EFFECTS OF *Aspergillus niger* AND *Trichoderma viride* ON THE NUTRIENT COMPOSITION OF GROUNDNUT HUSK

BY

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NIGERIA.

AUGUST, 2014.

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(B. Sc Biochemistry, A.B.U 2009)
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A THESIS SUBMITTED TO THE SCHOOL OF POSTGRADUATE STUDIES, AHMADU BELLO UNIVERSITY, ZARIA IN PARTIAL FULFILLMENT OF THE REQUIREMENT FOR THE AWARD OF MASTER OF SCIENCE (M. Sc) DEGREE IN BIOCHEMISTRY.

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AUGUST, 2014.

DECLARATION

I declare that the work in this thesis entitled "Effects of *Aspergillus niger* and *Trichoderma viride* on the Nutrient Composition of Groundnut Husk," has been carried out by me in the Department of Biochemistry under the supervision of Prof H. M. Inuwa and Dr P. A. Wuyep. The information derived from literature has been duly acknowledged in the text and a list of references provided. No part of this thesis was previously presented for another degree or diploma at any other institution.

_____ _____

ALIYU NAFISATU Date

CERTIFICATION

This project thesis entitled "EFFECTS OF *Aspergillus niger* AND *Trichoderma viride* ON THE NUTRIENT COMPOSITION OF GROUNDNUT HUSK" by Aliyu Nafisatu meets the regulation governing the award of the degree of Masters of Science of the Ahmadu Bello University, Zaria and is approved for its contribution to knowledge and literary presentation.

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DR P. A. WUYEP Member Supervisory Committee	_____ (Signature)	_____ Date
PROF I. A. UMAR Head of Department	_____ (Signature)	_____ Date
PROF A. A. JOSHUA Dean, School of Post Graduate Studies	_____ (Signature)	_____ Date

DEDICATION

This thesis is dedicated to Almighty Allah (S W T) for the gift of life, guidance and sound health.

ACKNOWLEGEMENT

All praises and thanks are for Almighty Allah, the Merciful, the Creator of the universe and source of all knowledge and wisdom, Who blessed me with good health, thoughts, talented teachers, helping friends and opportunity to complete this study. I offer my greetings of Salam to the Holy Prophet (Peace be Upon Him), whose moral and spiritual teachings enlightened my heart, mind and flourished my thoughts towards achieving high ideas of life.

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ABSTRACT

The effects of *Aspergillus niger* and *Trichoderma viride* on the nutritional composition of pre-treated groundnut husk (GH) with *Ustilago maydis* spores (biological, $_{B}$GH) and 2M NaOH (chemical, $_{C}$GH) were studied. The pre-treated groundnut husk were subjected to monoculture, co-culture and crude enzyme extract fermentation using *A. niger* and *T. viride* so as to evaluate its lignin and cellulose contents, quantify cellulase activities; determine proximate, anti-nutrient, mineral and amino acid contents. Enzymatic activities in all the pretreated GH increased steadily from day one and peaked off at day seven. The biological pretreated GH showed significant ($p<0.05$) increase in total cellulase and endoglucanase yield (activity) than the alkali pretreated GH. The proximate analysis of the cellulosic residues differed from one treatment to another, with chemical pretreated GH on which *T. viride* ($_{C}GH_{T.\ viride}$) was inoculated being the best as a result of 73% increase in protein with significant ($p<0.05$) decrease in anti-nutritional content; phytate (51.22g/100g) and alkaloid (7g/100g). Significantly ($p<0.05$) higher values of phosphorous, zinc and magnesium were observed for all fermented GH compared to untreated GH (control), while significantly ($p<0.05$) lower values of copper, sodium and iron were observed for all fermented GH compared to control. Except for aspartate, there was general decrease in amino acid content of all pretreated GH compared to the standard feed protein used to standardize the Amino acid analyser. Even though, the amino acid content of $_{C}GH_{A.\ niger}$ were slightly higher than $_{C}GH_{T.\ viride}$. The results of this study indicate that *T. viride* is a potentially viable microorganism for the production of enriched and safe animal feed.

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ABBREVIATIONS

SmF – Sub-merged fermentation

SSF – Solid state fermentation

GH – Groundnut husk

$_{B}$GH'- Biological pretreated groundnut husk (GH)

$_{B}$GH $_{A.\ niger}$ - Biological pretreated GH on which *A. niger* was inoculated

$_{B}$GH $_{T.\ viride}$ - Biological pretreated GH on which *T. viride* was inoculated;

$_{B}$GH $_{cc}$ - Biological pretreated GH on which both organisms was inoculated

$_{B}$GH$_{enz}$ - Biological pretreated GH on which crude enzyme from both organisms was inoculated

$_{C}$GH - Chemical pretreated GH

$_{C}$GH $_{A.\ niger}$ - Chemical pretreated GH on which *A. niger* was inoculated

$_{C}$GH $_{T.\ viride}$ - Chemical pretreated GH on which *T. viride* was inoculated

$_{C}$GH $_{cc}$ - Chemical pretreated GH on which both organism were inoculated

$_{C}$GH$_{enz}$ - Chemical pretreated GH on which crude enzyme from both organisms was inoculated

FPU – Filter paper unit

CMC – Carboxymethyl cellulose

CHAPTER ONE

1.0 INTRODUCTION

1.1 AGRICULTURE AND AGRO ALLIED INDUSTRIES

The development of new technologies in agriculture has resulted in an increase in production of many foods. It has given rise to wastes in large quantities thus leading to environmental pollution associated with several health hazards to mankind. Industrialization has helped in development of many types of food products from agricultural produce and there is a wide spread of food processing industries all over the world which produce a variety of foods from cereals, meat, milk, fruits and vegetables. In general, food wastes contain more than 70-80% water in addition to oil and biodegradable organic matters (Yun *et al*., 2000). The storage of food wastes is difficult for a long period before dumping due to sanitary reasons and putrefied odour associated with anaerobic decomposition. The waste material from food processing industries contains some useful organic substances like polysaccharides, sugars, acids, starch, pectic substances and other compounds of nutritional significance including fibers, vitamins and minerals (Wyman 2007). Among various forms of biomass, agricultural crop residues are particularly well suited for energy applications because of its large-scale availability, low cost and environmentally benign production (Chandra *et al*., 2011). As more people get involved in animal production, it becomes imperative to develop appropriate and cost effective feeding systems for the backyard animal producers. It has being opined that, the increasing scarcity of animal proteins and high cost of the conventional feedstuffs in developing countries can

be solved by the use of Agro-Industrial by-products (AIB) in formulating or incorporating it in feeds (Chen *et al*., 2002).

Agricultural residues are the most abundant ones present on earth, comprising 50% of all biomass with an estimated annual production of 50 billion tonnes. Large amount of AIBs from plants are produced by food and Agro Allied Industries. The production of sugar cane molasses has been reported to be 1.3 to 2.1 million tonnes during the last ten years (Kim *et al*., 2001). Corn is the third major cereal grain crop after wheat and rice. During wet milling of corn after steeping, a large quantity of corn liquor is produced as waste which contains nitrogen and water soluble vitamins. The sugar cane molasses and corn steep liquor contain a substantial amount of sugars and proteins, respectively. These may be good sources for animal nutrition as well as substrates for microbial fermentation.

In addition to traditional agro-food processing such as wine, beer, dairy products, bread and others, applications have been developed in the pharmaceutical sector (antibiotics, vitamins, and so on) and in the production of metabolites (enzymes and amino acids) (Kim *et al*., 2001). Many processes have been developed to produce microorganisms able to use organic material as a source of carbon and energy and to convert inorganic nitrogen into high-food value proteins. These can be used in human foodstuffs or animal feed to replace traditional plant or animal sources. Therefore, appropriate biotechnological approaches should be explored for bioconversion of these agro-based industrial wastes into value added products for inclusion of fibrous feed in the diet of livestock.

Lignocellulosic materials such as crop residues, grasses, wood chips, sawdust and animal waste (solid) can be considered as a potential source for large amount of low-cost utilizable products. Many physicochemical, structural and compositional factors hinder the hydrolysis of cellulose present in biomass to sugars and other organic compounds that can later be converted to feeds. Enzymatic hydrolysis is hindered by the following substrate-related factors: cellulose contains highly resistant crystalline structure, lignin and hemicellulose surrounding cellulose form a physical barrier and sites available for enzymatic attacks are limited (Kim *et al*., 2001). Pretreatment, an important tool for practical lignocellulose conversion processes is required to alter the structure of lignocellulosic biomass to make cellulose more accessible to the enzymes (Mosier *et al*., 2005). Pretreatment methods can be physical, chemical, physicochemical and biological processes. The goal of pretreatment is to make the cellulose accessible to hydrolysis for conversion to simple sugars. Various pretreatment techniques change the physical and chemical structure of the lignocellulosic biomass and improve hydrolysis rates.

Many microorganisms including fungi and bacteria had been found to degrade cellulose and other plant cell wall fibers. In nature, degradation of cellulosic biomass is performed by mixtures of hydrolytic enzymes collectively known as cellulases. Enzymes are among the most important bio-products and are being utilized in a large number of processes in the areas of industrial, environmental and food biotechnology. Moreover, current developments in biotechnology are yielding new applications for enzymes. Filamentous fungi are preferred for commercially important enzymes production, because the level of the enzymes produced by these cultures is higher than those obtained from yeast and bacteria (Ikram-ul-Haq *et al*., 2006).

Aspergillus and *Trichoderma* are the most important and safe microorganisms for industrial use and are more potent producers of cellulase (Camacho and Aguilar, 2003). The enzymatic degradation of waste cellulose by fungal cellulases has been suggested as a feasible alternate for the conversion of lignocellulosics into fermentable sugars and fuel ethanol (Elad, 2000). The cellulase system in fungi is considered to comprise of three hydrolytic enzymes: endo-1, 4- β -D- glucanase [carboxymethyl cellulase (EC.3.2.1.4)], which cleaves β-linkage randomly in the amorphous parts of cellulose; exo-1, 4- β -D-glucanase [cellobiohydrolase (EC.3.2.1.91)], which hydrolyzes cellobiose from either the non-reducing or the reducing end, generally from the crystalline parts of cellulose and β -glucosidase [cellobiase (EC.3.2.1.21)], which releases glucose from cellobiose and short chain cellooligosaccharides (cellodextrin) (Rajoka *et al*., 2004). Although β-glucosidase has no direct action on cellulose but it is regarded as a component of cellulase system because it completes cellulose hydrolysis (Chen and Stites, 2001). Although, a large diversity of microorganisms can produce cellulase enzymes, the cellulase system of the filamentous fungus *Trichoderma* has been found to be one of the most effective for hydrolysis of cellulosic materials. *Trichoderma* produces an extracellular, stable, and efficient cellulase enzyme system. *Trichoderma* is a partially catabolite repressed, hypercellulolytic mutant strain, widely studied, with improved enzyme production capabilities, when compared to the wild-type and some other strains (Juhász *et al*., 2005).

Microbial consortium consisting of two or more different microorganisms is known to be largely responsible for many biotransformations in natural environment. 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).

1.2 Statement of Research

Increasing cost and scarcity of conventional energy and protein feedstuffs is a limiting factor to small and large scale commercial animal production in Nigeria. The most important characteristics of the feeds biological value are protein concentration, composition of amino acids (qualitative and quantitative), level of energy, fats and carbohydrate, vitamins and mineral content and hazardous or toxic substances to the animal and these sources are also used in human diet.

Feed costs and animal competition with humans for feed items (grains) suggest strongly that alternative sources such as residues of crop harvests be used partially or totally to replace maize and other crops used in livestock diets so as to reduce cost, prevent pollution and enable cheaper meat production and making available major crops for human consumption.

1.3 Justification

Feed is the single largest cost associated with raising small ruminants, typically accounting for 60% or more of total production cost. It goes without saying that nutrient exert a very large influence on flock reproduction, milk production, kid and lamb growth. Livestock with higher growth potential have higher nutritional needs especially with regards to protein to enable them attain their genetic potential. Small ruminant require energy, protein, vitamins, mineral, fiber and water. Amongst all, protein is the most expensive hence the

need to improve the nutrient value of Agro Industrial by-products rich in protein such as groundnut shell, which contain materials that can be used for animal feeds through biodegradation or bio-processing using fungal spores/enzymes to enhance the protein quality. Agro-Industrial byproduct such as groundnut shell, maize stalk and cobs, millet straw, sugar cane bagasse and so on are potential animal feedstock that can be utilised efficiently after bioconversion with lower microorganisms such as bacteria and fungi to reduce the cost of animal breeding by local farmer. The final food product may contain approximately 30% w/w crude protein as opposed to the initial low protein and lignocellulosic content.

1.4 Aim and Objectives

The aim of this research is to improve the nutritional constituents of an Agro-Industrial residue, groundnut shell, for feed formulation. The objectives are;

1. To isolate and identify *Aspergillus niger* and *Trichoderma viride* spores from groundnut kernel and cultivated soil respectively.
2. To evaluate the effect of two pre-treatment methods on lignin and cellulose.
3. To quantify cellulase activities on fermenting groundnut husk.
4. To determine the potential of pretreated and fermented groundnut husk for use as animal feed by quantifying the proximate content, anti-nutritional content, mineral composition and amino acids profile.

CHAPTER TWO

2.0 LITERATURE REVIEW

2.1 BIOSYNTHETIC CAPABILITIES OF MICROORGANISMS / MICROBIAL TECHNOLOGIES

The uniqueness of microorganisms, their often unpredictable nature and biosynthetic capabilities, given a specific set of environmental and cultural conditions, has made them likely candidates for solving particularly difficult problems in the life sciences and other fields as well (Parr *et al*., 1994). The various ways in which microorganisms have been used over the past 50 years to advance medical technology, human and animal health, food processing, food safety and quality, genetic engineering, environmental protection, agricultural biotechnology, and more effective treatment of agricultural and municipal wastes provide a most impressive record of achievement. Many of these technological advances would not have been possible using straightforward chemical and physical engineering methods, or if they were, they would not have been practically or economically feasible.

Nevertheless, while microbial technologies have been applied to various agricultural and environmental problems with considerable success in recent years, they have not been widely accepted by the scientific community because it is often difficult to consistently reproduce their beneficial effects. Microorganisms are effective only when they are presented with suitable and optimum conditions for metabolizing their substrates including available water, oxygen (depending on whether the micro-organisms are obligate aerobes or facultative anaerobes), pH and temperature of their environment. Meanwhile, the various

types of microbial cultures and inoculants available today have increased rapidly because of these new technologies (Higa, 1991).

All life processes are the result of enzyme activity. In fact, life itself, whether plant or animal, involves a complex network of enzymatic reactions. An enzyme is a protein that is synthesized in a living cell, it catalyses a thermodynamically possible reaction so that the rate of the reaction is compatible with the numerous biochemical processes essential for the growth and maintenance of the cell. The synthesis of an enzyme is thus under tight metabolic regulations and controls that can be genetically or environmentally manipulated sometimes to cause the overproduction of an enzyme by the cell. Being a protein, an enzyme can lose its catalytic properties when subjected to agents such as heat, strong acids or bases, organic solvents, or other materials that denature the proteins. Each enzyme catalyzes a specific reaction or group of reaction with certain common characteristics. The high specificity of the catalytic function of an enzyme is due to its protein nature; that is the highly complex structure of a protein can provide both the environment for a particular reaction mechanism and the template function to recognize a limited set of substrate (Higa, 1991).

2.2 FERMENTATION; AN ASPECT OF BIOTECHNOLOGY

Biotechnology is the use of living organisms to make useful chemicals and products or to perform an industrial task. One of the oldest forms of biotechnology is the use of fermentation reaction, which is one of the most important technologies used for food processing such as the use of yeast to make beer and other alcoholic drinks, carbon dioxide produced by yeast is also used to make bread dough rise. Bioconversion is the most appropriate word to distinguish biocatalytic processes

involving the transformation of a defined starting material into specific products using either isolated enzymes or resting cells (Gabriel and Carrillo, 1999).

2.3 BIOCATALYTIC PROCESSES

The nature of a biocatalyst often defines its utility for industrial bioconversion and determines the conditions under which it can be used. For example the biocatalytic process must be stable enough for practical application and of sufficiently low cost to minimize the effect on the overall economics of the process. Biocatalysts come in many different forms including highly purified enzymes, enzyme mixtures and whole cells (Gabriel and Carrillo, 1999). The form used is often related to cost and performance considerations. Although a crude enzyme proportion might be relatively cheap, the presence of additional enzyme activity might affect the purity and yield of the product. Similarly, the need for cofactor recycling might weigh against the use of a purified enzyme in favor of a respiring whole cell. Another important factor relate to the exquisite selectivity of many enzymes. This has both advantages and disadvantages in that a particular enzyme might be too narrow in its substrate preference to allow the use of unnatural substrate, thus limiting the potential application of that enzyme (Kirk and Sawyer, 1991). Selection and development of the right biocatalyst for a given transformation is often the key for successful commercialization of a biocatalytic conversion.

Broadly defined, a biocatalytic process involves the acceleration of a chemical reaction by a biologically derived catalyst. In practice, the biocatalysts concerned are invariably enzymes and are used in a variety of forms. These include whole cell preparations, crude protein extracts, enzymes mixtures and highly purified enzymes,

both soluble and immobilized. The products of biocatalytic processes are varied ranging from the synthesis of fine chemicals and pharmaceuticals to polymer precursors, foodstuffs and fuel. The common theme however is the use of biocatalyst to either lowers the cost of production or facilitate the development of a new product.

