction (P□ 0.05) in the cellulose content of all pretreated *Indigofera arrecta* seeds as compared to the grinded sample in which the biologically pretreated sample gave the highest percentage reduction in the cellulose content (59%) followed by the combined steam and alkaline pretreatment with a reduction value of about 53%.

Significant reduction in lignin content was observed in all pretreated samples (P□ 0.05) when compared with the grinded sample. Biologically pretreated sample had the highest percentage reduction in lignin (72%) followed by the combined pretreatment and the alkaline pretreatment with percentage values of about 67% and 66% respectively.

Acid pretreated sample with a value of 5.58±0.42 had the highest percentage reduction of about 75% in the hemicellulose content.

Table 4.1 Effect of Pretreatment on the Hemicellulose, Cellulose and Lignin Content of Pretreated *Indigofera arrecta* seeds (%).

Pretreatment	Hemicellulose	Cellulose	Lignin
GdPS	22.11±0.03[a]	32.12±0.02[a]	26.32±0.21[a]
AlPS	18.51±0.44[b]	19.62±0.74[b]	9.12±0.12[d]
AcPS	5.58±0.42[e]	20.17±0.32[b]	18.3±0.13[b]
StPS	11.48±0.24[d]	13.45±0.38[d]	10.63±0.35[c]
BiPS	10.96±0.08[d]	13.13±0.16[d]	7.54±0.10[e]
CoPS	17.63±0.44[c]	15.06±0.07[c]	8.93±0.13[b]

Values are mean ± SD of triplicate determinations. Mean values with different superscripts down the column are significantly different ($p \leq 0.05$). Where GdPS= Grinded, AlPS= Alkaline pretreatment, AcPS= Acid pretreatment, StPS= Steam pretreatment, BiPS= Biological pretreatment and CoPS= Combined steam and alkaline pretreatment.

4.3 Chemical Composition of Grinded and Pretreated Seeds.

4.3.1 Proximate Composition of Pretreated *Indigofera arrecta* Seeds

Table 4.1 shows the proximate composition of the pretreated *Indigofera arrecta* seeds. It was observed that the biologically pretreated sample had the highest percentage protein, lipid and ash content with values of 28.050 ± 0.06, 8.867 ± 0.15 and 9.856 ± 0.14 respectively.

The steam pretreated sample had the lowest percentage crude protein, ash and total carbohydrate content with values of 16.27 ± 0.02, 2.65 ± 0.10 and 42.32 ± 0.02 respectively.

The combined steam and alkaline pretreated sample had the highest percentage total carbohydrate content and the lowest percentage crude fibre of 60.12 ± 0.29 and 5.60 ± 0.02 respectively. The percentage crude protein content did not differ significantly from that of the grinded sample.

The acid pretreated sample had the highest percentage crude fibre content (12.703 ± 0.05) while the alkaline pretreated sample had lowest percentage moisture content of 2.01 ± 0.01.

Table 4.2: Effect of Pretreatment on the Proximate Composition of *Indigofera arrecta* Seeds (%).

Pretreatment method	Moisture	Crude Protein	Crude fat	Ash	Crude fibre	Total carbohydrate
GdPS	6.150 ± 0.02[a]	22.277 ± 0.02[b]	8.150 ± 0.02[b]	3.867± 0.02[b]	10.730 ± 0.03[c]	50.627 ± 0.58[c]
AlPS	2.007 ± 0.01[d]	26.027 ± 0.05[ab]	7.113 ± 0.04[d]	3.067± 0.02[c]	7.347 ± 0.02[e]	54.467 ± 0.02[b]
AcPS	3.023 ± 0.02[d]	24.097 ± 0.08[ab]	7.040 ± 0.07[e]	2.983± 0.01[e]	12.703± 0.05[a]	50.060 ± 0.15[d]
StPS	3.633 ± 0.12[c]	16.267 ± 0.02[c]	7.827 ± 0.12[c]	2.650± 0.01[f]	11.383± 0.05[b]	42.317 ± 0.02[f]
BiPS	2.247 ± 0.01[e]	28.050 ± 0.06[a]	8.867 ± 0.15[a]	7.145± 0.01[a]	9.856 ± 0.14[c]	44.010 ± 0.10[e]
CoPS	4.167 ± 0.05[b]	22.980 ± 5.23[b]	7.373 ± 0.03[d]	3.060± 0.06[d]	5.597 ± 0.02[f]	60.117 ± 0.29[a]

Values are mean ± SD of triplicate determinations. Mean values with different superscripts down the column are significantly different ($p \leq 0.05$). Where; GdPS= Grinded sample, AlPS= Alkaline pretreated sample, AcPS= Acid pretreated sample, StPS= Steam pretreated sample, BiPS=Biologically pretreated sample and CoPS= combined steam and alkaline pretreatment.

4.3.2 Antinutritional Content of Pretreated *Indigofera arrecta*Seeds

Table 4.3 shows the antinutritional content of the grinded and pretreated *Indigofera arrecta* seeds. The biologically pretreated sample with values of 0.103 ± 0.03, 0.017 ± 0.01, 0.017 ± 0.01 and 2.157 ± 0.41had the highest reduction in tannin, lectin, trypsin and saponin content of the seeds respectively. The alkaline pretreated sample with values of 0.137 ± 0.03 and 0.063 ± 0.02 had the highest reduction in the phytate and oxalate content respectively when compared with the grinded.

There was an increase in the tannin, trypsin and lectin content of the acid pretreated sample while the tannin content of the steam pretreated sample also increased when compared with the grinded sample.

Table 4.3: Effect of Pretreatment on the Antinutritional Content of *Indigofera arrecta* Seeds (%).

Pretreatment method	% Tannin	% Phytate	%Alkaloid	%Saponin	%Oxalate	Trypsin (TIA/g)	%Lectin	Hydrogen cyanide (mg/100g)
GdPS	0.237± 0.04^{b}	0.303 ± 0.05^{a}	9.040 ± 0.11^{a}	4.173± 0.20^{a}	0.760± 0.01^{ab}	0.047± 0.01^{b}	0.033± 0.02^{a}	0.090±
AlPS	0.193± 0.02^{cd}	0.137 ± 0.03^{c}	2.313 ± 0.10^{b}	2.180± 0.25^{c}	0.063± 0.02^{d}	0.037± 0.02^{b}	0.020± 0.01^{c}	0.029± 0.02^{bc}
AcPS	0.267± 0.04^{a}	0.163 ± 0.01^{c}	0.670 ± 0.03^{e}	2.333± 0.07^{c}	0.137± 0.03^{c}	0.050± 0.03^{a}	0.040± 0.02^{a}	0.060± 0.03^{ab}
StPS	0.257± 0.01^{ab}	0.297± 0.15^{a}	0.817± 0.06^{d}	2.757± 0.01^{b}	0.447± 0.06^{b}	0.023± 0.01^{cd}	0.023± 0.01^{bc}	0.057± 0.02^{bc}
BiPS	0.103± 0.03^{e}	0.150± 0.03^{bc}	1.207± 0.10^{c}	2.157± 0.41^{d}	0.140± 0.04^{c}	0.017± 0.01^{d}	0.017± 0.01^{d}	0.027± 0.01^{c}
CoPS	0.143± 0.04^{d}	0.227 ± 0.04^{b}	0.157 ± 0.01^{f}	2.203± 0.21^{c}	0.073± 0.02^{cd}	0.033± 0.02^{bc}	0.027± 0.01^{bc}	0.027± 0.01^{c}

Values are mean ± SD of triplicate determinations. Mean values with different superscripts down the column are significantly different ($p \leq 0.05$).