Biocatalysts often enable one to perform chemical transformations with greater selectivity or under milder conditions than might be achieved through conventional chemical means. Large reduction in waste streams and agricultural by-products have been achieved in many instances.

2.3.1 Whole Cell Biocatalysis

Whole cell biocatalysis is a productive and practical style of conducting biocatalytic reactions. Such reactions are done with structurally intact cells, usually viable respiring cells. Whole-cell biotransformation use enzymes which are not normally excreted into the growth medium. Quite often enzymes which are normally intracellular are unstable outside the cell and quickly lose activity making them unsuitable for reactions without further stabilization work. Thus the specific productivity or total turn-over per mole of catalyst maybe severely compromised compared to that seen when used in the whole cell due to inactivation of the enzymes in the reaction conditions (Parr *et al*., 1994).

Whole cell biocatalytic reactions are most often used when the biotransformation to be conducted requires the input of energy. In biological systems this usually takes place in the form of reduced pyrimidine nucleotides or ATP but there can be a

number of reduced cofactors or modified reaction components. Using whole cells allows pre-formed cellular machinery to efficiently provide the required cofactors or component. In order to provide the energy to catalyse these reactions a source of reducing power is usually required. The co-oxidation of an oxidizable substrate such as glucose can provide this energy. The partitioning of the enzymes inside the cells from the bulk medium of the reaction also can be advantageous to let the cellular machinery keep the reaction medium around the enzymes constant and allow for the accumulation, removal or in other respect processing of the reaction products (Perez-Guerra *et al*., 2003).

A practical point of using whole cell biocatalysts is their inherent ease of preparation, use and removal. There is a variety of growth considerations, as with any fermentation, but the major ones with respect to the use in biotransformation surround the induction of the enzyme(s) of interest and the repression of enzymes which might compete with the desired process or degrade the desired catalyst. Generally, a medium can be used in which the cells are used directly after growth for the biotransformation. Some consideration needs to be given to the extraction of the product with respect to the growth medium. Often medium component form rich media used for fermentations can interfere with recovery or extraction procedures (Perez-Guerra *et al*., 2003). Having the resulting growth medium as much like water as possible simplifies downstream work. If the cells must be removed from the growth medium and re-suspended in a different biotransformation medium they can be removed by centrifugation or the medium exchanged by microfiltration. Once

the biotransformation is complete the catalyst can be removed by centrifugation or filtration.

2.4 FERMENTATION

The term fermentation is derived from the Latin verb "*fervere"*, to boil. Fermentation processes utilize microorganisms to convert solid or liquid substrates into various products. The fermentation technology now is being widely used to produce a wide range of products including microbial metabolites, microbial biomass, enzymes and isomers (Christine-Case, 2003).The substrates used varies widely to any material that supports microbial growth being a potential substrate. The action of micro-organisms during the preparation of cultured foods or in the digestive tract has been shown to improve the quantity, availability and digestibility of some dietary nutrients. Fermentation of food with lactic acid bacteria increases folic acid in yogurt, bifidus milk and kefir. Similarly, niacin and riboflavin levels in yogurt are increased with fermentation (Deeth and Tamime 1981; Alm 1982).

Fermentation has come to have different meanings to biochemists and to industrial microbiologists. Its biochemical meaning relates to the generation of energy by the catabolism of organic compounds, whereas, its meaning in industrial microbiology tends to be much broader. When fermentation is discussed, most of us assume the processes used to make wine or beer, i.e. production of alcohol by microorganisms, however, fermentation plays a much greater role in our lives. In fact, the fermentation process accounts for a large part of the foods we eat, such as sour cream, pickles, vinegar, sour dough, yogurt, sausage, cheese, bread, and sauerkraut have one thing in common (Christine-Case, 2003).

The major microorganisms used to carry out important chemical transformations (biocatalytic processes) are the micro fungi (yeast and moulds) and certain prokaryotes. Each microorganism has its own potential to utilize various substrates and synthesize nutrients like proteins, enzymes, essential amino acids, food additives, vitamins and many other useful products like organic acids (*i.e.* lactic acid, acetic acid, citric acid, ascorbic acid) as well as other organic compounds (Azab and Ammar, 1994). The fermentation technology has certain advantages over chemical synthesis because of an optically pure acid production through a suitable strain, whereas chemical synthesis always results in a racemic mixture of acids (Ryu *et al.* 2003). The acceptability of food products mainly depends on the flavour components, which are complex as well as type-specific. These flavour components are influenced by the presence of organic acids and other substances like sulphur compounds, lactones, methyl ketones, alcohols and phenolic substances (Seitz 1990; Urbach 1993). The important flavor substances are formed as a result of the hydrolysis of fatty acids or by the bacterial growth, or enhanced by the addition of acidulants during processing (Berry 2001).

2.4.1 Types of Fermentation

Various forms of fermentation are distinguished based on the processes involved. On the basis of sterility, fermentation can be differentiated into Septic and Aseptic processes. Septic fermentation needs cleaning along with clean environment, hence the need for sterilization, example include vaccine production, enzyme or vitamin production while aseptic fermentation, cleaning or sterilization is not a major issue, example of this include waste treatment, acidification of ethanol to acetic acid and

mushroom production. Fermentation can further be classified as mixed culture and monoculture fermentation.

Monoculture fermentations are those in which the inoculums always consist of one microorganism. Mixed-culture fermentations are those in which the inoculums always consist of two or more organisms. Mixed cultures can consist of known species to the exclusion of all others, or they may be composed of mixtures of unknown species. The mixed cultures may be all of one microbial group - all bacteria - or they may consist of a mixture of organisms of fungi and bacteria or fungi and yeasts or other combinations in which the components are quite unrelated. Mixed cultures are the rule in nature; therefore, one would expect this condition to be the rule in fermented foods of relatively ancient origin. Soil, for example, is a mixed-organism environment with protozoa, bacteria, fungi, and algae growing in various numbers and kinds, depending on the nutrients available, the temperature, and the pH of the soil. Soil microorganisms relate to each other - some as parasites on others, some forming substances essential to others for growth, and some having no effect on each other (Pierre-Yves, 2008).

2.5 SOLID-STATE FERMENTATION (SSF)

Solid-state fermentation (SSF) involves the growth of microorganisms on moist solid substrates in the absence of free flowing water and is an alternative cultivation system for the production of value added products from microorganisms, especially enzymes or secondary metabolites.

Solid-state fermentation (SSF) is traditionally defined as those processes in which microbial growth and products formation occur on the surfaces of solid substrates in the near absence of free water. Due to this low amount of water available in solid-state bio-processing, the class of microorganisms that are most commonly used is fungi (Zheng and Shetty, 2000). SSF is a valuable technique for utilization of agro-industrial by-product for production of value added product(s) of commercial importance. Several agro-industrial waste and by-products such as orange bagasse (Martins *et al*., 2002), sugar cane bagasse (Silva *et al*., 2002) wheat bran (Cavalitto *et al*., 1996) and other food processing waste (Zheng and Shetty, 2000) are effective substrates for depolymerizing enzyme production by solid-state fermentation. Recently, a large number of microorganisms, isolated from different materials, have been screened for their ability to degrade polysaccharide present in vegetable biomass. The technique involves inoculation and growth of microbes on porous particulate solid substrate maintaining low moisture content. The moisture content and nutrient present in the substrate support the growth of microorganism and the organism secrete useful enzymes while growing on solid substrate (Pandey *et al*., 2003).

SSF has been widely employed in industrial productions because of its advantages such as better process control, maximum substrate utilisation, lower chances of contamination, easy downstream processing and so on. Many bacteria and fungi have been utilized for production of industrially important products by SSF.

The production of utilizable products by solid state fermentation (SSF) has gained popularity as it involves lower media cost, stability of the product, increased yield and increased porosity. The higher yield associated with SSF is primarily due to increased mycelia density provided with an optimum moisture range between 60%-70%. The substrates used in fermentation include wheat bran, rice bran, non-glutinous rice, orange peels, grain husks (Jaivel and Marimuthu, 2010; Chanakya *et al*., 2011).

Solid State Fermentations (SSF) are widely used in pharma, food and agro industries. Their applications range from production of consumer products to bioremediation (Mitchell, 1992)

2.5.1 General Aspects of SSF

SSF is a microbial process occurring mostly on the surface of solid materials, which can absorb or contain water, in the presence or absence of soluble nutrients (Viniegra-Gonzalez *et al*., 2003). SSF comprises two very different modes. In the first one, a divided and humidified solid (organic material) acts as both support and nutrient source and the process essentially occurs in the absence of free water. In the second mode, a nutritionally inert solid (synthetic material), which exclusively acts as a support, is soaked in a nutrient solution (Murado *et al*., 1998; Pastrana *et al*., 1995).

In both cases, the success of the process is directly related to the physical characteristics of the support (particle size, shape, porosity, consistency), which

favour both gas and nutrient diffusion and the attachment of the microorganisms (Mitchell, 1992). Generally, smaller substrate particles provide a larger surface area for microbial colonisation but if they are too small may result in substrate agglomeration as well as poor growth. In contrast, larger particles provide better aeration but a limited surface for microbial colonisation. Therefore, a compromised particle size must be selected for each particular process (Pandey *et al*., 1999). Availability and cost are also criteria of great importance. At industrial-scale this SSF is carried out in large fed-batch reactors controlling temperature trajectories by manipulating fresh-air flow and/or recirculation ratio guaranteeing aerobic conditions. These temperature profiles are established based on restricted knowledge of the SSF phenomena (Straatsma *et al*., 2000). SSF has many advantages over submerged fermentation (SmF), including an economical use of space, that is required for fermentation, simplification of the fermentation media, superior yields and no requirement for complex machinery (Satyanarayana, 1994). However, SSF has some limitations such as a poor pool of microorganisms capable of growth under restricted conditions and the controlling and monitoring of parameters such as temperature, pH, humidity and air flow ((Rodríguez Couto and Sanromán, 2005).

2.6 AGRO-INDUSTRIAL RESIDUES

Nowadays, there is great political and social pressure to reduce the pollution arising from industrial activities. Almost all developed and underdeveloped countries are trying to adapt to this reality by modifying their processes so that their residues can be recycled. Consequently, most large companies no longer consider residues as

waste, but as a raw material for other processes. Most by-product feedstuffs (BPF) result from the processing of commercial crops, the food processing industry and the fiber industry. Consequently, BPF are becoming increasingly more important in the food and fiber system because they are available for use as livestock feeds at competitive prices relative to other commodities. Many by-products have a substantial potential value as animal feedstuffs. By-product feedstuffs, which contain little economic value as edible foods for human consumption have become major sources of dietary nutrients and energy in support of growth for livestock. This means that cereals can be largely replaced by these by-products. Consequently the competition between human and animal nutrition can be decreased. The utilization of agro-industrial by products may be economically worthwhile, since conventional feedstuffs are often expensive. The selection of raw material is important for utilizable product production, the selected raw material should be very cheap, easily available, and environment friendly (Pandey *et al*., 2003).

Agro-industrial residues are generally considered the best substrates for the fermentation processes, including enzyme production, based on SSF (Eliaiah *et al*., 2002). Castilho *et al*., (2000) state that 'the conditions in solid-state fermentation were closer to those in the natural habitat of fungi, which were, thus, able to grow better and excrete larger quantities of enzymes'. This can be of special interest in those processes where the crude fermented product may be used directly, as the enzyme source. The crop residues produced in Nigeria are mainly straws of wheat, millet, sorghum, oilseed crops, maize stalks and cobs, cotton stalks, sugar cane trash etc. The Agro Industrial residues like groundnut shells, rice husk, sugarcane

bagasse, cotton waste, coconut shells are used for feed formulation, production of chemicals like acetic acid, citric acid, enzyme production (tannase, cellulose, phytase and so on).

2.7 GROUNDNUT

Groundnut also known as peanut, (*Arachis hypogaea*), is a species in the legume or "bean" family (Fabaceae). Groundnut was first cultivated in the valleys of Peru. It is an annual herbaceous plant with mature fruit that develops into a legume pod that grows close to the ground and bears nuts below ground (hence the term "groundnuts" in some countries). It contains 1 to 4 oblong edible seeds with whitish to dark-purple seed coat.

Peanuts are known by many other local names such as earthnuts, ground nuts, goober peas, monkey nuts, pygmy nuts and pig nuts. Despite its name and appearance, peanut is not a "nut", but rather a legume belonging to the family as peas, broad beans and common beans. Peanuts come in four main varieties: Runner, Virginia, Spanish, and Valencia (Horn *et al*., 1999). Pound for pound groundnut have more protein, minerals and vitamins than beef liver, more fat than heavy ice cream and more calories than sugar. The peanut plant provides not only the peanut itself as food, but also offers many other uses, both as food for people and animals, and non-food items, such as personal care products (hair cream, cosmetics, soap, body lotion) and industrial materials (lubricating oil, furniture polish, insecticide). The plant is also useful in advanced farming techniques to keep soils rich and productive.

Groundnuts are rich in nutrients providing over 30 essential nutrients (magnesium, iron, calcium, phosphorous, potassium, zinc) and phytonutrients (saponin, alkaloid, oxalate and so on). Groundnuts are a good source of niacin, folate, fiber, magnesium, Vitamin E, manganese and phosphorous. They also have naturally free trans-fats and sodium, and contain about 25% protein (a higher portion than in any true nut) (Aletor, 1995).

Recent research in groundnut and nuts in general has found antioxidants and other chemicals that may provide health benefits due to the fact that groundnuts have been found to rival the antioxidant content of many fruits. Groundnuts are significant source of resveratrol, a chemical associated with but not proven to cause reduction in risk of cardiovascular disease and cancer (Sanders *et al*., 2000). Almost every part of groundnut is of commercial value.

2.7.1 Composition of Groundnut

Sensory studies have demonstrated that peanut composition affects peanut quality and shelf life (Sanders *et al*., 1990). High oil and protein content and lower percentages of carbohydrates and ash characterize peanut composition. In the four major market types (runner, virginia, valencia and spanish) total oil content was found to vary from 44–56 % (Ahmed and Young, 1982). Apparently there are no studies which indicate a direct correlation between the amount of oil in peanuts and their shelf life but many studies have indicated a positive correlation of oil content with maturity which in turn is associated with flavor and shelf life (Hinds, 1995).

The protein content of peanuts ranges from 22-33% with an average of about 25% (Ahmed and Young, 1982). Production location affects the total protein and oil content. Peanuts also contain approximately 50% moisture at the time of harvest, about 4.0% starch and 2.0-5.0% sucrose. Lower concentrations of glucose, fructose, raffinose and stachyose are also present (Pattee and Young, 1982). Pattee and Young, 1982, suggested that with increased moisture content, protein and starch hydrolysis could significantly change peanut composition.

Other important peanut components include minerals such as calcium, magnesium, phosphorous, sodium, potassium, iron, copper, zinc and manganese and vitamins A, B, C and E (Ahmed and Young, 1982). Peanut maturity has consistently been shown to be crucial in determining chemical composition, grade and oil stability (Sanders *et al*., 1993).

Almost every part of groundnut is of commercial value as listed below:

Groundnut oil: The groundnut oil has several uses but it is mainly used as cooking oil. It is used in many preparations, like soap making, fuel, cosmetics, shaving cream, leather dressings, furniture cream, lubricants, and so on. Groundnut oil is used in fatty acids manufacturing, as a medium of preservation for preparation of pickles, chutney, etc. The groundnut oil is used in making different types of medicated ointments, textile materials, plasters, syrups and medicated emulsion. It is also used to make various food preparations like butter, milk, candy and chocolate, chutney, groundnut pack, and so on.

Kernel: Whole kernels are used for table purpose by frying, soaking, roasting and boiling and in different types of recipes. Roasted groundnut is the most popular way of eating. Kernels are also used as a spice in vegetables and as sprouts for salad.

Groundnut cake: It is a good feed for animals and poultry due to its nutritive value and palatability.

Groundnut shell: Groundnut shell has great potential for commercial use. It is used as a fuel, filler in cattle feed, hard particleboard, cork substitute, activated carbon

In the past, peanut skins have been viewed as a low value byproduct of peanut processing and roasting. Recent studies have shown that these byproducts contain compounds including isoflavones, isorhamnetin, epicatechin, catechin, resveratrol, and quercetin. Previous research has revealed that these compounds exhibit high oxyradical quenching abilities. In addition, these natural antioxidants also have been reported to be responsible for health benefits in humans consuming wines and other plant materials. The identification of similar compounds in peanut kernel cover may result in similar consumer interest, improved sales, and increased value of peanuts. It is evident that peanut kernel cover (groundnut shell) may be a potential untapped source for the extraction of natural food antioxidants, nutracueticals, and even pharmaceuticals.

2.8 GROUNDNUT SHELL

Groundnut shell (GS), obtained after the removal of nuts from groundnut pod is an agricultural by-product and a valuable resource that can be a potential feedstock for production of fermentable sugars for animal feeding after processing due to high

amount of lignin, cellulose, fiber, protein, carbohydrate etc. These residues are often thrown away, burnt, ploughed into the soil to improve its nitrogen content, or used to form the principal feed in small scale farming systems during dry seasons. Recent research on groundnut shell has found its importance in production of substances such as ethanol and enzymes (tannase, cellulase). Peanut shell pellets contain crude protein, crude fiber, fat and kinds of vitamins. The content of crude fiber reaches the top of all kinds of feeds, closing to 60% hence its potential as a good material for feeds.

Table 2.1 Chemical composition (%) of groundnut shell

Component	**Quantity (%)**
Cellulose	65.7
Carbohydrates	21.2
Proteins	7.3
Minerals	4.5

Source: Jambunathan, 1991.

2.8.1 Shell Composition

The main components of groundnut shell are lignocelluloses. Three key structural polymers make it up: hemicelluloses (a group of heteropolymers, which includes mannans and xylans), lignin (α-complex polyphenolic polymer) and cellulose (α-homopolymer built of D-glucosyl residue).

2.8.2 Lignin

Lignin, introduced in 1819 and derived from Latin word *lignum,* is a highly branched heterogeneous aromatic polymer of phenylpropane units present as 10-35% of lignocellulose, found in plant cell walls, that has an important role in the physical characteristics of wood as well as in preventing lignocellulosic materials against biological attacks, example against cellulose degrading microorganisms. Lignin is particularly difficult to biodegrade, and reduces the bioavailability of the other cell wall constituents. Currently, Lignin is produced mainly as a byproduct of the paper industry, separated from trees by a chemical pulping process. The bio-conversion of plant lignocellulose to glucose is an important part of second generation biofuel production, but the resistance of lignin to breakdown is a major obstacle in this process, hence there is considerable interest in the microbial breakdown of lignin. White-rot fungi are known to break down lignin with the aid of extracellular peroxidase and laccase enzymes. There are also reports of bacteria that can degrade lignin (Tronchet *et al*., 2010).

2.10.3 Cellulose

Cellulose, a widely distributed long-chain polymeric and skeletal polysaccharide of β-glucose, is the most abundant renewable natural product that can be obtained in

the biosphere. It is also a major component in many the farm residues (Zhang *et al*., 2006; Spence *et al*., 2010).

Its potential as an alternative energy source has stimulated researches on converting cellulose to soluble sugars. One of the methods is pyrolysis (decomposition of complex molecules by heat) to biological methods such as the application of cellulase enzyme (Bharadwaj *et al*., 2004).

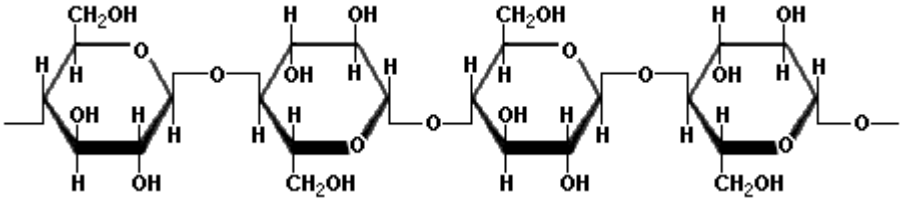


Fig. 2.1: Structure of Cellulose (Gibbons *et al.*, 2002)

2.8.3.1 Cellulose Degradation

The ability to breakdown cellulose is not possessed by mammals. Typically, this ability is possessed only by certain bacteria (which have specific enzymes) like *Cellulomonas*, or by fungi, which in nature are responsible for cycling of nutrients. The enzymes utilized to cleave the glycosidic linkage in cellulose are cellulases (glycoside hydrolases including endo-acting cellulases and exo-acting glycosidase). Such enzymes are usually secreted as part of multi-enzyme complexes that may include cellulose binding modules. The ability of cellulolytic microorganisms to degrade cellulose vary greatly with the physico-chemical characteristics of the substrate, such as, the size and permeability of cellulolytic enzymes and other reagent molecules, which are involved in relation to the size and surface properties of the grown fibrils and the space between microfibrils and cellulose molecules from amorphous region (Beguin and Aubert, 1994). The degree of crystallinity of cellulose is one of the most important structural parameters, which affect the rate of enzymatic degradation by hydrolysis. Therefore, the rate of degradation should be a function of the surface properties of cellulose, which makes possible the access of enzymes to polymeric molecules. The unit cell dimensions of cellulose, stereoscopic conformation and rigidity of the anhydrous-glucose units, degree of polymerization of cellulose molecules the nature of components with which cellulose is associated and the nature, concentration and distribution of substituted groups are the other physico-chemical characteristic.

Cellulase enzymes exist in complex system in order to efficiently harvest energy produced by polysaccharides. These enzymes are complex modular proteins that are

comprised of one or more catalytic domains and substrate binding domains, which hydrolyze a particular substrate. This enzyme complex breaks down cellulose to β-glucose. Cellulase enzyme complex consists of three major components that are endo-1, 4-β-glucanase (EC 3.2.1.4), exo-1, 4- β –glucanase (EC 3.2.1.91) and cellobiose or β -glucosidase (EC 3.2.1.21). Members of all these classes are necessary to degrade cellulose (Bhat, 2000). Cellulases are found in fungi and bacteria. Of commercial interest are fungal enzymes from *Aspergillus* and *Trichoderma* and a few bacterial enzymes

Endocellulase (β-1, 4-D glucan hydrolase) breaks internal bonds to disrupt the crystalline structure of cellulose and expose individual cellulose polysaccharide chains. Endoglucanase substrates are cellulose (nature), cellodextrin (particularly hydrolyse), phosphoric acid hydrated (swollen) cellulose, Carboxyl methyl (CM) cellulose (substrate normally used in cellulose assay system) and hydroxyethyl cellulose (Bhat, 1997).

Exocellulase also known as β-1, 4-D glucancellobiohydrolase scientifically, cleaves 2 - 4 units from the ends of the exposed chains produced by endoglucanase, resulting in the tetrasaccharides or disaccharide such as cellobiose. Cellobiohydrolase has strong affinity towards cellulose and able to hydrolyse the crystalline cellulose up to 80% (Gielkens *et al*., 1999).

Both endoglucanase and cellobiohydrolase will act synergistically to hydrolyse activated cellulose. Endoglucanase will act first on the amorphous region, which will be the starting point for cellobiohydrolase to act on cellulose. Synergistic

association is not strong when substrate is amorphous in nature (Juhasz, *et al*., 2005).

Cellobiase or β-glucosidase (β-D-glucosidaseglucohydrolase) hydrolyses the endocellulase product into individual monosaccharide known as β-glucose. Mainly symbiotic bacteria in the ruminating chambers of herbivores produce it. Besides the ruminants, most animals and humans do not produce cellulase. Therefore, unable to use most of the energy contained in plant material. Secretion of β-glucosidase is highly dependent on the culture conditions. A relatively high pH can enhance β-glucosidase production (Juhasz, *et al*., 2005).

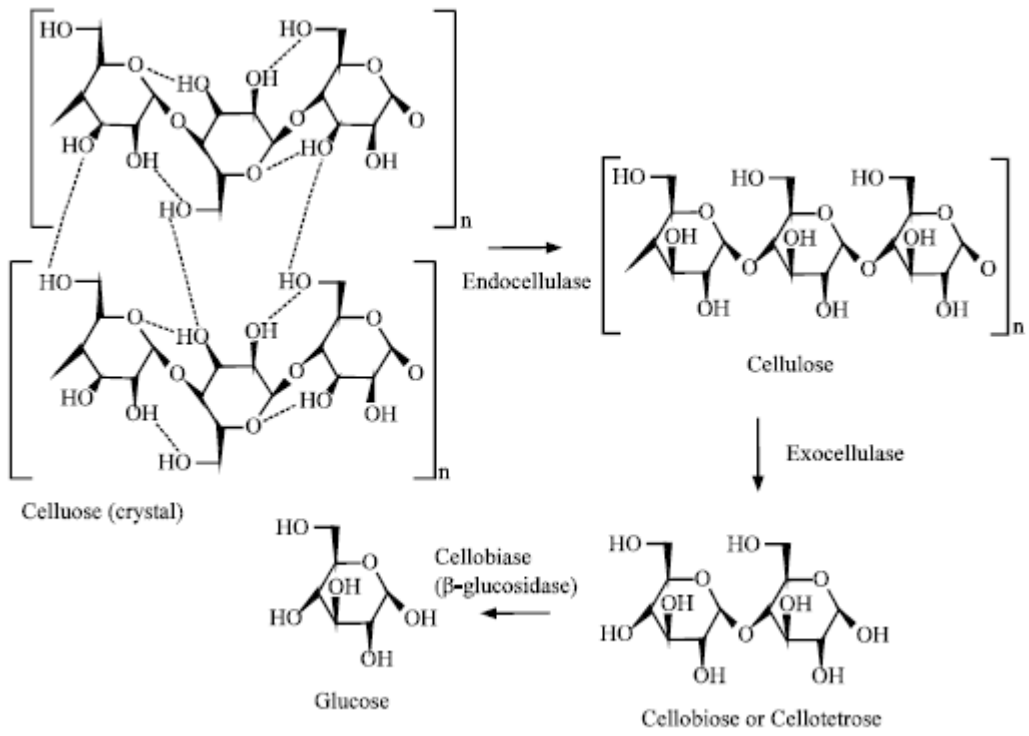


Fig. 2.2: Mode of action of various components of cellulase (Carcieri, 2010)

2.9 MICROORGANISM PRODUCING LIGNOCELLULOLYTIC ENZYMES

The major microorganisms used to carry out important chemical transformations (biocatalytic processes) are the micro fungi (yeast and moulds) and certain prokaryotes. Each microorganism has its own potential to utilize various substrates and synthesize nutrients like proteins, enzymes, essential amino acids, food additives, vitamins and many other useful products like organic acids (*i.e.* lactic acid, acetic acid, citric acid, ascorbic acid) as well as other organic compounds (Azab and Ammar 1994). Even though a large number of microorganisms can degrade cellulose, only a few of these microorganisms produce significant quantities of cell-free enzymes capable of completely hydrolysing crystalline cellulose in vitro.

Filamentous fungi secrete a plethora of important enzymes in the growth medium together with other secondary metabolites (Organic compounds that are not directly involved in the normal growth, development, or reproduction of organisms, though produced during culture). Most of these are hydrolytic in nature being employed in different food processing industries as well as in refinement of fodder quality. Edible filamentous fungi producing these enzymes present an added advantage for their use in food and feed formulation.

Many fungi from *Asco-* and *Basidiomycetes* can produce extracellular enzymes that enable them to break down polysaccharides such as celluloses and convert these polymeric compounds into sugars.

2.9.1 Phylogenetic Classification of Fungi

The term fungus (pl. Fungi) was derived from the Latin word "*fungus",* meaning mushroom. Fungi are eukaryotic, spore-bearing organism that reproduce asexually/sexually and are saprophytic in nature (Bruinsma *et al*., 1990). The *Ascomycota*, commonly known as sac fungi or Ascomycetes, constitute the largest taxonomic group within the Eumycota. These fungi form from meiotic spores called ascospores, which are enclosed in a sac-like structure called an *ascus*. Prominent and important genera of filamentous ascomycetes include *Aspergillus, Penicillium, Fusarium* and *Claviceps.*

Members of Basidiomycota, commonly known as the club fungi or basidiomycetes, produce meiospores called basidiospores on club-like stalks called basidia. Basidiomycetes are the most efficient lignin-degrading organisms that produce mainly laccases (EC 1.10.3.2), lignin peroxidase (EC 1.11.10.14) and manganese peroxidase (EC 1.11.1.13). These enzymes present a non-specific biocatalyst mechanism and have been used for bioremediation process due to their ability to degrade azo, heterocyclic, reactive and polymeric substances (Bruinsma *et al*., 1990).

Lignocellulolytic enzymes-producing fungi are widespread, and include species from the ascomycetes (e.g. *Trichoderma viride, T. reesei*, *Aspergillus niger*), basidiomycetes including white-rot fungi (e.g. *Ustilago maydis, P. chrysosporium*), brown-rot fungi (e.g. *Fomitopsis palustris*) and finally a few anaerobic species (e.g. *Orpinomyces sp.*) which degrade cellulose in gastrointestinal tracts of ruminant animals (Yoon, 2007). Biomass degradation by these fungi is performed by

complex mixtures of cellulases, hemicellulases (Ljungdahl, 2008) and ligninases (Sanchez, 2009), reflecting the complexity of the materials. Cellulases and most hemicellulases belong to a group of enzymes known as glycoside hydrolases.

Fungal fermentations are complex systems into which the operating conditions, the broth rheology, the enzyme production, the morphology of the microorganisms, and their physiological state are all interrelated (Lecault *et al*., 2007). The morphology of a filamentous fungus developing in any fermentation system could be considered as a final result of competing influences, equilibrium between forces of cohesion and disintegration (Papagianni, 2004). Many filamentous fungi, including *T. reesei*, show a strong tendency to decrease the pH of their culture medium during growth on carbohydrate substrates. Kadam and Keutzer (1995) and Juhász *et al*. (2004) have reported the influence of medium supplementation with buffers on cellulase production, however the effect and possible relations between this systems and fungal morphology was not evaluated.

2.10 Aspergillus niger

Aspergillus niger is a fungus and one of the most common species of the genus *Aspergillus*. It causes a disease called black mold on certain fruits and vegetables such as grapes, onions, and peanuts and is a common contaminant of food. It is ubiquitous in soil and is commonly reported from indoor environments. *A. niger* is cultured for the industrial production of many substances. Various strains of *A. niger* are used in the industrial preparation of citric acid (E330) and gluconic acid (E574) and have been assessed as

acceptable for daily intake by the World Health Organisation. *A. niger* fermentation is "generally recognized as safe" (GRAS) by the United States Food and Drug Administration under the Federal Food, Drug and Cosmetic Act.

Many useful enzymes are produced using industrial fermentation of *A. niger*. For example, *A. niger* glucoamylase is used in the production of high fructose corn syrup, and pectinases are used in cider and wine clarification.

2.10.1 Morphology

Macroscopically, colonies on potato dextrose agar at 25°C are initially white, quickly becoming black with conidial production. Reverse (of the plate) is pale yellow and growth may produce radial fissures in the agar. Microscopically, hyphae are septate and hyaline. Conidial heads are radiate initially, splitting into columns at maturity. The species is biseriate (vesicles produces sterile cells known as metulae that support the conidiogenous phialides). Conidiophores are long (400-3000 µm), smooth, and hyaline, becoming darker at the apex and terminating in a globose vesicle (30-75 µm in diameter). Metulae and phialides cover the entire vesicle. Conidia are brown to black, very rough, globose, and measure 4-5 µm in diameter (de Hoog *et al*., 2000).

2.11 Trichoderma viride

Trichoderma is a fungal genus that was described in 1794, including anamorphic fungi isolated primarily from soil and decomposing organic matter. Strains within this genus

include a wide spectrum of evolutionary solutions that range from very effective soil colonizers with high biodegradation potential, to non-strict plant symbionts that colonize the rhizosphere.

Most *Trichoderma* species are morphologically very similar and were considered for many years as a single species: *T. viride*. Strains of *Trichoderma* may also be aggressive biodegraders (Wardle *et al*., 1993), the strong biodegradation and substrate colonization performances of *Trichoderma viride* (*T. viride*) is the result of an amazing metabolic versatility and a high secretory potential which leads to the production of a complex set of hydrolytic enzymes (Kubicek *et al.*, 2001). Among others, chitinases, β-1,3- glucanases (De la Cruz *et al*., 1995b), β-1,6-glucanases (De la Cruz *et* al., 1995a), α-1,3-glucanases (Ait-Lahsen *et al*., 2001) and proteases have been described as important components of the multi-enzymatic system of Trichoderma strains.

2.12 PRETREATMENT OF LIGNOCELLULOSIC MATERIALS

Lignocellulosic materials such as agricultural residues (e.g. wheat straw, sugarcane bagasse and corn stover), forest products (hardwood and softwood) and dedicated crops (switch grass, salix) are renewable sources of energy. These raw materials are sufficiently abundant and generate very low net greenhouse emissions. Approximately 90% of the dry weight of most plant materials is stored in the form of cellulose, hemicellulose, lignin, and pectin (Yat *et al*., 2008). The presence of lignin in lignocelluloses leads to a protective barrier that prevents plant cell destruction by fungi and bacteria for conversion to fuel. 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. Pretreatment must meet

the following requirements: improve the formation of sugars or the ability to subsequently form sugars by hydrolysis, avoid the degradation or loss of carbohydrate, avoid the formation of byproducts that are inhibitory to the subsequent hydrolysis and fermentation processes and be cost-effective.

Pretreatment methods can be roughly divided into different categories: physical (milling and grinding), physicochemical (steam pretreatment/auto-hydrolysis, hydro-thermolysis, and wet oxidation), chemical (alkali, dilute acid, oxidizing agents and organic solvents), biological, electrical, or a combination of these. The following pretreatment technologies have promise for cost-effective pretreatment of lignocellulosic biomass for biological conversion to fuel, chemicals and other utilizable products (Galbe and Zacchi, 2007).

2.12.1 Physical Pretreatment

2.12.1.1 Mechanical Comminution

Comminution of lignocellulosic materials through a combination of chipping, grinding, and/or milling can be applied to reduce cellulose crystallinity. The size of the materials is usually 10-30 mm after chipping and 0.2-2 mm after milling or grinding. Vibratory ball milling was found to be more effective than ordinary ball milling in reducing cellulose crystallinity of spruce and aspen chips and in improving their digestibility (Ohgren *et al*., 2006). The final particle size and biomass characteristics determine the power requirement for mechanical comminution of agricultural materials.