Where; GdPS= Grinded sample, AlPS= Alkaline pretreated sample, AcPS= Acid pretreated sample, StPS= Steam pretreated sample, BiPS=Biologically pretreated sample and CoPS= Combined steam and alkaline pretreatment.

4.3.3 Mineral Content of Pretreated *Indigofera arrecta*Seeds

The contents of micro and macro elements varied in different pretreated samples. The biologically pretreatment with values of 21.813 ± 0.01, 20.03 ± 0.02, 10.557 ± 0.00, 50.641 ± 0.01, 10200 ± 0.01 and 4333 ± 0.02 for calcium, magnesium, iron, zinc, potassium, and phosphorus respectively, had the highest increase in the mineral composition of the seeds.

Alkaline pretreatment had the highest percentage increase of 4000 ± 0.01 in sodium content while the combined steam and alkaline pretreated sample had the highest reduction in iron, potassium and phosphorus content.

The acid pretreated sample had the lowest calcium and magnesium content and the steam pretreated sample had the lowest zinc content of about 1.63 ± 0.03.

There was a general decrease in magnesium and potassium content of the entire pretreated sample except for the biologically pretreated sample while the sodium content of all pretreated sample increased when compared to the grounded sample. On the other hand, the phosphorus content of the alkaline, acid, and steam pretreated sample did not differ significantly when compared with the grinded sample.

Table 4.4: Effect of Pretreatment on the Mineral Composition of *Indigofera arrecta* Seeds

Pretreatment method	Ca (ppm)	Mg(ppm)	Fe (ppm)	Zn (ppm)	K(ppm)	P(ppm)	Na(ppm)
GdPS	18.657± 0.00[c]	11.609± 0.02[b]	1.481± 0.00[e]	1.665± 0.01[e]	6600± 0.01[b]	2200± 0.01[b]	233± 0.01[d]
AlPS	16.389± 0.00[d]	9.347± 0.02[c]	2.638± 0.00[b]	2.311± 0.01[d]	2767± 0.03[d]	2300± 0.03[b]	4000± 0.01[a]
AcPS	11.154± 0.00[f]	5.075± 0.02[f]	1.566± 0.04[d]	2.829± 0.01[b]	2433± 0.03[e]	2000± 0.02[b]	1500± 0.02[c]
StPS	15.962± 0.00[e]	8.258± 0.05[d]	2.452± 0.04[c]	1.631± 0.03[f]	4333± 0.01[c]	2100± 0.02[b]	567± 0.01[d]
BiPS	21.813± 0.01[a]	20.03± 0.02[a]	10.557± 0.00[a]	50.641± 0.01[a]	10200± 0.01[a]	4333± 0.02[a]	2500± 0.05[b]
CoPS	19.320±	6.230±	1.259±	2.343±	500±	1267±	1467±

	0.00^{b}	0.02^{e}	0.00^{f}	0.01^{c}	0.01^{f}	0.01^{c}	0.01^{c}

Values are mean ± SD of triplicate determinations. Mean values with different superscripts down the column are significantly different ($p \leq 0.05$).

Where; GdPS= Grinded sample, AlPS= Alkaline pretreated sample, AcPS= Acid pretreated sample, StPS= Steam pretreated sample, BiPS= Biologically pretreated sample and CoPS= combined steam and alkaline pretreatment.

4.4 Pretreatment- fermentation combination

Based on the results illustrated on Table 4.1, 4.2, 4.3 and 4.4, the alkaline and biologically pretreated samples were chosen for further treatments.

4.4.1 Proximate Composition of the Alkaline and Biologically Pretreated Seeds as Affected by Mono and Co-Culture Fermentation

Table 4.5 shows the proximate composition of the treatment combinations on the seeds of *Indigofera arrecta.* The BiPS - *Tri h* treatment combination had significantly ($P<0.05$) lower percentage crude protein than each of the other five treatment combinations. The AlPS- *Asp.n* and *Tri.h* treatment combination had the highest percentage crude protein content with a value of 42.450 ± 0.70 which did not differ significantly from the BiPS – *Asp.n* treatment combination with a value of 42.277 ± 0.42. It was observed that the AlPS – *Asp.n* treatment combination had the highest percentage lipid composition while the BiPS – *Asp.n* and *Tri.h* significantly reduced the lipid content of the seeds. The BiPS – *Tri.h* treatment combination had the lowest moisture content (1.13 ± 0.01) while the AlPS – *Asp.n* and *Tri.h* treatment combination witha value of 4.187 ± 0.02 had the highest percentage moisture content. The highest percentage ash content was noticed in the BiPS – *Tri.h* treatment combination with a value of 9.903 ± 0.03 followed closely by the BiPS – *Asp.n* and BiPS – *Asp.n* and *Tri.h* treatment combination with values of 8.300 ± 0.01 and 8.193 ± 0.02 respectively. The lowest percentage ash content (3.820 ± 0.05) was recorded for the AlPS – *Asp.n* treatment combination.AlPS – *Asp.n* and AlPS – *Tri.h*

treatment combination with values of 15.086 ± 0.10and 15.180 ± 0.02 had the highest crude fibre composition while the BiPS – *Asp.n* and BiPS – *Tri.h* treatment combination had the lowest crude fibre content. The co-cultures of both AlPS and BiPS treatment combination with values 27.750 ± 0.13 and 28.157 ± 0.02 reduced the carbohydrate content of the seeds.