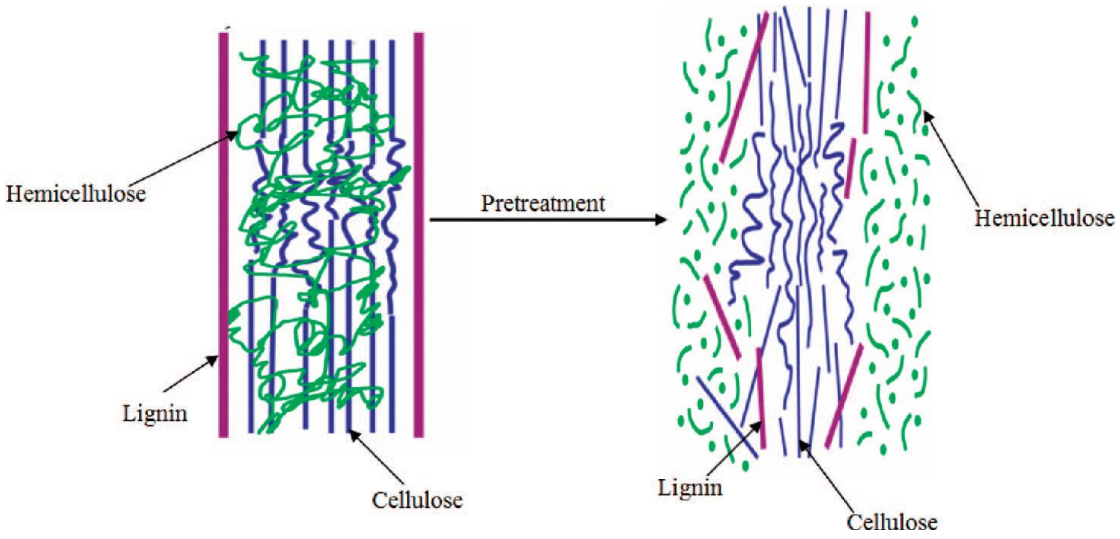


Figure 2.3: Schematic conversion of lignocellulosic biomass to simple sugars.

2.14.1.2 Pyrolysis

Pyrolysis has also been used for the pretreatment of lignocellulosic materials. Cellulose rapidly decomposes to gaseous products and residual char when biomass is treated at temperatures greater than 300 °C. At lower temperatures, the decomposition is much slower, and the products formed are less volatile. Zwart *et al*., (2006) reported that 'mild acid hydrolysis (1 N H_2SO_4, 97°C, 2.5 h) of the products from pyrolysis pretreatment resulted in 80-85% conversion of cellulose to reducing sugars with more than 50% glucose'. The pyrolysis process is enhanced when carried out in the presence of oxygen.

2.12.2 Chemical Pretreatment

2.12.3.1 Acid Hydrolysis

Concentrated acids such as H_2SO_4 and HCl have also been used to treat lignocellulosic materials. Pretreatment with acid hydrolysis can result in improvement of enzymatic hydrolysis of lignocellulosic biomasses to release fermentable sugars. Although they are powerful agents for cellulose hydrolysis, concentrated acids are toxic, corrosive, hazardous, and thus require reactors that are resistant to corrosion, which makes the pretreatment process very expensive. In addition, the concentrated acid must be recovered after hydrolysis to make the process economically feasible (Sun and Cheng, 2002).

Dilute-acid hydrolysis has been successfully developed for pretreatment of lignocellulosic materials. Sulfuric acid at concentrations usually below 4% has been of the most interest in such studies as it is inexpensive and effective. Dilute H_2SO_4 is mixed with biomass to hydrolyze hemicellulose to xylose and other sugars and then continues to break xylose down to form furfural (Mosier *et al*., 2005). The dilute H_2SO_4 pretreatment can achieve

high reaction rates and significantly improve cellulose hydrolysis. Dilute acid effectively removes and recovers most of the hemicellulose as dissolved sugars, and glucose yields from cellulose increase with hemicellulose removal to almost 100% for complete hemicellulose hydrolysis.

2.12.3.2 Alkaline Pretreatment

Mild alkali treatment of lignocellulosic biomass is an effective pretreatment method, which improves enzymatic hydrolysis without the presence of inhibitory product. The use of dilute alkali (NaOH) pretreatment followed by enzyme saccharification of cereal residues for their potential to serve as feedstock in the production of product such as next-generation utilizable products. Generally, pretreatment conditions at elevated temperatures led to highly digestible material enriched in both cellulose and hemicellulose components.

CHAPTER THREE

MATERIALS AND METHODS

3.1 MATERIALS

3.1.1 Chemicals: All chemicals used were of analytical grade. Sulphuric acid, hydrochloric acid, nitric acid, sodium hydroxide, sodium citrate, citric acid, magnesium sulphate, zinc chloride, potassium nitrate, iron chloride, calcium chloride and so on.

3.1.2 Equipment

Hemocytometer 0.25mm (New Berger), Muffle furnance (model SXL), Gallenkamp Incubator (modelIH-150), Oven (Memmert Type HE 500), Microscope (Max-Bino 060614), Amino Acid Analyser, Gallenkamp shaker (Lab-line orbit environ-shaker 18 No. 3527-1/34).

3.2 METHODS

3.2.1 Collection of Groundnut shell

Dried samples of groundnut pods were obtained from a local farmer in Layin Zomo, Zaria, and identified by the Herbarium at Department of Biological Science, Ahmadu Bello University, Zaria. The pods were shelled to obtain the shells. The shells were dried, milled and sieved with a mesh of 0.20mm pore size and a particle size of less than 0.20mm.

3.2.2 Agar Preparation (Titan media)

Potato dextrose agar (PDA) (39g) was dissolved in 1000ml of distilled water and boiled vigorously. 20ml each was dispensed into 40 glass special bottles, autoclaved at 121^0C for 15min and slanted at 60^0C to solidify, these served as slants on which pure strains were preserved. The remaining volume of PDA was autoclaved and then dispensed until the PDA covered the base of the plates. The PDA was dispensed into 10 sterile petri dishes and allowed to solidify

3.2.3 Culturing of Microorganisms

The method according to Grace and Jaronski (2005) with modification was used.

3.2.3.1 Isolation of A. niger

Two groundnut seeds were dropped in 1ml distilled water and agitated for 2minutes thus washing off microbes on the seed into the water, this was used for A. *niger* isolation. A sterile syringe was used to measure 0.5ml of the suspension and injected onto two PDA plates. The technique is called Pour plating. The plates were incubated at room temperature (28^0C) for 72hrs to allow for profuse growth.

3.2.3.2 Isolation of T. viride

Soil (0.25g) was dissolved in 2ml distilled water and vortexed for 2 minutes, for isolation of *T. viride*. Serial dilution technique was used to reduce the microbial load by measuring 1ml of the stock solution into 9ml distilled water. 1ml of this was also diluted in 9ml distilled water, this was diluted to 10^4. Each of the diluted

solution (0.5ml) was inoculated on two PDA plates by pour plating technique, these were incubated at 37^0C for 72hrs to obtain spores of *T. viride.*

3.2.3.2 Isolation of Ustilago maydis

Pre-harvest maize (matured with seeds) was soaked in distilled water over night, after which 0.5ml of it was pour plated on a PDA plate and incubated at 28^0C. The organism of interest (*Ustilago maydis*) obtained was cultured on another plate for growth and morphological identification.

The organisms were microscopically identified by dropping spores picked with sterile wire loop onto spirit sterilized glass slide staining with Methylene blue. A cover slip (glass) was placed on the stained organism, de-stained by pouring water over the cover slip and then viewed under the microscope.

Each organism was sub-cultured on PDA slant, by picking some spores with sterile wire loop and inoculating on slants, after comparing the organisms obtain with that in a standard Fungal Atlas, so as to obtain a pure strain.

3.2.4 Inocula Preparation

The inoculum of each of the three isolates were prepared by measuring 10ml of sterile distilled water into each agar slants and using sterile wire loop to wash the sporophore into the water. Each isolate was subsequently diluted with more sterile distilled water until a sporophore count of approximately 5×10^6 per ml is obtained using a Haemocytometer (Onilude and Oso, 1999).

Potato Dextrose (PD) broth was prepared by dissolving 2g of glucose in 1L of distill water used to boil 200g of peeled potatoes (sweet potatoes) for 1hr. The resulting solution was filtered through muslin cloth and made up to 1L with Basal medium consisting of the different salts (Appendix I). PD broth (25ml) was measured into 150ml conical flasks corked with cotton wool and foil paper. These were autoclaved at 121^0C for 15minutes before use.

Sporophores of *A. niger* and *T. viride* (1ml) each were inoculated into 25ml PD broth and placed in a shaker at 200rpm for 5 days, after which 10ml was centrifuged at 4^0C 3000rpm for 15minutes in a refrigerated centrifuge to separate the mold from the supernatant. The supernatant (3ml) was used as source of enzyme and stored at 4^0C.

Ustilago maydis inoculum (isolate three), after counting, the spores were not subjected to growth in PD broth as above but were inoculated directly on the substrate (groundnut husk) and allowed to grow for a period of 14 days (Grace and Jaronski, 2005).

3.2.5 Pre-treatment of Groundnut husk (GH)

Apart from milling, chemical and biological pre-treatments were carried out.

Chemical pre-treatment: 100g of milled sample was treated with Alkaline (500ml 2M NaOH), for 2hours. This was then washed with distilled water until the pH of the filtrate is 7 then dried in the oven at 60^0C.

Biological pre-treatment:40ml Basal medium was used to moisten 20g of groundnut husk in a 250ml conical flask by thorough mixing with a glass rod, corked and

autoclaved. 3ml of 10^6 spores of *Ustilago maydis* was inoculated into the groundnut husk, incubated at 28^0C for 14 days after which the groundnut husk was autoclaved. This was carried out in four conical flasks.

3.3 SAMPLE FERMENTATION

3.3.1 Monoculture fermentation

Spores (in form of flocs) from growing fungi in broth (3.2.4) were inoculated directly on moistened and autoclaved groundnut husk (GH) in a sterile environment by opening a small part of the flask.

Chemical and biologically pre-treated GH, 10g each were weighed into eight 250ml conical flasks and sterilized in an autoclave for 15minutes at 121^0C. They were then moistened with 40ml Basal medium as prepared in Appendix I.

Two flasks of biologically pre-treated GH were inoculated with 3ml spores suspension of each isolate ($_BGH_{A.\ niger}$, $_BGH_{T.\ viride}$). Same was repeated for chemical pretreated GH ($_CGH_{A.\ niger}$, $_CGH_{T.\ viride}$). All flasks were sealed with cotton wool and incubated at 28^0C for 14days after which they were oven dried at 60^0C for 24hours to stop further action of the spores. All fermentations were carried out in duplicate.

3.3.2 Enzyme fermentation

Spores of actively growing fungus of each organism in broth (3.2.4) was centrifuged at 3000rpm for 5minutes and 5ml each of the supernatant was added to

10g of moistened GH, chemically ($_{C}GH_{enz}$) and biologically pre-treated ($_{B}GH_{enz}$) respectively.

3.3.3 Co-culture fermentation

Spore suspension of each microorganism (1.5ml) was co-cultured in 10g of moistened and autoclaved GH, chemically ($_{C}GH_{cc}$) and biologically pre-treated ($_{B}GH_{cc}$) separately.

3.3.4 Unfermented GH

An equal amount of GH was also autoclaved at 121^{0}c for 15min but without enzymes or spores added, this was tagged undegraded GH (uGH) and used as control.

Samples from all three sets were incubated at 28^{0}C in an incubator for 14days. After 24hours of inoculation as above, an aliquot of fermenting GH was taken from each of the flasks for enzyme extraction. Citrate buffer 0.05M (3ml) was added and vigorously shaken on the rotary shaker at 200rev/min for 30 minutes. The solid biomass was separated from the suspension by centrifuging at 3000 rpm for 5minutes. The supernatant was used as the source of enzyme. The procedure was repeated with each batch of organism after every 24hours to assay for the presence of cellulase enzymes for the period of 14 days after which the samples were

analysed for proximate composition, anti-nutrient content, minerals, cellulose and lignin content.

3.4 MEASUREMENT OF ENZYME ACTIVITY

The method of Ghose (1987) was used for both assay.

3.4.1 Filter Paper Assay

Total cellulase activity was carried out based on the method of filter paper assay for saccharifying cellulase. 1ml of 0.05M citrate buffer was added to the test tube containing one Whatman No.1 filter paper strip (1 cm x 6 cm). After that, 0.5ml of sample solution (supernatant) also pre-warmed to 40^0C was added to the citrate buffer solution. The resulting solution was mixed thoroughly and then transferred to a water-bath maintained at 50^0C. After 60minutes (reaction step) the test tubes were removed from the water bath, and 3ml of 3, 5-dinitrosalicylic acid (DNS) solution was added and mixed thoroughly to stop the enzymatic reaction. Tubes were covered and placed in a boiling water bath for 5 minutes. All the tubes were cooled to room temperature in a cooling water bath. The absorbance was taken at 540 nm against water blank. Enzyme activity was expressed as FPU/ml (Amount of reducing sugar released per ml of filtrate per minute).

3.4.2 Carboxymethyl Cellulase (CMC-ase)

Carboxymethyl cellulose, 2%, in 0.05M Sodium citrate buffer, pH 4.8 was used as the substrate for endoglucanase activity assay. Enzyme extract (supernatant from centrifugation of aliquot from each set of fermentation) (0.5ml) was diluted in 0.5ml 0.05M citrate buffer in a test tube of 25ml capacity and temperate to 50^0C. Carboxymethyl cellulose, 0.5ml, was added, mixed well and incubated at 50^0C for 30minutes. Dinitrosalicylic acid (DNS), 3ml, was added and mixed. These were transferred to a rack on the table, after which the test tubes (24) were boiled in

vigorously boiling water for exactly 5minutes. All samples, enzyme blanks, glucose standards and spectro zero were boiled together. After boiling, they were immediately transferred to a cold water bath and 20ml distilled water was added, mixed by completely inverting the tube so that the solution leaves the bottom of the tube. The colour formed was measured against the spectro zero at 540nm. Enzyme activity was expressed as IU/ml that is one unit of endoglucanase is the amount of enzyme that released one micromole of reducing sugars per minute under the assay conditions (pH 4.8, 50ºC).

3.5 LIGNIN AND CELLULOSE DETERMINATION

The method of Van Soest *et al.*, (1991) was used for both determination

3.5.1 Lignin Content

One gram (1g) of GH from each set of fermentation was hydrolysed with 5ml 1.25%$^{v}/_{v}$ $H_2SO_4$and allowed to stand for 2hrs, after which the supernatant was decanted, then 5ml of 72%$^{v}/_{v}$ H_2SO_4 was added and allowed to stand for 4hrs.

The residue was filtered with Whatman no.1 filter paper and washed with distill water until the filtrate is colorless.

The residue was oven dried at 105^0C for constant weight.

$$\text{Lignin}(\%) = \frac{\text{weight of residue}}{\text{weight of sample}} \times 100$$

3.5.2 Cellulose Content

Acetic acid, 3ml, was added to 1g GH in a test tube and mixed in a vortex mixer. The tube was placed in a water-bath at 100^0C for 30minutes. This was cooled and centrifuged for 20minutes. The supernatant was discarded, the residue was washed with 10ml distilled water by adding water, shaken vigorously and decanted.

Ten milliliter (10ml) of 67% Sulphuric acid was added and allowed to stand for 1hour, 1ml was then diluted to 100ml with 99ml distilled water. Anthrone reagent (10ml) was added to 1ml of the diluted solution, mixed well and heated in a boiling water-bath for 10minutes. The test tube was cooled and absorbance measured at 630nm. A blank was set using Anthrone reagent and distilled water.

Cellulose, 100mg, was weighed into a test tube, 10ml of 67% Sulphuric acid was added and allowed to stand for 1hour, 1ml was then diluted to 100ml with distilled water. Series of volume (0.4, 0.8, 1.2, 1.6, 2.0ml) were measured, distilled water was added to the volumes less than 2.0ml to make them up to 2ml and corresponding absorbance taken at 630nm to plot standard graph from which cellulose concentration of GH samples were extrapolated.

3.6 DETERMINATION OF PROXIMATE COMPOSITION

The method of AOAC (2006) was used.

3.6.1 Moisture Content

Crucibles were washed, dried to a constant weight in an oven at 100^0C and they were later removed and cooled in a dessicator and weighed (W_1). Sample was

weighed (2g) into pre-weighed crucible (W_2). The sample was kept in the oven at 100^0C for 3hrs, removed, cooled and weighed (W_3). This was repeated until the weight is constant. The moisture was calculated as:

$$\% \text{ Moisture} = \frac{W2 - W3}{W2 - W1} \times 100$$

3.6.2 Ash Content

Crucibles were cleaned, dried in an oven, cooled in a dessicator and weighed (W_1). Sample was weighed (2g) into crucible and weighed (W_2). This was transfer into a furnace set at 550^0C, then removed, cooled in a dessicator and weighed (W_3). The ash content was calculated thus:

$$\% \text{ Ash } = \frac{W3 - W1}{W2 - W1} \times 100$$

3.6.3 Fiber Content

Two grams (2g) of sample and beaker was weighed (W), 1.2ml H_2SO_4 was added and boiled for 30minutes. The solution was filtered, washed with hot water and the residue transferred to a beaker containing 100ml of 0.3M NaOH and boiled for 30minutes. This was filtered, the residue washed with hot water, dried in an oven and weighed (C_2). The weighed sample was incinerated in a furnance at 550^0C after which it was brought out, cooled and weighed (C_3). The fiber content was calculated thus:

$$\% \text{ Fiber } = \frac{C3 - C2}{W} \times 100$$

3.6.4 Lipid (Fat) Content

Clean, dry boiling flask (250ml) was weighed (W_1). Sample was weighed (2g), wrapped in filter paper and dropped into the thimble of soxhlet apparatus. The boiling flask was filled with 300ml petroleum ether. The soxhlet apparatus was fitted to the flask and allowed to reflux for 8hrs, after which the thimble was removed and the petroleum ether was allowed to evaporate. The flask was transferred to the oven to dry properly, then cooled and weighed (W_2).

$$\% \text{ Fat } = (W2 - W1) \times 100$$

3.6.5 Crude Protein Content

3.6.5.1 Digestion

Two gram (2g) of sample was weighed into Kjeldahl flask containing 25ml concentrated sulphuric acid and catalyst (Copper) added. This was heated in a fume cupboard until the colour of the solution turned green. The digest was cooled and transferred with several washings with distilled water into 250ml distillation flask.

3.6.5.2 Distillation

The distillation apparatus was steamed for 15minutes then a conical flask containing 5ml Boric acid was placed under the condenser. Steam through for 7minutes to collect enough ammonium sulphate.

3.6.5.3 Titration

The distillate was titrated with 0.01M hydrochloric acid, nitrogen and protein content were calculated, nitrogen content was calculated first before protein content;

$$N = \frac{\text{Molar mass} \times \text{molarity of acid} \times \text{volume of digest (Blank} - \text{Titre)}}{\text{weight of sample} \times \text{volume taken from distillate}}$$

Protein content = 6.25 × Nitrogen content

3.6.6 Carbohydrate Content

This was calculated by subtracting the other estimated fractions from 100, that is

$$\text{Carbohydrate Content} = 100 - (\%\text{moisture} + \%\text{Ash} + \%\text{Protein} + \%\text{Fat})$$

3.7 ANTI-NUTRIENTS DETERMINATION

3.7.1 Saponin Content

Groundnut husk (2g) was folded in a filter paper and put in a thimble and extracted by refluxing in a soxhlet extractor. Extraction was with acetone in a 250cm^3 capacity round bottomed flask for 3hr after which the apparatus was dismantled and another 150cm^3 capacity flask containing 100cm^3 methanol was fitted to the extractor and extraction sustained for another 3hrs.

Weight of the flask before (B) and after the second extraction (A) was taken to note any change in weight. Methanol was recovered by distillation after the second extraction and the flask oven dried, allowed to cool at room temperature, weighed and saponin calculated thus;

$$\text{Saponin} = \frac{\text{A} - \text{B}}{\text{Sample mass}} \times 100$$

3.7.2 Alkaloid Content

Five grams (5g) of powdered sample was dispersed in 50ml of 10% acetic acid in ethanol. It was filtered and the filtrate was evaporated to 15ml of its original volume in a water bath. Alkaloid was precipitated with a drop of ammonium hydroxide and the precipitate was recovered by centrifugation at 100rpm for 10minutes, dried at 70^0C for 30minutes in a vacuum oven.

$$\% \text{ Alkaloid } = \frac{\text{weight of dried precipitate}}{\text{weight of original sample}} \times 100$$

3.7.3 Oxalate Content

Groundnut shell (2g) was dispersed in 190ml of distilled water in 250ml volumetric flask. Ten milliliter (10ml) of 6M HCl was added, then digested at 100^0C for 1hr, cooled and made to volume before filtration. The filtrate was precipitated with ammonium hydroxide and the precipitate was dissolved in 10ml of 20% sulphuric acid. The solution was titrated with 0.05M potassium permanganate.

$$\text{Calcium oxalate } = \frac{\text{T } \times (\text{Vme})(\text{DF}) \times 105}{\text{ME } \times \text{Mf}}$$

Where:

T – Titre value of potassium permanganate

Vme – Volume-mass equivalent (i.e 1ml of Potassium permanganate = 0.00225 anhydrous oxalic acid).

DF – Dilution factor

ME – Molar equivalent of potassium permanganate

Mf – Mass of sample

3.7.4 Phytate Content

Four grams (4g) of GH was soaked in 100ml of 2% HCl for 5hrs and filtered. To 25ml of the filtrate, 5ml 0.3% ammonium thiocyanate solution was added. The mixture was then titrated with Iron (III) chloride solution until a brownish-yellow colour that persisted for 5min was obtained.

Phytate phosphorus = Iron equivalent × 1.95g of titer

Phytate = Phytate phosphorus × 3.65g

3.7.5 Tannin Content

Two grams (2g) of GH weighed into a beaker containing 50ml distilled water, heated to 60^0C and filtered. Ten milliliter (10ml) of 4% copper acetate solution was added to the hot filtrate and boiled again for 10minutes. The precipitate was filtered and the filtrate discarded. The residue was dried using filter paper after which the dried GH scraped from filter paper into a pre-weighed crucible (W_1). The crucible (which contained the GH) was incinerated in a muffle furnance at 550^0C, cooled in a dessicator and then reweighed as W_2. The difference between the weight of sample before ashing and ash residue after incineration is the tannin content;

$$\text{Tannin Content} = W_2 \times W_1$$

3.8 DETERMINATION OF MINERAL CONSTITUENTS

Mixture from Appendix I; Reagent for Digestion, (20ml) was added to 1g of GH sample, heated continuously with the addition of 25ml distilled water for 3hrs then filtered into a clean sample bottle and made up to 100ml with distilled water

(digest). The minerals content in the digest were taken from Absorption Atomic spectroscopy (AAS).

3.9 DETERMINATION OF AMINO ACID PROFILE

Amino Acid profile in the sample was determined using methods described by Benitez (1998). The sample at dried constant weight, defatted, hydrolyzed, evaporated in a rotary evaporator and loaded into the Technicon sequential Multi-Sample Amino Acid Analyzer (TSM).

3.9.1 Defatting Sample

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

3.9.2 Nitrogen Determination

Sample was weighed (0.2g), wrapped in Whatman filter paper (No. 1) and put in the Kjeldhal digestion flask. Concentrated sulphuric acid (10ml) was added. 0.5g catalyst mixture containing sodium sulphate (Na_2SO_4), copper sulphate ($CuSO_4$) and selenium oxide (SeO_2) in the ratio 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 3hours until the liquid turned light green. The digested sample was cooled and diluted with distilled water to 100ml in standard volumetric flask. 10ml 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 4drops of bromocresol green/methyl

red indicator until about 70ml of distillate was collected. The distillate was then titrated with standardize 0.01N hydrochloric acid to grey colour

$$\text{Percentage Nitrogen} = \frac{(a - b) \times 0.01 \times 14 \times V \times 100}{W \times C}$$

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.

3.9.3 Hydrolysis of sample

Defatted GH sample was weighed (1g) into glass ampoule. 7ml of 6M HCl 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 22hours. The ampoule was allowed to cool before broken open at the tip and the content was filtered to remove the residue. 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 acetate buffer (pH 2.0) and stored in plastic specimen bottles, which were kept in the freezer.

3.9.4 Loading hydrolysate into TSM analyser

The sample (5µl) was dispensed into the cartridge of the analyzer. The TSM analyzer was designed to separate and analyzes free acidic, neutral and basic amino acids of the hydrolysate. The period of an analysis lasted for 76minutes.

3.9.5 Calculating Amino Acid Values from the Chromatogram Peaks

The net height of each peak produced by the chart recorder of TSM (each representing an Amino acid) 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 with a calibrated ruler and recorded. Approximate area of peak was 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:

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

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

Where $S_{std} = NE_{std} \times$ Molecular weight $\times$ μMAA_{std}

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

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

Where $$C = \frac{\text{Dilution} \times 16}{\text{Sample wt(g)} \times \text{N\%} \times 10 \times \text{Volume loaded}} \div \mathrm{NH} \times \mathrm{W\ (Nle)}$$

NH = Net height

W = Width @ half height

Nle = Norleucine

3.10 STATISTICAL ANALYSIS

SPSS v20 statistical package was used to analyse data. The results were expressed as mean ± standard deviation (SD) and data was analysed by one-way analysis of variance (ANOVA). The difference between various fermentation and pretreatment were computed using DUNCAN range multiple test. p value less than 0.05 were considered significant ($p<0.05$).

CHAPTER FOUR

4.0 RESULTS

4.1 ISOLATION AND IDENTIFICATION OF *Aspergillus niger* AND *Trichoderma viride*

4.1.1 Isolation

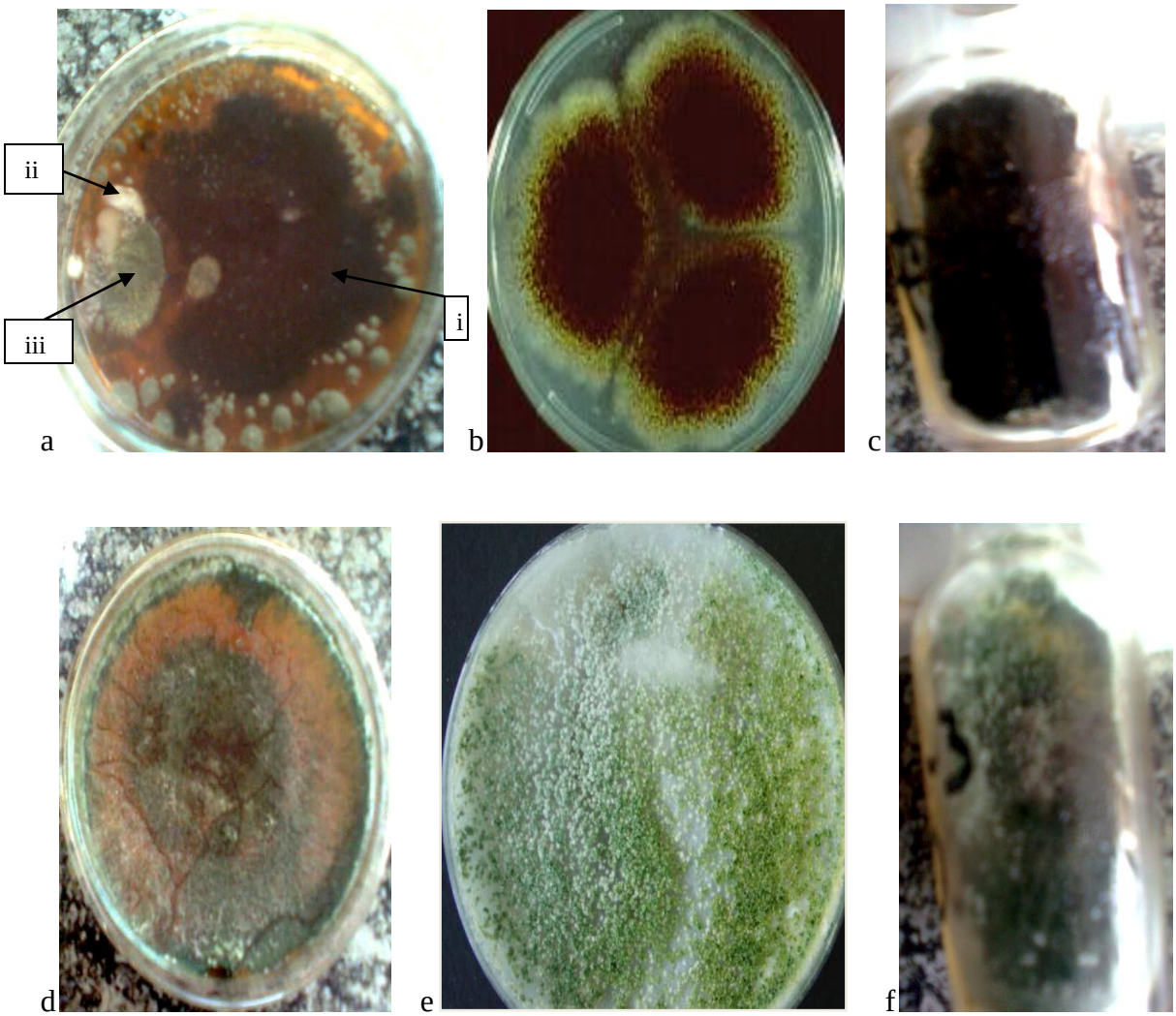
Three different colonies of microorganisms with different colours grew on the PDA plates (Figure 4.1a) initially inoculated after serial dilution of groundnut kernel suspension. Different microorganisms also grew on the plates (Figure 4.1d) inoculated after serial dilution of soil suspension. Black (*A. niger*) and blue green (*T. viride*) colonies from the former and latter plates were sub-cultured on different plates (fresh) to observe their pattern of growth as described below (4.1.2).

4.1.2 Identification

Isolates viewed under light microscope were identified based on morphological structures through comparison of structure obtained with that from Atlas of Fungi (Robert *et al*., 2004). Black isolate of *A. niger* was observed to possess distinct conidiophores which terminate by a swollen vesicle shaped phialide. The spore is black on the surface, white underneath and powdery in texture, which is typical of *A. niger* (Plate 4.1b).

The second isolate (*T. viride*) was whitish in colour with blue-green patches, which become visible as the conidia are formed and form concentric rings however on the reverse plate (when plate is turned upside down) the colour was yellowish. Growth rate was rapid (within 72 h) and colonies are initially wooly becoming compact with time. Microscopically, Conidiophores appear in pyramidal arrangements which are

branched and translucent. Conidia are round, green in color, smooth-walled and are grouped in sticky heads at the tips of the phialides (Plate 4.1e).



Key: ‘a’ Organisms present on groundnut kernel,

‘b’ Pure isolate of *A. niger*;

‘c’ Pure strain of *A. niger*;

‘d’ Organisms present in soil

‘e’ Pure isolate of *T. viride;*

‘f’ Pure strain of *T. viride*;

‘g’ Co-culture of pure strains of both organisms (*A. niger* and *T. viride*).

Plate 4.1: Isolates of Microorganisms (identified) from different sources.

4.2 TOTAL CELLULASE AND ENDOGLUCANASE ACTIVITIES

Total cellulase and endoglucanase activities at different incubation time (day: 1, 7 and 13) during fermentation of pretreated GH are shown in Table 4.1. Filter Paper Unit (FPU) (FP-ase) and Carboxymethyl cellulase (CMC) assays (CMC-ase) were respectively used for the assays above. For biological pretreated GH ($_{B}$GH), total cellulase activity when *A. niger* was inoculated was observed to increase significantly ($p<0.05$). *T. viride* activity was observed to fluctuate however the activity increased significantly for co-culture while no significant ($p<0.05$) change in activity was observed as incubation time increases in enzyme fermentation. For the chemical pretreatment, total cellulase activity of flask inoculated with *A. niger* was observed to decrease significantly ($p<0.05$), however the activity in co-culture fermentation fluctuate with significant decrease at the end of the fermentation period consequently, activity in the enzyme fermentation was observed to significantly decrease from 0.07 to 0.04 IU/ml/min, although the activity of *T. viride* decreased significantly ($p<0.05$) as days increased.

A. niger fermenting biologically pretreated GH was observe to have the best activity trend for total cellulase activity.

The Endoglucanase activity of biological pretreated GH on which *T. viride* ($_{B}GH_{T. viride}$) and co-culture spores ($_{B}GH_{cc}$) were inoculated was observed to significantly ($p<0.05$) decrease (0.19 to 0.03 IU/ml/min) and increase (0.02 to 0.06 IU/ml/min) respectively however the activity in *A. niger* increase rapidly within 6 days (0.04 to 0.17 IU/ml/min) after which it remained relatively constant and activity in enzyme fermentation increased gradually with no significant ($p<0.05$) difference. In

comparison with the latter assay, activities of *A. niger* and enzyme treatments of chemical pretreated GH ($_{C}$GH) decrease significantly ($p<0.05$) with time, activities in *T. viride* and co-culture treatments increased with *T. viride* activity increasing drastically (Table 4.1). Appendix II - V shows the graphical activity trend for total cellulase and Endoglucanase for the various microbial actions for the 14 days fermentation.

Table 4.1 Total cellulase (FPU) and Endoglucanase (CMC) activities on Pretreated GH

		Biol. Pretreated GH			**Chem. Pretreated GH**		
Treatment	Assay	Day 1	Day 7	Day 13	Day 1	Day 7	Day 13
A. niger	FPU (IU/ml/min)	0.09 ± 0.001^{b}	0.11 ± 0.001^{b}	0.10 ± 0.001^{c}	0.07 ± 0.001^{b}	0.08 ± 0.001^{c}	0.03 ± 0.00^{a}
T. viride		0.19 ± 0.003^{d}	0.09 ± 0.001^{a}	0.08 ± 0.002^{b}	0.14 ± 0.001^{c}	0.07 ± 0.001^{a}	0.06 ± 0.00^{e}
Co-culture		0.16 ± 0.002^{c}	0.09 ± 0.001^{a}	0.12 ± 0.001^{d}	0.05 ± 0.001^{a}	0.08 ± 0.001^{c}	0.04 ± 0.00^{b}
Enzyme extract		0.08 ± 0.002^{a}	0.09 ± 0.003^{a}	0.04 ± 0.002^{a}	0.07 ± 0.001^{b}	0.08 ± 0.001^{b}	0.04 ± 0.00^{d}
A. niger	CMC (IU/ml/min)	0.04 ± 0.002^{b}	0.17 ± 0.001^{d}	0.08 ± 0.001^{d}	0.034 ± 0.001^{c}	0.028 ± 0.001^{c}	0.01 ± 0.00^{a}
T. viride		0.19 ± 0.001^{c}	0.09 ± 0.002^{b}	0.03 ± 0.002^{b}	0.01 ± 0.001^{a}	0.03 ± 0.001^{bc}	0.03 ± 0.00^{b}
Co-culture		0.02 ± 0.003^{a}	0.10 ± 0.000^{c}	0.06 ± 0.001^{c}	0.03 ± 0.001^{b}	0.02 ± 0.001^{a}	0.03 ± 0.00^{b}
Enzyme extract		0.02 ± 0.001^{a}	0.02 ± 0.001^{a}	0.02 ± 0.003^{a}	0.03 ± 0.001^{b}	0.02 ± 0.001^{b}	0.01 ± 0.00^{a}

Mean ± SD. Values in the same column in different treatment with different superscript are significantly different ($p<0.05$)

4.3 EFFECT OF PRETREATMENTS ON LIGNIN AND CELLULOSE CONTENTS

It was observed that pretreatment has a reductive effect on the lignin content of GH with respect to control (Figure 4.1). However no significant change in lignin content was observed between chemical pretreated GH and biologically pretreated GH.