Table 4.5: Effect of Mono and Co-Culture Fermentation on the Proximate Composition of Alkaline and biologically Pretreated *Indigofera arrecta* Seeds

Treatment Combination	Moisture	Crude Protein	Crude fat	Ash	Crude fibre	Total carbohydrate
AlPS–*Asp.n*	1.990 ± 0.03[c]	38.937 ± 0.90[b]	9.363 ± 0.06[a]	3.820 ± 0.05[f]	15.086 ± 0.10[a]	30.320 ± 0.49[c]
AlPS–*Tri.h*	2.207 ± 0.02[b]	35.983 ± 0.98[c]	8.330 ± 0.07[c]	6.010 ± 0.03[e]	15.180 ± 0.02[a]	32.577 ± 0.46[b]
AlPS-*Asp.n* and *Tri.h*	4.187 ± 0.02[a]	42.450 ± 0.70[a]	8.787 ± 0.02[b]	6.563 ± 0.06[d]	10.393 ± 0.02[c]	27.750 ± 0.23[e]
BiPS-*Aspn*	2.233 ± 0.01[b]	42.277 ± 0.42[a]	8.287 ± 0.25[c]	8.300 ± 0.01[b]	9.847 ± 0.04[d]	28.993 ± 0.02[d]
BiPS–*Tri.h*	1.133 ± 0.03[e]	34.620 ± 0.27[d]	8.080 ± 0.03[d]	9.903 ± 0.03[a]	9.847 ± 0.04[d]	36.070 ± 0.06[a]
BiPS–*Asp.n* and	1.983 ±	38.067 ±	7.990 ±	8.193 ±	14.203 ±	28.157 ±

Tri.h	0.03^{d}	0.22^{b}	0.01^{e}	0.02^{c}	0.21^{b}	0.30^{e}

Values are mean ± SD of triplicate determinations. Mean values with different superscripts down the column are significantly different ($p \leq 0.05$). Where;

AlPS= Alkaline pretreated sample,BiPS= Biologically pretreated sample, *Asp.n = Aspergillus niger, Tri.h =Trichoderma harzanium*

4.4.2 Antinutritional Content of the Alkaline and Biologically Pretreated Seeds as Affected by Mono and Co-Culture Fermentation

Table 4.6 shows the antinutritional content of the AlPS and BiPS treatment combination. There was significant reduction ($p<0.05$) in the tannin content of theAlPS – *Tri.h* and AlPS – *Asp.n* and *Tri.h* treatment combination followed closely by the BiPS – *Asp.n* and *Tri.h* treatment combination with value of 0.048 ± 0.01. The AlPS – *Asp.n* and *Tri.h* treatment combination had the highest reduction in phytate content with a value of 0.073 ± 0.03. There was significant reduction in the alkaloid content of BiPS – *Tri.h* and BiPS – *Asp.n* and *Tri.h* treatment combination while the saponin content of the AlPS – *Asp.n* and *Tri.h* treatment combination had the highest significant reduction with a value of 1.403 ± 0.33. BiPS – *Asp.n* treatment combinationhad the highest percentage of oxalate content of 0.110 ± 0.02. AlPS – *Asp.n* had the highest percentage trypsin, lectin and hydrogen cyanide content with values 0.027 ± 0.01, 0.030 ± 0.01 and 0.047 ± 0.01 respectively while BiPS – *Asp.n* and *Tri.h* treatment combination had the highest percentage reduction in trypsin, lectin and hydrogen cyanide content when compared with the other five combinations.

Generally, there was significant decrease in in the alkaloid, saponin, trypsin, lectin and hydrogen cyanide content of the BiPS-*Asp.n* and *Tri.h* treatment combination while the AlPS-*Asp.n* and *Tri.h* significantly decreased the tannin and phytate content.

Table 4.6: Effect of Mono and Co-Culture Fermentationon theAntinutritional Content of Alkaline and biologically Pretreated *Indigofera arrecta* Seeds

Treatment Combination	% Tannin	% Phytate	%Alkaloid	%Saponin	%Oxalate	Trypsin (TIA/g)	%Lectin	Hydrogen cyanide (mg/100g)
AlPS–*Asp.n*	0.157± 0.01^{b}	0.120 ± 0.02^{ab}	2.113 ± 0.11^{a}	1.927 ± 0.14^{b}	0.027 ± 0.01^{cd}	0.027± 0.01^{a}	0.030 ± 0.01^{a}	0.047 ± 0.01^{a}
AlPS–*Tri.h*	0.287± 0.01^{d}	0.093 ± 0.02^{bc}	2.077 ± 0.08^{a}	1.920 ± 0.12^{b}	0.019 ± 0.01^{d}	0.019 ± 0.00^{ab}	0.019 ± 0.00^{b}	0.037 ± 0.00^{ab}
AlPS-*Asp.n* and *Tri.h*	0.028 ± 0.00^{d}	0.073 ± 0.03^{c}	1.990 ± 0.01^{a}	1.403 ± 0.33^{c}	0.017 ± 0.01^{d}	0.016 ± 0.00^{b}	0.017 ± 0.00^{b}	0.029 ± 0.00^{bc}
BiPS-*Aspn*	0.197 ± 0.02^{a}	0.150 ± 0.04^{a}	1.343 ± 0.32^{b}	2.870 ± 0.10^{a}	0.110 ± 0.02^{a}	0.013 ± 0.00^{b}	0.006 ± 0.00^{c}	0.020 ± 0.01^{cd}
BiPS–*Tri.h*	0.060 ± 0.01^{c}	0.107± 0.01^{bc}	0.867 ± 0.11^{c}	1.147± 0.20^{c}	0.077 ± 0.25^{ab}	0.013 ± 0.00^{b}	0.005 ± 0.00^{c}	0.017 ± 0.01^{cd}

BiPS–*Asp.n* and *Tri.h*	0.048± 0.01^{cd}	0.080± 0.01^{bc}	0.610 ± 0.09^{c}	0.543± 0.20^{d}	0.060 ± 0.04^{bc}	0.012 ± 0.00^{b}	0.004 ± 0.00^{c}	0.012 ± 0.00^{d}

Where; Values are means ± SD of triplicate readings down the column are significantly different ($p \leq 0.05$). Mean values with different superscripts

AlPS= Alkaline pretreated sample, BiPS= Biologically pretreated sample, *Asp.n = Aspergillus niger, Tri.h =Trichoderma harzanium*

4.4.3 Mineral Content of the Alkaline and Biologically Pretreated Seeds as Affected by Mono and Co-Culture Fermentation

The result on Table 4.7 shows the mineral composition of the AlPS and BiPS treatment combination. It was observed that BiPS – *Asp.n* treatment combinationhad the highest content of calcium and magnesium with values 24.438 ±0.00 and 23.986 ± 0.02 when compared with the other treatment combination. BiPS – *Asp.n* and *Tri.h* treatment combination had the highest content of iron with a value of 19.919 ± 0.00. BiPS – *Tri.h* treatment combination had the highest content of zinc and potassium with values 45.887 ± 0.58and 15,533.33 ± 57.74respectively. AlPS– *Asp.n* and *Tri.h* and BiPS – *Asp.n* treatment combination had the highest content of phosphorus which did not differ significantly from that of BiPS – *Tri.h* with a value of 5666.67 ±305.51. The BiPS– *Asp.n* and *Tri.h* treatment combination had the highest significant reduction in phosphorus and sodium content with values 2566.67 ±57.74and 2266.67 ±152.75respectively while AlPS– *Asp.n* and *Tri.h* had the highest increase in sodium content with a value of 7335.33 ±378.59when compared with the other five treatment combination.