Cellulose content increased significantly ($p<0.05$) in both pretreated sets where biological pretreated GH has the highest cellulose content.

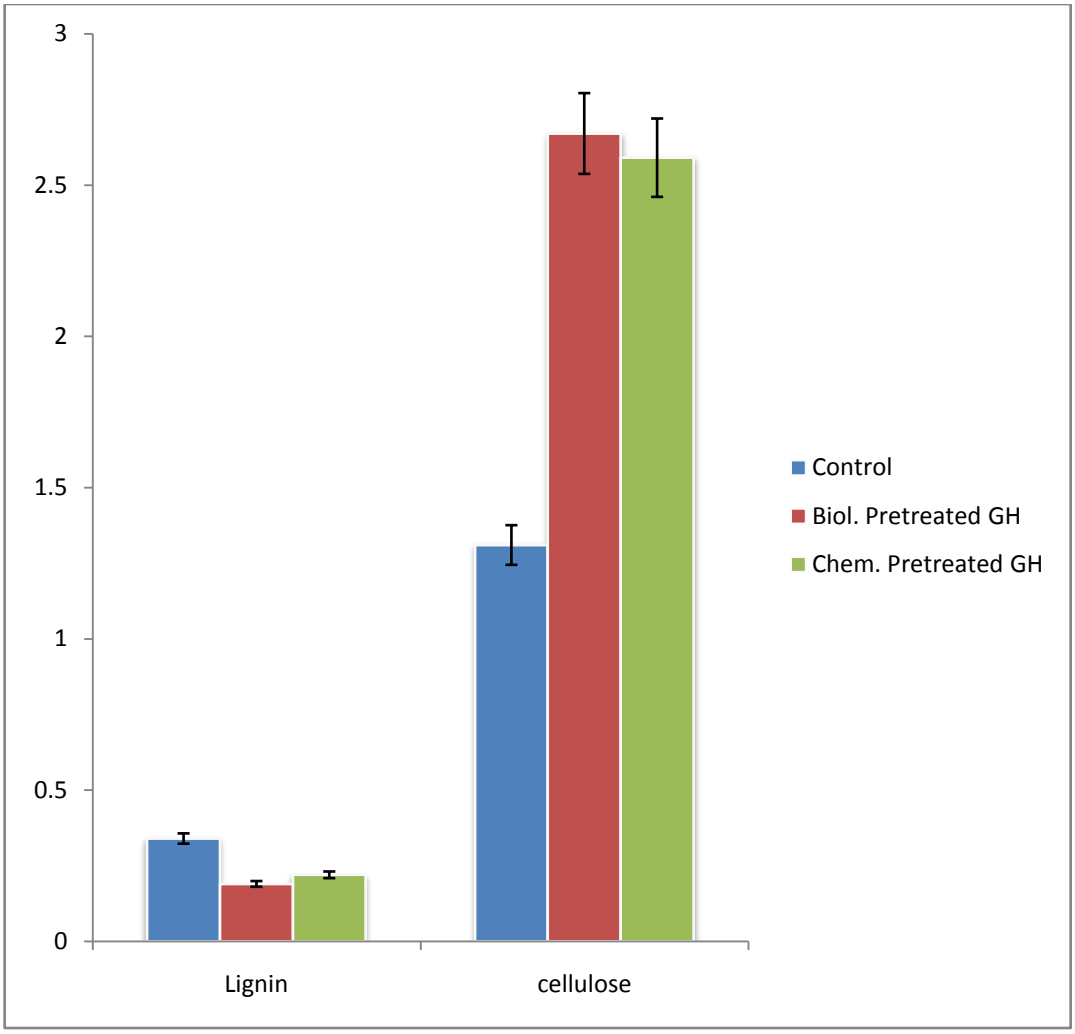


Figure 4.1: Effect of Pretreatment on Lignin and Cellulose content of GH samples.

4.4 CHEMICAL COMPOSITION

4.4.1 Proximate Composition

Percentage change in proximate composition of untreated, pretreated and treated groundnut husk (GH) is shown in Table 4.2. The moisture and fat contents of all fermented GH treatments decreased significantly ($p<0.05$) when compared with untreated GH. $_{C}GH_{A.\ niger}$ have the least fat value, 0.4% and chemical pretreated GH on which *T. viride* acted on ($_{C}GH_{T.\ viride}$) having the highest fat value (2.13%), as shown in Appendix VI of percentage change in proximate composition with respect to the control (uGH). Ash content also differs, with biological pretreated GH having significant increase compared to chemical pretreated GH although ash content within biological pretreatment was not significantly ($p<0.05$) different. The ash content for chemical pretreated GH with respect to the control (uGH) slightly decreased, with $_{C}GH_{A.\ niger}$ having the least value (1.35%). No difference was observed in protein content of untreated GH (uGH) and chemically pretreated GH ($_{C}GH$) however the content in other fermentations were observe to increase, 73% and 13% for $_{C}GH_{T.\ viride}$ and $_{C}GH_{enz}$ respectively. Significant ($p<0.05$) increase in protein was observed in the co-cultured fermentations; 6.95 and 6.64 for $_{B}GH_{cc}$ and $_{C}GH_{cc}$ respectively. There was decrease in fiber content of all treatments compared to untreated GH. Table 4.2 shows no significant ($p<0.05$) difference in carbohydrate content in $_{B}GH_{T.\ viride}$, $_{B}GH_{A.\ niger}$ and $_{C}GH_{A.\ niger}$ with respect to control. However significant ($p<0.05$) decrease in carbohydrate was observed for $_{C}GH_{T.\ viride}$.

Table 4.2: Proximate Composition (%) of untreated, pretreated and fermented groundnut husk

Sample	Moisture	Fat	Ash	Protein	Fiber	Carbohydrate
GH	5.61 ± 0.000^{h}	2.39 ± 0.007^{g}	2.53 ± 0.010^{cd}	4.87 ± 0.035^{a}	71.28 ± 0.725^{c}	84.82 ± 0.14^{bc}
$_{B}$GH	1.76 ± 0.020^{a}	1.53 ± 0.071^{b}	3.79 ± 0.025^{f}	4.73 ± 0.015^{a}	67.61 ± 0.610^{b}	85.19 ± 0.03^{c}
$_{B}$GH$_{A.\ niger}$	2.94 ± 0.049^{e}	1.86 ± 0.014^{d}	3.84 ± 0.015^{f}	7.81 ± 0.170^{d}	20.09 ± 0.140^{a}	83.56 ± 0.180^{bc}
$_{B}$GH$_{T.\ viride}$	2.29 ± 0.007^{c}	1.53 ± 0.014^{b}	3.75 ± 0.04^{f}	8.95 ± 0.170^{e}	19.02 ± 0.230^{a}	83.46 ± 0.01^{bc}
$_{B}$GH$_{cc}$	2.30 ± 0.000^{c}	1.72 ± 0.014^{c}	3.82 ± 0.03^{f}	6.95 ± 0.020^{c}	19.66 ± 0.285^{a}	85.21 ± 0.020^{c}
$_{B}$GH$_{enz}$	2.27 ± 0.028^{c}	2.51 ± 0.000^{h}	4.83 ± 0.02^{h}	5.06 ± 0.370^{b}	20.78 ± 0.170^{a}	85.00 ± 0.12^{c}
$_{C}$GH	2.81 ± 0.283^{d}	2.11 ± 0.021^{f}	2.47 ± 0.055^{c}	4.87 ± 0.170^{a}	83.23 ± 2.230^{e}	85.76 ± 0.32^{c}
$_{C}$GH$_{A.\ niger}$	4.20 ± 0.014^{f}	0.48 ± 0.354^{a}	1.35 ± 0.150^{a}	9.37 ± 0.140^{e}	72.78 ± 1.020^{c}	84.60 ± 0.66^{bc}
$_{C}$GH$_{T.\ viride}$	5.40 ± 0.014^{g}	2.13 ± 0.021^{f}	2.76 ± 0.025^{d}	10.45 ± 0.000^{f}	78.67 ± 0.135^{d}	79.27 ± 1.22^{a}
$_{C}$GH$_{cc}$	3.51 ± 0.480^{e}	1.99 ± 0.014^{e}	4.05 ± 0.000^{e}	6.64 ± 0.280^{c}	65.28 ± 0.200^{b}	82.37 ± 1.49^{ab}
$_{C}$GH$_{enz}$	3.25 ± 0.000^{d}	3.02 ± 0.010^{i}	4.15 ± 0.140^{e}	5.53 ± 0.000^{b}	66.25 ± 0.560^{b}	85.07 ± 1.94^{c}

Results are presented as Mean ± SD. Values in the same column with different superscript are significantly ($p<0.05$) different

Key: $_{B}$GH- Biological pretreated GH;

$_{B}$GH$_{A.niger}$-Biological pretreated GH on which *A. niger* is inoculated;

$_{B}$GH$_{T.viride}$-Biological pretreated GH on which *T. viride* is inoculated;

$_{B}$GH$_{cc}$- Biological pretreated GH on which both organism were inoculated;

$_{B}$GH$_{enz}$ -Biological pretreated GH on which crude enzyme from both organisms were inoculated;

$_{C}$GH - Chemical pretreated GH

$_{C}$GH$_{A.\ niger}$- Chemical pretreated GH on which *A. niger* is inoculated

$_{C}$GH$_{T.\ viride}$ - Chemical pretreated GH on which *T. viride* is inoculated

$_{C}$GH$_{cc}$- Chemical pretreated GH on which both organism were inoculated

$_{C}$GH$_{enz}$- Chemical pretreated GH on which crude enzyme from both organisms was inoculated.

4.4.2 Anti-Nutrient Content

Table 4.3 shows the Anti-nutritional content of untreated, pretreated and fermented GH. Alkaloid, Phytate and Saponin contents of all fermented GH were observed to significantly ($p<0.05$) decrease with respect to untreated GH, with $_{C}GH_{T.\ viride}$ having the least value (0.17mg) for saponin and $_{B}GH_{T.\ viride}$ (3212mg) been the least phytate content. Significant ($p<0.05$) decrease, in alkaloid content was observed although $_{C}GH_{cc}$ have high alkaloid content (1.79mg). Tannin contents of biological pretreated GH on which different fermentation was carried out increased significantly ($p<0.05$) compared to chemical pretreated GH fermentations and control, however no significant change in oxalate was observed for uGH, $_{B}GH$ and biological pretreated GH fermentations and uGH (22.09mg) and $_{C}GH_{A.\ niger}$ (22.03mg) while $_{C}GH_{T.\ viride}$ (32.80mg) and $_{C}GH_{cc}$ (32.79) increased significantly ($p<0.05$). Percentage change in minerals is shown in Appendix VII.

Anti-nutrient contents were observed to be relatively lower in $_{C}GH_{T.\ viride}$ with respect to the control.

Table 4.3: Anti-nutrient Content of untreated, pretreated and fermented groundnut husk

Sample	Saponin mg/100g	Alkaloid mg/100g	Oxalate mg/100g	Phytate g/100g	Tannin g/100g
GH	0.80 ± 0.100^{b}	1.03 ± 0.020^{f}	22.09 ± 0.315^{a}	53.42 ± 98.0^{h}	1.32 ± 0.25^{c}
$_{B}GH$	2.53 ± 0.100^{e}	0.65 ± 0.005^{a}	22.15 ± 0.250^{a}	41.65 ± 5.00^{d}	3.29 ± 1.59^{h}
$_{B}GH_{A.\ niger}$	1.88 ± 0.005^{d}	0.39 ± 0.010^{b}	27.94 ± 0.060^{b}	51.17 ± 2.50^{g}	1.93 ± 5.63^{e}
$_{B}GH_{T.\ viride}$	1.04 ± 0.035^{c}	0.35 ± 0.002^{b}	21.99 ± 0.010^{a}	32.12 ± 12.00^{a}	2.30 ± 1.64^{g}
$_{B}GH_{cc}$	0.99 ± 0.025^{c}	1.69 ± 0.030^{h}	22.25 ± 0.150^{a}	43.18 ± 1.50^{e}	1.31 ± 0.87^{c}
$_{B}GH_{enz}$	0.99 ± 0.005^{c}	0.99 ± 0.005^{ef}	22.40 ± 0.000^{a}	37.47 ± 3.00^{b}	2.02 ± 0.89^{f}
$_{C}GH$	4.13 ± 0.040^{f}	0.75 ± 0.010^{c}	28.03 ± 0.025^{b}	40.05 ± 5.50^{c}	3.29 ± 1.59^{h}
$_{C}GH_{A.\ niger}$	1.89 ± 0.025^{d}	1.13 ± 0.015^{g}	22.03 ± 0.370^{a}	47.98 ± 1.50^{f}	1.64 ± 0.79^{d}
$_{C}GH_{T\ .viride}$	0.17 ± 0.015^{a}	0.97 ± 0.010^{e}	32.80 ± 0.800^{d}	41.63 ± 3.50^{d}	0.98 ± 0.96^{b}
$_{C}GH_{cc}$	1.02 ± 0.030^{c}	1.79 ± 0.025^{i}	32.79 ± 0.810^{d}	51.22 ± 2.00^{g}	0.85 ± 2.61^{a}
$_{C}GH_{enz}$	1.00 ± 0.000^{c}	0.82 ± 0.000^{d}	30.41 ± 0.405^{c}	40.47 ± 3.0^{c}	0.99 ± 0.62^{b}

Results are presented as Mean ± SD. Values in the same column with different superscript are significantly ($p<0.05$) different.

Key: $_{B}GH$- Biological pretreated GH; $_{C}GH$ - Chemical pretreated GH

$_{B}GH_{A.niger}$-Biological pretreated GH on which *A. niger* is inoculated; $_{C}GH_{A.\ niger}$- Chemical pretreated GH on which *A. niger* is inoculated

$_{B}GH_{T.viride}$-Biological pretreated GH on which *T. viride* is inoculated; $_{C}GH_{T.\ viride}$ - Chemical pretreated GH on which *T. viride* is inoculated

$_{B}GH_{cc}$- Biological pretreated GH on which both organism were inoculated; $_{C}GH_{cc}$- Chemical pretreated GH on which both organism were inoculated

$_{B}GH_{enz}$ -Biological pretreated GH on which crude enzyme from both was inoculated; $_{C}GH_{enz}$- Chemical pretreated GH on which crude enzyme from both organisms organism inoculated.

4.4.3 Mineral Composition

Copper, Iron, Sodium and Manganese contents in all fermentations were observed to decrease significantly with Copper having the least value (0.02ppm for $_{C}GH_{T.\ viride}$ and $_{C}GH_{A.\ niger}$) followed by Iron (30ppm for $_{C}GH_{T.\ viride}$), these are shown in Table 4.4 of mineral composition of untreated, pretreated and fermented groundnut husk (GH). However Calcium, Magnesium, zinc and Phosphorous content increased significantly ($p<0.05$), with $_{B}GH$ having the highest value (4.6ppm) for Manganese and 72% increase (10.3ppm) in magnesium for $_{B}GH_{cc}$ (Appendix VIII). Significant increase in phosphorous content for $_{B}GH$ was observed although there was a decrease after fermentation while no significant difference for control and $_{C}GH$.

Table 4.4: Mineral Composition (ppm) of untreated, pretreated and fermented groundnut husk

Sample	Cu	Zn	Mn	Mg	Fe	Ca	Na	P
GH	0.12 ± 0.001^{d}	1.40 ± 0.16^{c}	0.81 ± 0.00^{e}	6.94 ± 0.10^{e}	5.93 ± 0.02^{g}	15.58 ± 0.08^{e}	880 ± 20^{g}	774.99 ± 14.31^{a}
$_{B}$GH	0.10 ± 0.006^{c}	11.50 ± 0.01^{e}	4.55 ± 0.18^{g}	3.98 ± 0.78^{c}	5.86 ± 0.04^{g}	8.36 ± 0.05^{c}	470 ± 20^{cd}	1334.71 ± 4.60^{c}
$_{B}$GH$_{A.\ niger}$	0.03 ± 0.003^{ab}	0.31 ± 0.00^{a}	0.11 ± 0.12^{ab}	0.31 ± 0.00^{a}	0.63 ± 0.19^{b}	11.27 ± 0.01^{d}	70 ± 20^{a}	1033.27 ± 67.05^{c}
$_{B}$GH$_{T.\ viride}$	0.02 ± 0.003^{ab}	20.31 ± 0.07^{f}	0.64 ± 0.01^{de}	7.75 ± 0.26^{f}	4.01 ± 0.05^{f}	37.51 ± 0.13^{h}	260 ± 10^{b}	818.05 ± 68.05^{b}
$_{B}$GH$_{cc}$	0.02 ± 0.003^{a}	21.99 ± 0.14^{g}	0.28 ± 0.00^{abc}	11.96 ± 0.49^{i}	12.30 ± 0.07^{i}	25.21 ± 0.12^{f}	800 ± 50^{f}	1119.43 ± 39.21^{c}
$_{B}$GH$_{enz}$	0.12 ± 0.001^{d}	23.50 ± 0.00^{h}	0.91 ± 0.01^{e}	9.38 ± 0.25^{g}	6.75 ± 0.02^{h}	5.213 ± 0.51^{b}	400 ± 20^{c}	1033.27 ± 67.05^{c}
$_{C}$GH	0.20 ± 0.000^{a}	3.01 ± 0.23^{d}	0.46 ± 0.22^{cd}	1.54 ± 0.06^{b}	1.89 ± 0.01^{c}	33.17 ± 0.13^{g}	670 ± 10^{e}	774.99 ± 14.31^{a}
$_{C}$GH$_{A.\ niger}$	0.02 ± 0.001^{a}	20.42 ± 0.19^{f}	0.32 ± 0.01^{bc}	5.35 ± 0.022^{d}	3.18 ± 0.01^{e}	33.73 ± 0.13^{g}	620 ± 30^{e}	774.99 ± 14.31^{a}
$_{C}$GH$_{T.\ viride}$	0.02 ± 0.004^{ab}	0.76 ± 0.00^{b}	0.00 ± 0.00^{a}	0.18 ± 0.001^{a}	0.11 ± 0.02^{a}	0.794 ± 0.26^{a}	30 ± 0.0^{a}	301.39 ± 21.10^{a}
$_{C}$GH$_{cc}$	0.11 ± 0.003^{d}	22.27 ± 1.40^{g}	0.70 ± 0.01^{de}	10.27 ± 0.474^{h}	2.19 ± 0.02^{d}	82.59 ± 1.34^{i}	770 ± 20^{f}	1334.71 ± 4.60^{c}
$_{C}$GH$_{enz}$	0.03 ± 0.009^{b}	1.13 ± 0.00^{c}	2.15 ± 0.08^{f}	1.71 ± 0.026^{b}	0.56 ± 0.01^{b}	8.322 ± 0.05^{c}	480 ± 10^{d}	731.94 ± 41.43^{b}

Results are presented as Mean ± SD. Values in the same column with different superscript are significantly ($p<0.05$) different.