Treatment Combination	Ca (ppm)	Mg(ppm)	Fe (ppm)	Zn (ppm)	K(ppm)	P(ppm)	Na(ppm)
AlPS–*Asp.n*	23.446 ± 0.00[b]	16.162 ± 0.02[e]	8.455 ± 0.00[d]	9.681 ± 0.01[c]	533.330 ± 57.74[c]	4300.00 ± 173.21[b]	6233.330 ± 57.74[b]
AlPS–*Tri.h*	14.606 ± 0.01[c]	15.774 ± 0.00[f]	5.248 ± 0.00[f]	3.010 ± 0.09[d]	12600.00 ± 100.00[b]	1800.00 ± 100.00[d]	5500.00 ± 100.00[c]
AlPS-*Asp.n* and *Tri.h*	11.193 ± 0.00[d]	19.851 ± 0.00[d]	5.578 ± 0.00[e]	2.217 ± 0.01[e]	15500.00 ± 100.00[a]	5833.330 ± 115.47[a]	7533.330 ± 378.59[a]
BiPS-*Aspn*	24.438 ± 0.00[a]	23.986 ± 0.02[a]	12.757 ± 0.00[c]	43.971 ± 0.00[b]	12866.670 ± 208.17[b]	5833.330 ± 115.47[a]	4433.330 ± 152.75[d]
BiPS–*Tri.h*	10.125 ± 0.00[e]	20.321 ± 0.01[c]	14.760 ± 0.00[b]	45.887 ± 0.58[a]	15533.330 ± 57.74[a]	5666.670 ± 305.51[a]	2700.00 ± 100.00[e]

BiPS–*Asp.n* and *Tri.h*	8.633 ± 0.00^{f}	20.425 ± 0.00^{b}	19.919 ± 0.00^{a}	45.657 ± 0.00^{a}	13300.00 ± 100.00^{b}	2566.670 ± 57.74^{c}	2266.670 ± 152.75^{f}

Table 4.7: Effect of Mono and Co-Culture Fermentation on the Mineral Content of Alkaline and Biologically Pretreated *Indigofera arrecta* Seeds

Where; Values are means ± SD of triplicate readings down the column are significantly different ($p \leq 0.05$). Mean values with different superscripts AlPS= Alkaline pretreated sample, BiPS= Biologically pretreated sample, *Asp.n = Aspergillus niger, Tri.h =Trichoderma harzanium*

4.4.4 Changes in Amino Acid Composition of Alkaline and Biologically Pretreated *Indigofera arrecta* Samples as Affected by Mono and Co- Culture Fermentation

Result of the amino acid profile determination reveals that seventeen amino acids werepresent in the alkaline and biologicallypretreated fermented *Indigofera arrecta* samples namely: lysine, histidine, arginine, aspartic acid, threonine, serine, glutamic acid, proline, glycine, alanine, cysteine, valine, methionine, isoleucine, leucine, tyrosine and phenylalanine (Table 4.8 and 4.9).The amino-acid tryptophan was either not present in the pretreated fermented seeds protein or not in detectable level.

Table 4.8 shows the essential amino-acid (EAA) composition of the AlPS and BiPS treatment combination. TheAlPS – *Tri h* treatment combination had the least values of methionine (0.59±0.13) and phenylalanine (3.17±0.06).AlPS – *Asp n* and *Tri h* treatment combination had significantly ($P < 0.05$) high values of valine (4.08±0.47) when compared with the other five treatment combinations. It also had the highest values of threonine, methionine and leucine content which did not differ significantly ($P < 0.05$) from that of BiPS –*Asp n* contents of the same amino acids while lysine and isoleucine content of BiPS –*Asp n* with values 4.29±0.39 and 3.06±0.03 respectively had values significantly ($P < 0.05$) higher than the other five treatment combinations.

Significantly high values of lysine, histidine, arginine, threonine, valine, methionine, isoleucine, leucine and phenylalanine were obtained in the AlPS co-culture

fermented samples increased appreciably compared with its monoculture treatment combination.

Table 4.9 shows the non-essential amino-acid (NEAA) composition of the AlPS and BiPS treatment combination.

Serine, prolineand cysteinein the BiPS- *Asp n* treatment-combination samples with values 3.43±0.38, 3.25±0.40 and 1.18±0.26 respectively were the only amino-acids which hadvalues significantly ($P < 0.05$) higher thanthe other five treatment combinations while AlPS - *Asp n* treatment combination had significantly ($P < 0.05$) high alanine (4.35±0.481) value.

BiPS- *Tri h* treatment combination with values 2.20±0.01, 1.97±0.08, 2.39±0.26, 2.53±0.03, 0.76±0.04 and significantly ($P < 0.05$) decreased the levels of serine, proline, glycine, alanine, cysteinerespectively.

AlPS – *Asp n* and *Tri h* treatment combination had significantly ($P < 0.05$) high values in glutamic acid (10.17±0.06) when compared with the other five treatment combinations. It also had the highest values in glycine content which did not differ significantly ($P < 0.05$) from that of BiPS –*Asp n* contents of the same amino acids while the high aspartic acid found in this treatment combination did not significantly differ from the other treatment combinations.

Aspartic acid,serine, glutamic acid, proline, glycine, cysteine and tyrosine increased appreciably in the AlPS co-culture fermented samples when compared with its monoculture treatment combination while that of the BiPS co-culture combination increased the level of aspartic acid when also compared with its monoculture combination.

Table 4.8Effect of Mono and Co-Culture Fermentation on the Essential Amino Acid Composition of *Indigofera arrecta* seeds

Essential Amino Acid Composition (g/100g Protein)						
Amino acid	AlPS *Asp.n*	AlPS-*Tri.h*	AlPS-*Asp.n* and *Tri. h*	BiPS-*Asp.n*	BiPS-*Tri.h*	BiPS-*Asp.n* and *Tri.h*
Arginine	5.71 ± 0.12^{c}	5.17 ± 0.08^{a}	6.70 ± 0.32^{a}	6.66 ± 0.50^{a}	4.98 ± 0.06^{c}	6.36 ± 0.21^{a}
Histidine	2.06 ± 0.02^{a}	2.13 ± 0.23^{a}	2.35 ± 0.04^{a}	2.49 ± 0.27^{a}	1.56 ± 0.18^{b}	2.37 ± 0.25^{a}
Isoleucine	2.12 ± 0.30^{cd}	2.48 ± 0.30^{bc}	2.91 ± 0.02^{ab}	3.06 ± 0.03^{a}	2.00 ± 0.02^{d}	2.76 ± 0.31^{ab}
Leucine	6.25 ± 0.02^{b}	7.23 ± 0.74^{ab}	8.31 ± 0.02^{a}	8.19 ± 0.86^{a}	6.08 ± 0.64^{b}	7.18 ± 0.06^{ab}
Lysine	3.09 ± 0.04^{b}	2.88± 0.04^{d}	3.55 ± 0.08^{b}	4.29 ± 0.39^{a}	2.43 ± 0.01^{d}	3.52± 0.33^{b}
Methionine	0.87 ± 0.15^{abc}	0.59 ± 0.13^{c}	1.16 ± 0.13^{a}	1.03 ± 0.19^{a}	0.64 ± 0.12^{bc}	1.01 ± 0.17^{ab}
Phenylalanine	3.51 ± 0.06^{a}	3.17 ± 0.06^{d}	4.61 ± 0.06^{a}	3.76 ± 0.30^{bc}	3.27 ± 0.33^{cd}	3.84 ± 0.28^{b}
Threonine	2.99 ± 0.02^{b}	2.54 ± 0.02^{bc}	3.60 ± 0.02^{a}	3.51 ± 0.37^{a}	2.50 ± 0.29^{c}	3.01 ± 0.01^{b}
Valine	3.24 ± 0.02^{ab}	2.98 ± 0.02^{b}	4.08 ± 0.47^{a}	3.70 ± 0.47^{ab}	2.63 ± 0.02^{b}	3.62 ± 0.80^{ab}

Total EEA	3.15 ± 1.80[a]	3.24 ± 1.91[a]	4.14 ± 2.19[a]	4.07 ± 2.15[a]	2.90 ± 1.67[a]	3.68 ± 1.93[b]

Values are means ± SD of duplicate readings. Mean values with different superscripts in the same column are significantly different ($p \leq 0.05$). Mean values were separated (ranked) using Duncan Multiple Range Test. Where; AlPS= Alkaline pretreated sample, BiPS= Biologically pretreated sample, *Asp.n = Aspergillus niger, Tri.h =Trichoderma harzanium*

Table 4.9Effect of Mono and Co-Culture Fermentation on the Non- Essential Amino Acid Composition of Pretreated *Indigofera arrecta* Seeds

Non-Essential Amino Acid Composition (g/100g Protein)						
Amino acid	AlPS-*Asp.n*	AlPS-*Tri.h*	AlPS-*Asp.n* and *Tri.h*	BiPS-*Asp.n*	BiPS-*Tri.h*	BiPS-*Asp.n* and *Tri.h*
Alanine	4.35 ± 0.48[a]	3.03 ± 0.03[cd]	3.88 ± 0.03[ab]	3.55 ± 0.38[bc]	2.53 ± 0.03[d]	3.24 ± 0.33[bcd]
Aspartic acid	8.55 ± 0.91[a]	8.22 ± 0.87[a]	9.73 ± 0.09[a]	9.03 ± 0.02[a]	7.75 ± 0.02[a]	9.59 ± 0.95[a]
Cysteine	0.97 ± 0.05[abc]	0.79 ± 0.19[bc]	1.16 ± 0.04[ab]	1.18 ± 0.26[a]	0.76 ± 0.04[c]	0.90 ± 0.15[abc]
Glutamic acid	8.72 ± 0.11[b]	9.01 ± 0.62[b]	10.17 ± 0.06[a]	9.47 ± 0.06[ab]	9.51 ± 0.71[ab]	8.87 ± 0.11[b]
Glycine	2.71 ± 0.01[bc]	2.70 ± 0.28[bc]	3.98 ± 0.36[a]	3.76 ± 0.02[a]	2.39 ± 0.26[c]	3.10 ± 0.01[b]

Proline	2.29 ± 0.08^{bc}	2.18 ± 0.08^{bc}	2.92 ± 0.07^{ab}	3.25 ± 0.40^{a}	1.97 ± 0.08^{c}	2.66 ± 0.58^{abc}
Serine	2.58 ± 0.02^{bc}	3.01 ± 0.01^{ab}	3.05 ± 0.38^{ab}	3.43 ± 0.38^{a}	2.20 ± 0.01^{c}	3.10 ± 0.332^{ab}
Tyrosine	2.25 ± 0.00^{b}	2.01 ± 0.11^{b}	3.30 ± 0.11^{a}	3.31 ± 0.35^{a}	1.85 ± 0.11^{b}	2.90 ± 0.45^{b}
Total NEAA	4.05 ± 2.97^{a}	3.87 ± 3.02^{a}	4.77 ± 3.31^{a}	4.29 ± 2.52^{a}	3.62 ± 3.62^{a}	4.29 ± 3.14^{a}

Values are means ± SD of duplicate readings. Mean values with different superscripts in the same column are significantly different ($p \leq 0.05$). Mean values were separated (ranked) using Duncan Multiple Range Test. Where; NEAA= Non-Essential AminoAcid, AlPS= Alkaline pretreated sample, BiPS= Biologically pretreated sample, *Asp.n = Aspergillus niger, Tri.h =Trichoderma harzanium*

4.5 Enzyme Assay

A time course study (0 to 7 days) in which all of the optimized parameters were used was conducted to determine when the enzyme yield was at the highest.

Table 4.10 shows changes in tannase activities in mono and co-culture combination of *Aspergillus niger*and *Trichoderma harzanium* of the alkaline and biologically pretreated *Indigofera arrecta* seeds assayed during incubation for 7 days. Maximum tannase activity was found in the simultaneous co-culture of *Aspergillus niger*and

Trichoderma harzanium of the alkaline and biologically pretreated seeds after 3days of incubation with an enzyme activity of 13.68µmol/min and 11.55µmol/min respectively. Enzyme activity of mono-culture of *A. niger*was maximum on the fourth day (96hrs) of incubation with an activity of 6.90µmol/min and 7.30µmol/min

	Treatment

while that of *T. harzianum* was also maximum on the fourth day of incubation, with a value 11.02µmol/min and 11.12µmol/min for the alkaline and biologically pretreated sample respectively. Maximum phytase production(Table 4.11) for co-culture fermentation, 3.26µmol/min and 5.97µmol/min for the alkaline and biological pretreated sample respectively were observed at 72hrs (3 days) of incubation. Maximum yield for monoculture of *Aspergillus niger* gave values of 3.12µmol/min and 5.68µmol/min for the alkaline and biological pretreated sample respectively while incubation with *Trichoderma harzanium* gave values of 3.22µmol/min and 5.76µmol/min for the alkaline and biological pretreated sample respectively after 4days of incubation.