Key:

$_{B}$GH- Biological pretreated GH;

$_{B}$GH$_{A.niger}$-Biological pretreated GH on which *A. niger* is inoculated;

$_{B}$GH$_{T.viride}$-Biological pretreated GH on which *T. viride* is inoculated;

$_{B}$GH$_{cc}$- Biological pretreated GH on which both organism were inoculated;

$_{B}$GH$_{enz}$ -Biological pretreated GH on which crude enzyme from both organism was inoculated;

$_{C}$GH - Chemical pretreated GH

$_{C}$GH$_{A.\ niger}$- Chemical pretreated GH on which *A. niger* is inoculated

$_{C}$GH$_{T.\ viride}$ - Chemical pretreated GH on which *T. viride* is inoculated

$_{C}$GH$_{cc}$- Chemical pretreated GH on which both organism were inoculated

$_{C}$GH$_{enz}$- Chemical pretreated GH on which crude enzyme from both organisms was inoculated.

4.4.4 Amino Acid Composition

Amino acids (G, A, V, L, I, S, T, C, M, D, Y, R, E, K, H, P and F) were assayed and depicted in spectra in Appendix IX-XVI while Table 4.5 show the calculated values for each amino acid. Concentration of amino acids was represented in peaks with Norleucine (synthetic amino acid) as internal standard used for quantitative determinations of other amino acids. Aspartic acid was observed to be highest in all fermented GH samples, with $_{C}GH_{A.\ niger}$ sample been the highest (13.60g/100g) and 10.26g/100g for $_{C}GH_{cc}$ been the least. Glutamic acid, proline and leucine were observed to have a higher concentration (longer peak) than other amino acids, $_{C}GH_{T.\ viride}$ and $_{B}GH_{A.\ niger}$ were observed to have glutamic acid concentration of 10.50 and 8.67g/100g respectively. The highest concentration (8.58g/100g) of proline was recorded for $_{C}GH_{A.\ niger}$ with $_{B}GH_{A.\ niger}$ having the least value (2.78g/100g). Leucine was observed to increase in $_{B}GH_{A.\ niger}$ with respect to the control (GH_{u}) however there was a slight decrease in the other samples. Lysine was observed to be low in all treatments with the exception of $_{B}GH_{A.\ niger}$ which has a high concentration of 10.28mg/100g protein. Tyrosine, threonine, arginine and histidine contents were all observed to be low in concentration in all fermented GH with the exception of $_{B}GH_{A.\ niger}$. Glycine was observed to be higher in $_{B}GH_{A.\ niger}$ than the standard and control. However, sulphur containing amino acids such as Cysteine and Methionine were not detected in all GH samples.

Table 4.5 Amino acid concentration of untreated and fermented GH

Amino Acid (g/100g)	Standard	GH (Control)	$_{B}GH_{A.\ niger}$	$_{B}GH_{T.\ viride}$	$_{B}GH_{cc}$	$_{C}GH_{A.\ niger}$	$_{C}GH_{T.\ viride}$	$_{C}GH_{cc}$
Lys	13.20	4.56	10.28	4.32	8.28	5.51	4.00	3.22
His	15.28	2.41	6.00	4.38	4.57	2.32	2.38	1.68
Arg	9.10	3.15	1.62	3.00	2.62	2.98	2.98	2.21
Asp	8.68	13.60	6.93	13.16	10.26	10.41	13.00	10.00
Thr	7.81	3.29	4.00	3.01	2.24	3.21	3.01	2.19
Ser	8.08	4.47	2.91	4.17	2.70	4.35	4.17	2.40
Glu	11.40	10.87	8.67	10.52	8.53	10.30	10.52	8.50
Pro	15.95	8.82	2.78	8.24	5.34	8.58	8.24	5.28
Gly	6.16	4.76	6.71	4.53	3.33	4.71	4.53	3.30
Ala	9.97	4.47	4.23	4.39	3.40	4.23	4.39	3.35
Val	9.76	4.90	4.02	4.02	3.01	4.78	4.72	3.01
Ile	8.97	3.69	4.00	4.00	2.02	3.69	3.52	2.00
Leu	8.07	6.34	6.57	6.51	5.08	6.16	6.28	5.01
Tyr	15.93	1.32	1.65	1.59	1.16	1.16	1.16	1.11
Phe	10.59	4.05	3.26	3.30	2.99	3.96	3.78	2.99

Key:

$_{B}GH$- Biological pretreated GH;

$_{B}GH_{A.niger}$-Biological pretreated GH on which *A. niger* is inoculated;

$_{B}GH_{T.viride}$-Biological pretreated GH on which *T. viride* is inoculated;

$_{B}GH_{cc}$- Biological pretreated GH on which both organism were inoculated;

$_{B}GH_{enz}$ -Biological pretreated GH on which crude enzyme from both organism was inoculated;

$_{C}GH$ - Chemical pretreated GH

$_{C}GH_{A.\ niger}$- Chemical pretreated GH on which *A. niger* is inoculated

$_{C}GH_{T.\ viride}$ - Chemical pretreated GH on which *T. viride* is inoculated

$_{C}GH_{cc}$- Chemical pretreated GH on which both organism were inoculated

$_{C}GH_{enz}$- Chemical pretreated GH on which crude enzyme from both organism was inoculated.

CHAPTER FIVE

5.0 DISCUSSION

The overlap of growth of the two microorganisms (*A. niger* and *T. viride*) indicate that both organisms can co-habit in a co-culture (mixed) fermentation without antagonism, however this study is consistent with that by Ikram-ul-Haq *et al.*, (2006), who reported that simultaneous mixing of both fungi have 59-66% more cotton saccharifying activity as compared to their pure cultures and other combinations. Hence *A. niger* and *T. viride* were co-cultured (Figure 4.1g) however the co-culture of these microorganisms, enzyme extract and metabolic product of one will not antagonize the other.

It was observed that activity profile for all fermentation of both pre-treatments increased within the first day, then gradually decreased for total cellulase activity. Both pretreatments had highest activity after day seven, 0.111IU for biological pretreatment and 0.084IU for chemical pretreated GH. This might be due to structural changes in cellulose as reported by Subramaniam (2007), suggesting that these changes will increase the substrate in susceptibility to microbe and enzyme attack. Changes in cellulose structure involve pore structure, particle size, lignin and hemicellulose association, crystallinity and degree of polymerization. Similar report was made by Damisa *et al*., (2008) using *A. niger* on 2M NaOH pretreated bagasse, corn cob and corn straw which had 0.067, 0.049 and 0.504 IU enzyme activities respectively. Another study by Reddi and Narasimha, (2011) reported the total

cellulolytic activity of *A. niger*GNEM7 on 1N HCl pretreated pea seed husk to be 9.13IU/ml.

Endoglucanase activity was observed to be highest (0.191 IU/ml/min) in the biological pretreated sample when compared to the chemical pretreated sample (0.026IU/ml/min). These occurred at day one (1) and seven (7) respectively after inoculation of *T. viride* indicating that cellulose content of biological pretreated GH is readily available than that of chemical pretreated GH hence the high activity. This may also be attributed to increase in surface area and less crystalline structure of biological pretreated GH. Endoglucanase activity of $_{C}GH_{T.viride}$ was not significantly different for days 7 and 13. This might be due to the absence of β–glucosidase repression mechanism present in *A. niger* that stops further breakdown of cellobiose to simple sugars as a result of excess glucose. This study is in accordance with reports by Kocher *et al*., (2008), who had maximum cellulase production (FPase 0.09 and CMCase 0.12 IU/ml/min) after 8 days by *T. harzianum*8230 using rice straw as carbon source. Abd El-Zahel and Fadel (2010) reported maximum activity (1.17 IU/g) on rice straw pretreated with 1% NaOH using *T. reesei* F418.

It was also observed that the total cellulase and endoglucanase activities produced by simultaneous co-culture fermentation were slightly low when compared with the values of monoculture fermentations. This may be attributed to lack of synergy between both organisms due to fast growth of *T. viride* over *A. niger* as suggested by Ikram-ul-Haq *et al*., (2005).

The trend in the enzyme activity of the crude extract from both microorganisms is consistent with the report of Moses *et al*., (2012). They reported that a high level of β-glucosidase is necessary to avoid the accumulation of cellobiose, which is a strong inhibitor of endoglucanase and exoglucanase. In order to overcome the deficiency of β-glucosidase in a *Trichoderma sp.* derived enzyme system, the fungus can be co-cultured with another fungus, *Aspergillus* species, which is a good source of β-glucosidase. This was observed in the present study for the co-culture sets, where there was a gradual increase in total cellulase activity and a dwindling value in Endoglucanase activity from high to low and then high thus indicating a boost in activity by the co-cultured organism (*A. niger*). Moses *et al*., (2012) reported maximum cellulase activity on the 6^{th} day (18.5IU/ml) with β-glucosidase activity of 21.8IU/ml. It was postulated that cellulases from different microorganisms are closely related to each other and that endoglucanase enzyme component from one fungal species can operate with exoglucanase of another at least when both fungi have the "endoglucanase-exoglucanase" system.

The chart of cellulose and lignin show a negative correlation (Appendix XVIII) that is a decrease in lignin content led to an increase in cellulose content. This might be attributed to the fact that the pretreatment methods; alkali (2M NaOH) and biological (action of *U. maydis* spores), disrupt the compact nature of lignin which encapsulates macro and micro nutrients.

The results of proximate composition and analyzed fibre components in all treatments show an increase in the protein contents. The increase in crude protein value was partly due to the ability of the enzymes to increase the bioavailability of the protein hitherto encapsulated in the cell. Many workers have reported similar increase in protein content; Bachtar (2005) reported increase in crude protein when *A. niger* was inoculated on Sago fibre and cassava fibre, resulting to 16.5 and 18.5% protein increase, respectively. The author repeated it for cocoa shell and 21.9% increase in crude protein was recorded. The decrease in carbohydrate may be due to the ability of *A. niger* and/or *T. viride* to break the polysaccharides into monomer sugars which are then easily available for assays and for utilization by the organisms, this was also reported by Iyayi and Aderolu (2004). The improvement seen in the minerals (ash, manganese, calcium, phosphorous and magnesium) available may be explained by the ability of the organisms to elicit enzymes like phytase which increases the availability of phosphorus and this may invariably lead to improvement in the availability of other minerals such as zinc, manganese, calcium, copper and iron that are susceptible to chelation with phytate (Lawal *et al.*, 2012). Reduction of some anti-nutrients such as alkaloids, tannins and phytate in various treatments are consistent with reports of Vijayakumari *et al*., (1995), Wyss *et al*., (1999) and Nithya *et al*., (2006) that a significant reduction of various anti-nutritional components were observed during processing which dislodge the complexes form with proteins and metal ions therefore decreasing their dietary availability.

Amino acid composition in the samples was observed to decrease with respect to the standard although the concentration of glycine was higher in $_{B}GH_{A.\ niger}$ than both the standard and control. The decrease in amino acid content can be attributed to the uptake of these amino acids by the fungi for proper metabolism and growth, however the protein content of fungi is often small and they are deficient in sulfur-containing amino acids, this study is in accordance with report of Boze *et al*., 1992. High synthesis of acidic amino acids (aspartate and glutamate) may be due to the fact that aspartic acid prefers generally to be on the surface of proteins exposed to aqueous environment and thus easily accessible. When buried within the protein, aspartic acid (and glutamic acid) are frequently involved in salt-bridges, where they pair with a positively charged amino acid (such as Arginine) to create stabilizing H-bond that can be important for protein stability.

Comparing both pretreatments, biological pretreated GH produced a high concentration of essential amino acids such as lysine, histidine, phenylalanine, leucine, isoleucine, threonine, tryptophane and valine than chemical pretreated GH, this may be due to the synthesis of these amino acids by the organism used for pretreatment. High content of lysine in $_{B}GH_{A.niger}$ can be ascribed to increase in conversion of lipid to energy and absorption of calcium for fast and adequate growth of the organism.

Therefore, the treatment with high proximate content, less anti-nutrient content, relatively high amino acid content and relatively low mineral content, $_{B}GH_{\ T.\ viride}$ is considered the best for feed formulation.

CHAPTER SIX

6.0 SUMMARY, CONCLUSION AND RECOMMENDATIONS

6.1 SUMMARY

Bioconversion of lignocellulosic agro-residues like groundnut shell through mushroom cultivation and single cell protein production offer the potential for converting these residues into protein-rich palatable food and reduction of the environmental impact of the wastes.

A. niger and *T. viride* (isolated from groundnut seed and soil respectively) were used in monoculture fermentation, co-culture fermentation and crude enzyme extract fermentation from both organisms.

Groundnut husk (GH) was pretreated independently with 2M sodium hydroxide (Chemical) and spores of *Ustilago maydis* (Biological), after which biologically pretreated GH was found to significantly ($p<0.05$) decrease lignin content with consequent increase in cellulose.

Total cellulase and Endoglucanase activities were assayed for fourteen fermentation days to observe the trend of substrate utilization. Biological pretreated GH has a significantly ($p<0.05$) high activity, 0.19IU for total cellulase and Endoglucanase while chemical pretreated GH has 0.14IU and 0.034IU as highest activity for total cellulase and Endoglucanase respectively.

To ascertain which treatment is best for incorporation into non-ruminant feed proximate, anti-nutrients, minerals and amino acid contents were quantified. Chemical pretreated GH on which *T. viride* ($_{C}GH_{T.\ viride}$) was inoculated indicated significant increase in protein content (73%), decrease anti-nutrients and also increase in some minerals which are paramount in metabolic function and energy generation.

Significant ($p<0.05$) increase in threonine, histidine, aspartate, serine, glycine, lsoleucine, leucine and tyrosine were observed when comparing the control and some fermented GH treatments.

6.2 CONCLUSION

The results of this study indicate that action of microorganisms on pretreated GH is suitable and effective in increasing protein content, reducing various anti-nutrients without affecting the nutritional quality and quantity. Biological pretreatment was found to be more effective in decreasing lignin and increasing cellulose although Chemical pretreated GH on which *T. viride* fermented ($_{C}GH_{T.\ viride}$) was best in terms of increase in protein content and decrease in anti-nutrients. Although the mineral content is low and the amino acid content decreased with respect to the control ($_{u}GH$) and thus are not up to standard. Unlike other researches, this study contributes the fact that the quantity of essential amino acids increased when GH was pretreated biologically than chemically.

Hence such economic and potential processing methods could be adapted for versatile utilization of groundnut husk treated with *T. viride* as protein source. Incorporation of such fermented groundnut husk in non-ruminant diet will clearly reduce over dependence on common cereals or legumes for meeting protein source.

6.2 RECOMMENDATIONS

Sequel to results obtained from this study, $_{C}GH_{T.\ viride}$ is recommended for incorporation into or substitution as protein source in non-ruminant feed. Further research should be directed towards;

i. Screening microorganisms (*A. niger* and *T. viride*) by genetic modification to enable them degrade anti-nutrients such as phytate and tannins and also improve mineral content during fermentation.

ii. Feeding poultry and/or mice with upgraded GH to ascertain effect on growth rate and bioavailability of nutrients.

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APPENDIX I

Preparation of Basal Medium

The method of Subramaniam, 2007 with slight modification was used.

Basal medium was prepared by dissolving 5mg KNO_3, 2g KH_2PO_4, 0.5g $MgSO_4$ $7H_2O$, 3.5mg $FeSO_4$ $4H_2O$, 0.002g $ZnCl_2$, 0.002g $FeCl_2$, 0.05g $CaCl_2$, 0.28g $(NH_4)_2SO_4$, 0.06g Urea in 1000ml distilled water. This was used to moisten groundnut husk before use.