	Alkaline Pretreated Sample				Biologically Pretreated Sample		
Day	***Asp.n***	***Tri.h***	***Asp.n/Tri.h***		***Asp.n***	***Tri.h***	***Asp.n/Tri.h***
1	0.73±0.03^{f}	2.47±0.05^{f}	1.52±0.03^{f}		1.76±0.01^{g}	1.76±0.01^{f}	1.99±0.01^{e}
2	2.46±0.06^{d}	5.05±0.04^{d}	5.51±0.01^{d}		2.78±0.01^{e}	4.06±0.06^{d}	4.48±0.04^{d}
3	3.72±0.08^{c}	7.42±0.01^{b}	13.68±0.17^{a}		5.04±0.01^{c}	5.16±0.07^{b}	11.55±0.01^{a}
4	6.90±0.16^{a}	11.02±0.06^{a}	10.15±0.03^{b}		7.30±0.01^{a}	11.12±0.02^{a}	9.56±0.01^{b}
5	4.87±0.19^{b}	6.78±0.01^{c}	7.80±0.14^{c}		6.20±0.12^{b}	4.27±0.01^{c}	5.52±0.01^{c}
6	2.53±0.04^{d}	2.83±0.06^{c}	2.26±0.06^{c}		4.06±0.07^{d}	2.24±0.04^{e}	5.22±0.02^{c}
7	2.01±0.01^{e}	2.01±0.01^{g}	1.54±0.01^{f}		2.26±0.01^{f}	1.54±0.05^{g}	4.76±0.38^{d}

Table 4.10Tannase Activity (µm Min^{-1}) of Alkaline and Biologically Pretreated Sample after 7 Days of Incubation with Mono and Co-Culture of *Aspergillus niger* And *Trichoderma harzanium*

Values are means ± SD of duplicate readings. Mean values with different superscripts in the same column are significantly different ($p \leq 0.05$). Mean values were separated (ranked) using Duncan

Multiple Range Test. Where; AlPS= Alkaline pretreated sample, BiPS= Biologically pretreated sample, *Asp.n = Aspergillus niger, Tri.h =Trichoderma harzanium*

Table 4.11 Phytase Activity (µm Min^{-1}) of Alkaline and Biologically Pretreated

	Treatment

Sample after 7 Days of Incubation with Mono and Co-Culture of *Aspergillus niger*And *Trichoderma harzanium*

	Alkaline Pretreated Sample				Biologically Pretreated Sample		
Day	***Asp.n***	***Tri.h***	***Asp.n/Tri.h***		***Asp.n***	***Tri.h***	***Asp.n/Tri.h***
1	0.33±0.01 f	0.34±0.00 f	0.33±0.01 f		0.36±0.01 f	0.44±0.01 g	0.88±0.01 e
2	1.11±0.01 c	1.12±0.01 c	1.72±0.01 c		1.33±0.01 d	1.41±0.01 d	2.49±0.01 b
3	1.21±0.01 b	1.21±0.01 b	3.26±0.01 a		3.19±0.04 b	3.25±0.06 b	5.97±0.01 a
4	3.12±0.01 a	3.22±0.01 a	1.86±0.01 b		5.68±0.01 a	5.76±0.01 a	2.55±0.01 b
5	0.88±0.01 d	0.89±0.02 d	1.49±0.01 d		1.53±0.01 c	2.62±0.01 c	1.52±0.01 c
6	0.52±0.03 e	0.42±0.00 e	0.80±0.01 e		1.04±0.01 e	1.18±0.01 e	1.03±0.01 d
7	0.22±0.02 g	0.24±0.01 g	0.24±0.01 g		0.34±0.01 f	1.00±0.01 f	0.88±0.01 e

Values are means ± SD of duplicate readings. Mean values with different superscripts down the column are significantly different ($p \leq 0.05$). Where; AlPS= Alkaline pretreated sample, BiPS= Biologically pretreated sample, *Asp.n = Aspergillus niger, Tri.h =Trichoderma harzanium*

CHAPTER 5

DISCUSSION

Invitro interaction of the two different fungi grown adjacently as shown in Plate 1C and the reverse in 1D showed that the hyphal growth of *Aspergillus niger*and *Trichoderma harzanium*interacted mutually at the touching point but did not precede long distance into each other as much as mutual compatible cultures should.Mycelium growth and synergetic or antagonistic characteristics of *Aspergillus niger*and *Trichoderma harzanium* shown on Plate 1(A and B) were categorized based on literature review (Porter, 1924; Skidmore and Dickinson, 1976; Molla *et al*., 2001; Alam *et al*., 2003). The interaction of *Aspergillus niger* with*Trichoderma harzanium* was identified as partial intermingling.

Lignocellulose matter is highly recalcitrant and needs suitable pretreatment to degrade lignin and ease the way for cellulose and hemicellulose digestion of the seed. In the plant cell wall the lignin forms a matrix that surrounds cellulose and hemicellulose. Lignin has long been recognized as the major barrier to rumen digestion of cell wall structural polysaccharides (Kerley *et al.,*1988); removal of lignin by chemical and biological treatment enhances fibre digestibility but to different extent (Jung *et al.,*1992; Karunnananda *et al.,* 1992). *Degradation* of lignin is a prerequisite for hydrolysis of cellulose and hemicelluloses and thus for the usage of the main carbon and energy sources by the fungi. According to Mosier *et al*., (2005), the pretreatments areefficient if their action releases the lignin, hemicellulose and cellulose, the polymers which constitute the lignocelluloses (Weil *et al*., 1994).

The seedof *Indigofera arrecta* is known for its hardness. Hardness and total fibre content are related. From studies, when the total fibre content is over 9%, the seeds are classified as hard and practically inedible (Melgarejo*et al*.,2000). Grinding and milling are the primary physical pretreatment operations but in combination with other methods can produce more desirable results as observed in this study. Findings showed a significant decrease ($P<0.05$) in the lignin, hemicellulose and cellulose content of all pretreated sample as shown in Table 4.1.

The alkaline pretreatment and combined steam and alkaline pretreatment also greatly reduced the amount of lignin composition in the sample and this can play a significant role in exposing the cellulose to hydrolysis as the major action of NaOH is to degrade the lignin by breaking ester bond cross linking, thus creating porosity in biomass (Rebecca *et al.,* 2007). The result of the combined steam and alkaline pretreated sample showed that for good results, temperature elevation is not necessary in addition to alkaline pretreatment. It appears that harsh conditions are not required for corresponding pretreatment as in the case of pretreatment of *Indigofera arrecta* seeds as it did not cause any significant decrease in lignin and hemicellulose composition when compared to the alkaline pretreated sample but rather further solubilized part of the cellulose content. It is possible that part of the already formed fermentable sugars is simultaneously being degraded during the pretreatment time. From the results, conversely to acid or steam processes, alkaline-

based methods are more effective for lignin solubilization exhibiting only minor cellulose and hemicellulose solubilization.

Steam pretreatment significantly decreased (P□ 0.05)the cellulose and hemicellulose content of the seed (Table 4.1).Steam pretreatment affects cell structure by solubilization of hemicellulose. The advantage of the steam explosion method is that no chemicals are used which can reduce both environmental and economic cost of the process. A major disadvantage is that the lignin-cellulose matrix is incompletely disrupted. The glucose liberated from the cellulose can be degraded to the 5-hydroxymethyl furfural and formic acids, while the pentoses liberated from the hemicellulose can be converted to furfural and formic acid. These products act as inhibitors in the later stages of enzymatic hydrolysis and fermentation (Klinke *et al*., 2003).

From the result on Table 4.1, the most promising pretreatment option for hemicellulose breakdown is dilute acid, which significantly increases susceptibility to hydrolysis for cellulose. However, it produces fermentation inhibitors, and acid utilization is a critical and expensive issue, resulting in a waste disposal problem, and thereby requiring use of expensive corrosion-resistant equipment. Apart from furans and weak acids, a wide range of phenolic compounds formed from lignin

breakdown and carbohydrate degradation during acid hydrolysis, most of which were considered potential fermentation inhibitors as well.

The biological pretreated sample had the highest reduction in lignin content. The ligninolytic fungi, which preferentially attack the lignin, was employed in the pretreatments due to their capacity to liberate oxidases and hydrolases (Perez *et al*., 2002), which are specialized enzyme systems to attack, depolymerize and degrade the lignocellulosic matrix to release the lignin and other polymers. There was also significant decrease in the amount of cellulose and hemicellulose recovered as it got utilized by the organism for its growth. The use of the fungi has the added advantage that it increases the crude protein content of the residues and avoids the problems associated with chemical pretreatment.

Chemical Composition of the Pretreated Samples

From this study, it was observed that there was a significant difference (P□ 0.05) in the proximate composition between the different pretreatments methods (Table 4.2).The biologically pretreated sample had the highest protein, lipid and ash content as the white-rot fungi was able to degrade recalcitrant molecules like lignin thereby increasing accessibility of the microorganisms. The increase in protein content could also be attributed to the increase in the biomass brought about by the microorganisms which resulted in the increase in crude fibre content. Increase in the

ash content reflected in the increase in the concentrations of some major minerals like P and Ca while increase in the lipid content could be as a result of solubilization of some component of the sample. The decrease in carbohydrate content could be as a result of degradation of cellulose and hemicellulose fraction by the micro-organism. Compared to other pretreatment alternatives, the fungal treatment requires a long pretreatment time, but the energy requirement of the process is low and the treatment conditions are mild (Sun and Cheng 2002). Wan and Li (2011) also stated that although considerable improvement of cellulose digestibility has been reported from lignocellulosic biomass pretreated with white rot fungi, long pretreatment time and loss of holocellulose still remain as major weaknesses of biological pretreatment.

The decrease in the protein content of the steam pretreated sample could be as a result of the effective elimination of much of the heat-sensitive indigenous micro flora (El Faki *et al*., 1991).

Lignin is removed only to a limited extent during steam pretreatment but is redistributed on the fiber surfaces as a result of melting and depolymerization/repolymerization reactions (Li *et al*., 2007).The removal and redistribution of hemicellulose and lignin increase the volume of the pretreated sample and this could be responsible for the high fibre content of the steam pretreated sample.

Decrease in the lipid content of all the pretreated sample apart from the biologically pretreated sample could be as a result of the fact that there isneed for the pretreated biomass to be washed with water to remove materials that are inhibitory to microbial growth and fermentation as this could also lead to washing off of the soluble lipid component of the sample.

A significant reduction (P □ 0.05) in the anti-nutrient composition was also observed after pretreatment. The high reduction in tannin, lectin, trypsin and saponin content of the biologically pretreated sample could be as a result of the ability of the micro-organism to secrete enzymes that could degrade these antinutrients. The reduction in the antinutritional factors of the other pretreatment techniques may be attributed to the action of chemicals which could inactivate some of the antinutrients, leaching out of soluble antinutrients during washing of the pretreated sample and also due to the known heat labile nature of some antinutrients.

Potassium was found to be the most abundant mineral in the ground sample (Table 4.4). The abundance of potassium is in close agreement with the observation of Aremu *et al*., (2006) that potassium was the most predominant mineral in agricultural products.

The high mineral content of the biologically pretreated sample could be as a result of the ability white-rot fungi to degrade recalcitrant molecules like lignin. This increase

could also be attributed to the ability of the fungi to degrade the antinutrients or the complexes they form with these ions led to the significant increases in their concentrations.

There was significant reduction in the calcium content of the acid and steam pretreated sample. One plausible explanation is that calcium ions extensively cross-linked lignin molecules and prevented lignin solubilization thereby decreasing the amount of available calcium ion in the sample. The decrease in mineral content for most of the pretreated sample could be as a result of the washing process of the pretreated seeds in order to remove materials that are inhibitory to microbial growth and fermentation.

Based on these results, the alkaline and the biologically pretreated samples were selected for further fermentation treatments.

Pretreatment -Fermentation Combination

Increase in crude protein on Table 4.5of AlPS – *Asp.n* and *Tri.h* and BiPS – *Asp.n* treatment combination could be attributed to the addition of mycoprotein (single cell protein) which may be due to the addition of non-protein nitrogen from amide and nucleic acid synthesis by fungal cells (Oboh, 2006). This increase could also be as attributed to the ability of the micro-organism to secrete some extracellular enzymes (proteins) which degrade the materials during fermentation, Oseni and Ekperigin

(2007). Increase in the ash content of BiPS-*Tri.h*treatment combination reflected increase in the concentrations of some major minerals. This increase could also be due to contribution by fermenting micro-organism (El Faki *et al*., 1991). It is also possible that there was breakdown of some organic molecules within the fermented sample and consequently the release of the mineral elements from the organic phase into the inorganic phase.

Contrary to expectation, there was an increase in crude fibre content (Table 4.5) of the BiPS - *Asp.n* and *Tri.h* treatment combination. This may be due to the formation of resistant starch together with condensed tannin-protein complex which could contribute to the increase (Perez-Hildalgo *et al*., 1997). It could also be due to the production of some dietary fibre-rich substances such as component parts of mycoprotien deposition in the fermented sample.

The decrease in the carbohydrate content of both AlPS-*Asp.n* and *Tri.h* and BiPS-*Asp.n* and *Tri.h* treatment combination could be attributed to the ability of fermenting micro floral to hydrolyze and metabolize carbohydrate as carbon source in order to synthesize cell biomass (Madigan *et al*., 2002).

The significant decrease in lipid content for BiPS-*Asp.n* and *Tri.h* treatment combination when compared with the other treatment combinationmay be an indication that the fungus utilized the lipid as source of carbon and energy for growth and perhaps for the synthesis of protein.

The significant decrease in in the tannin, phytate,alkaloid, saponin, trypsin, lectin and hydrogen cyanide contenton on Table 4.6 of the AlPS and BiPS co-culture treatment combination as compared to the mono culturetreatment combination could be attributed to the fact that the synergistic interactions between the compatiblemicroorganisms in co-culture fermentation might have resulted in degradation of the antinutrients.

The significant reduction in magnesium, iron and zinc content on Table 4.7 of all AlPS treatment combination could be as a result of leaching or effect of the fermentation inhibitors on the micro-organisms. Increase in mineral content for some of the treatment combinations probably mean that fermentation with the funguscaused the breakdown of the antinutrients or the complexes they form with these ions leading to significant increases in their concentrations. The reduction in the mineral content during fermentation could also be attributed to increase in biomass. Minerals are also used by microbes as nutrients source during growth.

The amino acid profile shown in Table 4.8 and 4.9 showed that the AlPS and BiPS treatment combinations affected the overall amino acid profile of the seeds.The essential amino acids are lysine, histidine, arginine, threonine, valine, methionine,

isoleucine, leucine and phenylalanine while the non-essential amino acids include glycine, alanine, serine, cysteine, tyrosine, aspartic acid, glutamic acid and proline. Since asparagine and glutamine do hydrolyze to their acids (aspartic acid and glutamic acid respectively) under acidic or basic medium, they might have been present in the seed protein but converted to their acids (Lehninger, 1975).The relatively higher levels of aspartic acid and glutamic acid with the absence of asparagine and glutamine in this study for the AlPS and BiPStreatment-combination of the seeds could be attributed to these conversions. The high amino acid content of some of the treatment combination samples could be due to the fact that some of the microorganisms are capable of synthesizing amino acids themselves. The result shows that the samples contain nutritionally useful quantities of most of the essential amino acids.It was observed that some of treatment combinations decreased the amino acid composition of the alkaline and biologically pretreated seedsand these findings could be attributed to the complexmetabolic process during which the lipids, carbohydrates, and storage proteins within the seedsare broken down.

There was a significant increase in the non-essential amino acids over the essential aminoacids in all the samples. Similar increase in non-essential amino acids over essential aminoacids was reported by Muhammad and Oloyede, (2009) in *Terminalia catappa* seed mealfermented by *Aspergillus niger*. This may be an indication that the non-essential amino acidsand nucleic acids have been synthesized

at the expense of the essential amino acidsthus the reduction in the essential amino acids (Muhammad and Oloyede, 2009).

Enzyme Production

Time course of tannase and phytase production was studied to determine the optimum incubation time required for maximum tannase production.Enzyme activity was initially detected at 24hr and progressively increased with time. The organism had different time for optimal phytase and tannase yield. Decreased enzyme yield on prolonged incubation could be due to reduced nutrient level of the medium. It has been reported that tannase activity (Table 4.10) decreases after reaching maximum level, due to inhibition or degradation of enzyme (Suseela *et al*., 1983). Lekha and Lonsane (1997) and Sabu *et al*. (2005) also reported maximum extracellular tannase production by *A.niger* in 96 hrs. Similar observations have been reported for tannase production by many researchers (Banerjee *et al*., 2007; Costa *et al*., 2008; El-Fouly1 *et al*., 2010; Selwal *et al*., 2011).

For phytase production (Table 4.11), simultaneous co-culture incubation of *Aspergillus niger*and *Trichoderma harzanium* ofthe alkaline and biological pretreated sample showed the highest activity after 72hrs (3 days)whereas maximum yield for monoculture of *Aspergillus niger*and *Trichoderma harzanium*was after

4days of incubation.Short incubation period for enzyme production offers the potential for inexpensive production of enzyme (Sonjoy *et al.*, 1995).

Further increase in incubation time also resulted in a decline in enzyme yields. This could be due to the onset of stationary phase in fungal culture (Tarafdar *et al.*, 2003). Isoenzymes released by different fungi may affect the efficiency per unit of enzyme to hydrolyze phytic acid. This could also be attributed to the depletion of micro and macronutrients in the fermentation medium, which effected directly on the pH value of the medium and resulted in the inactivation of enzyme synthesis machinery with the passage of time (Nochure *et al*., 1993). In addition, the medium components were initially more susceptible to fungal digestion and made a rapid rise in enzyme biosynthesis but with the prolongation of incubation time, the susceptible portions were completely hydrolyzed by microorganisms which inhibited the enzyme secretion pathway (Reese, E., 1977).

The high phytase and tannase activity of the co-culture incubation of both pretreated samples could be as a result of the synergistic interaction between the two compatible strains of microorganisms thereby increasing the rate and amount of enzyme produced.

The general decrease in both phytase and tannase activity of the alkaline pretreated sample as compared with the biologically pretreated sample could be due to incorporation of salts into the biomass during the pretreatment reactions which might

have led to the formation of degradation products and release of natural biomass fermentation inhibitors.

CHAPTER 6

Summary, Conclusion and Recommendation

6.1 Summary

This work has revealed that:

i. The mycelium growth and synergetic interaction of *Aspergillus niger*and *Trichoderma harzanium* was identified as partial intermingling.

ii. Pretreatment has significant effect on the hemicellulose, cellulose and lignin composition of *Indigofera arrecta seeds.* Biological pretreatment with a value of 7.54 ± 0.10, greatly decreased the tannin content of the seed while biological and alkaline pretreatment increased the protein content of the seeds with values of 28.050 ± 0.062 and 26.027± 0.027 respectively. Most of the pretreatment methods reduced the antinutrients content of the seed while biological pretreatment significantly increased all the mineral content of the seed.

iii. Mono and co-culture fermentation increased the protein content of the pretreated seed and decreased the antinutrients content of the seed. It also resulted to significant changes in the mineral content.

iv. Co-culture fermentation increased the enzyme activity and decreased the incubation time required for maximum tannase and phytase production as compared to the mono culture incubation.

6.2 Conclusion

The pretreatment, fermentation and combination of both treatments decreased some of the antinutrients, lignin, hemicellulose and cellulose content of the seeds. It also increased some of the proximate, mineral and amino-acids contents of the seeds of *Indigofera arrecta*. These treatments may therefore be used to increase the nutritive value of *Indigofera arrecta* seeds for use as animal feeds.

Diverse advantages have been reported for most of the pretreatment methods, which make them interesting for agricultural applications however, none of those can be declared a "winner" because each pretreatment has its intrinsic advantages and disadvantages. While methods such as dilute acid, dilute alkaline, steam, combined steam and alkaline pretreatment are capital-intensive, lead to formation of sugar degradation products and fermentation inhibitors, some other methods such as biological pretreatment are extremely slow.

Strain compatibility is the determining factor for successful mixed culture fermentation. In the present study, observations revealed that the simultaneous mixing of the cultures did not only greatly decrease the antinutrients but also increase the crude protein content of the pretreated seed suggesting it to have good potentials as protein source. It also increased the phytase and tannase production capacity of the two micro-organisms.

The use of different treatment combination can be used to improve the nutritional value of animal feed.

6.3 Recommendations

- There is therefore a need for optimization of pretreatment conditions of the*Indigofera arrecta*seeds to ensure effective degradation of the recalcitrant lignocellulosic materials with the production of minimal fermentation inhibitors.

- Incorporation of phytase and tannase enzymes into the pretreated seeds to enhance the nutritional value in addition to yield improvement by the application of biotechnology.

- Understanding the combination of the pretreatments and the structural changes of the seeds during the pretreatment.

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Appendix 1: Standard Curve of Sodium Phosphate for Phytase Activity Assay