Preparation of Sodium Citrate Buffer

Citric acid (2.40g) was dissolve in 50ml of distilled water, 3.67g of Sodium citrate was also dissolved in 50ml distilled water. Both solutions were transferred into 400ml volumetric flask and made up to the mark to obtain 0.05M sodium citrate buffer.

Preparation of 67% v/v Sulphuric acid (H_2SO_4) Solution

To prepare 67% v/v of Sulphuric acid (H_2SO_4), the formula below was used

$$C_1V_1 = C_2V_2$$

Where;

C_1 is concentration of concentrated acid (100% H_2SO_4)

V_1 is the volume of concentrated acid

C_2 is concentration of H_2SO4 needed

V_2 is the volume of acid needed

$$V1 = \frac{C2 \times V1}{C1}$$

$$V1 = \frac{67 \times 160}{100}$$

$$= 107.2 \text{ ml}$$

Hence 107.2ml of Sulphuric acid was added to 52.8ml of distilled water to form 67% sulphuric acid. This procedure was used to prepare 72% and 1.25% v/v sulphuric acid.

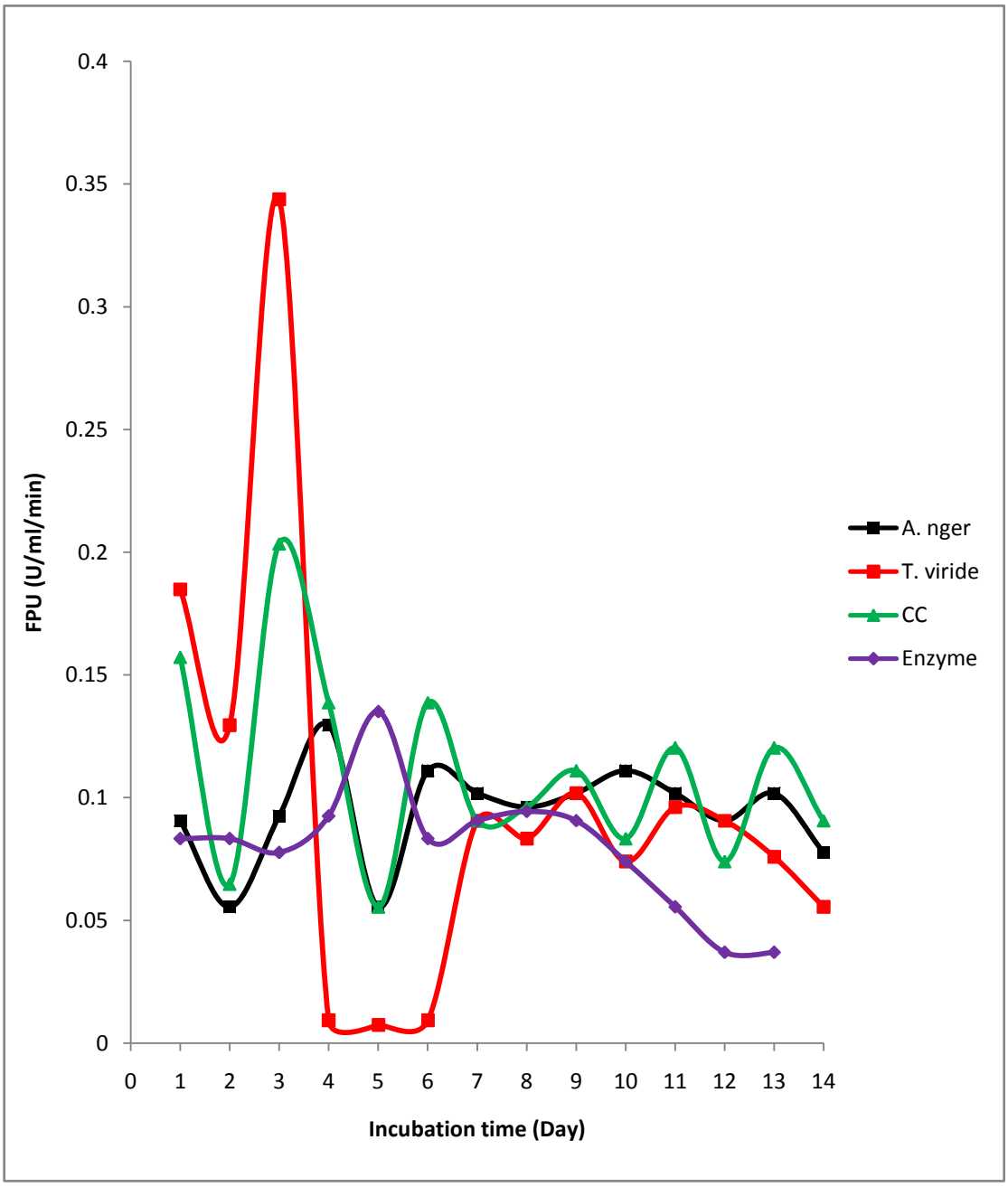
Preparation of Anthrone Reagent

Anthrone reagent (0.2g) was dissolve with 100ml of concentrated sulphuric acid (H_2SO_4) for use in cellulose assay.

Preparation of Reagent for Digestion

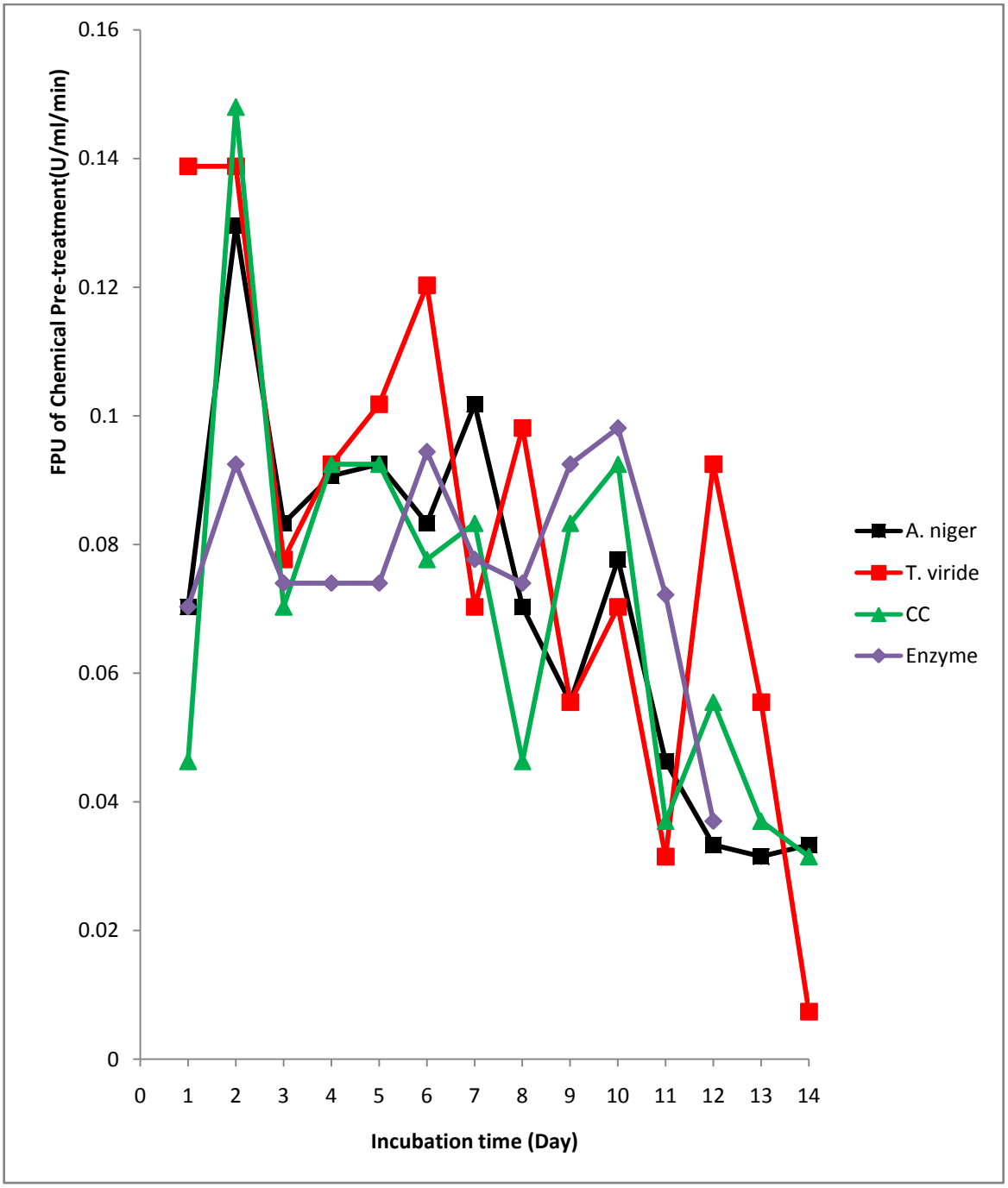
A 3:1 ratio of Hydrochloric acid (HCl): Nitric acid (HNO_3) was prepared by measuring 165ml of Hydrochloric acid (HCl) into 55ml of Nitric acid (HNO_3) to form a 220ml solution.

APPENDIX II



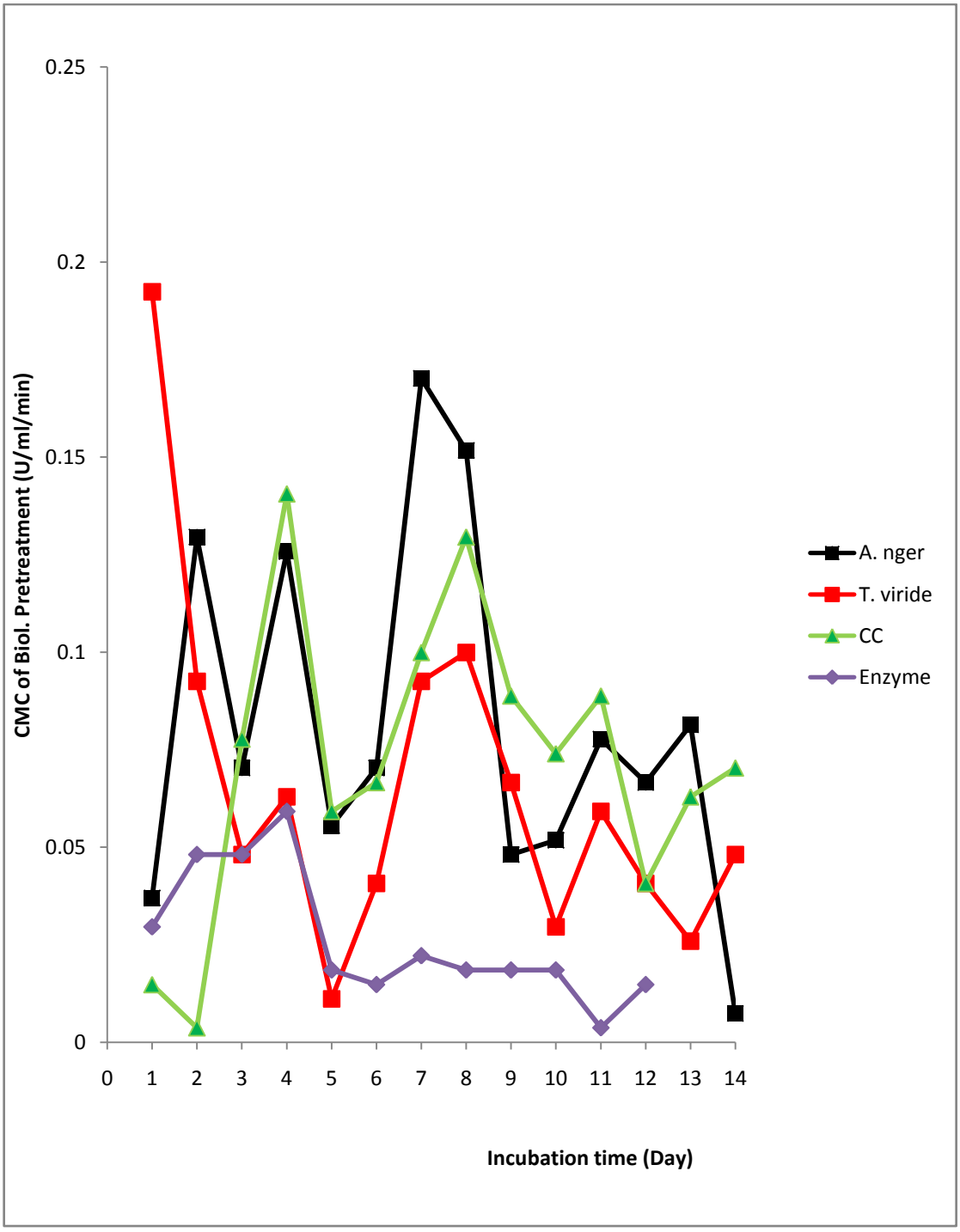
Total Cellulase activity of Biologically Pre-treated GH

APPENDIX III



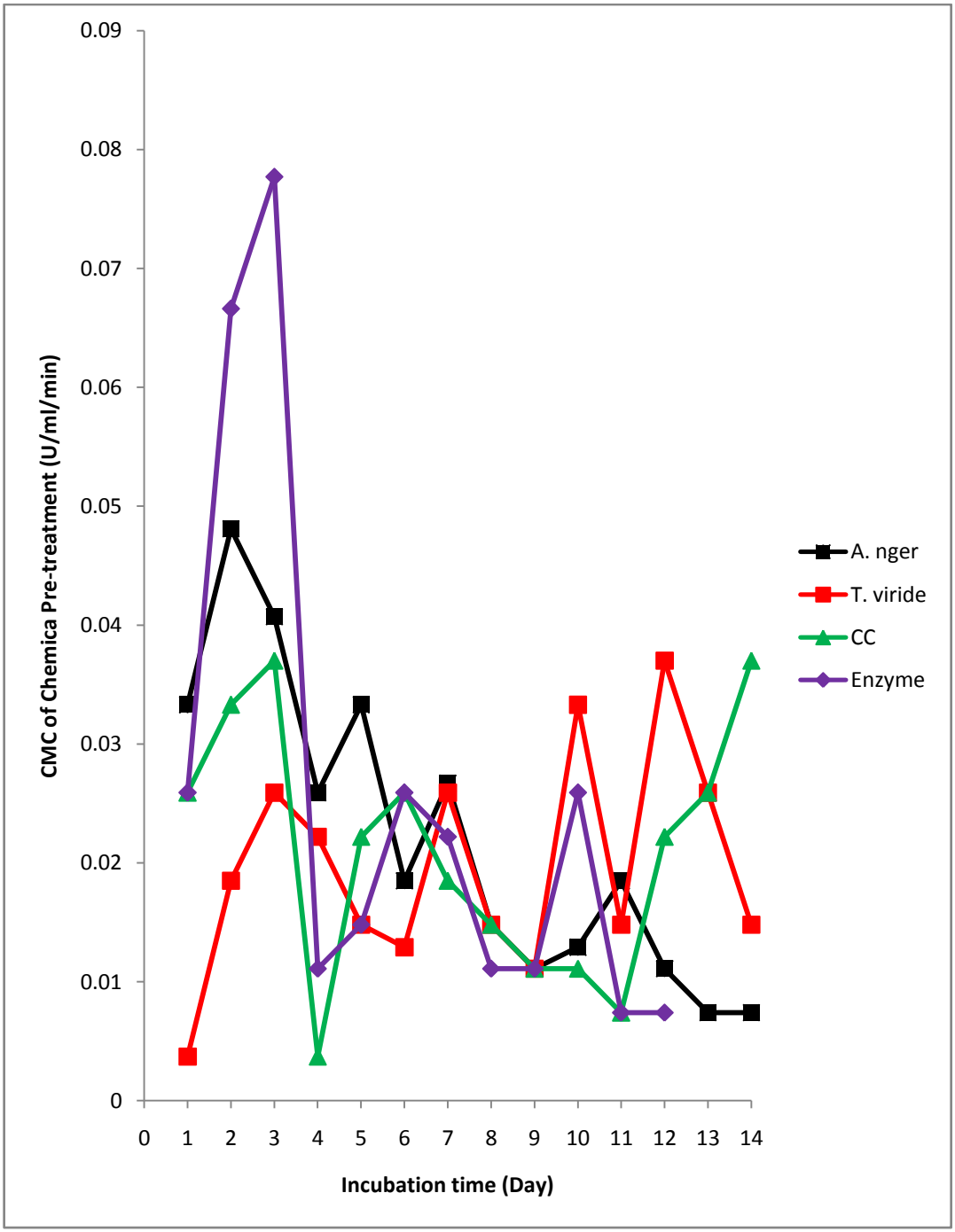
Total Cellulase activity of Chemically Pre-treated GH

APPENDIX IV



Endoglucanase activity Chart of Biologically pre-treated GH

APPENDIX V



Endoglucanase activity Chart of Chemically pre-treated GH

APPENDIX VI

Percentage Change of Proximate Composition With Respect to Control

Treatment	Moisture	Fat	Ash	Protein	Fiber	Carbohydrate
$_{B}GH$	68.63 ±0.50	36.33 ± 0.05	54.88 ± 7.46	23.43 ± 0.34	1.82 ± 7.24	0.31 ± 7.283
$_{B}GH_{A.\ niger}$	50.36 ± 4.66	22.34 ± 0.18	51.58 ± 0.01	19.41 ± 4.31	71.81 ± 0.67	0.12 ± 1.223
$_{B}GH_{T.\ viride}$	59 ± 0.13	36.12 ± 0.20	48.23 ± 3.07	63.83 ± 0.22	73.31 ± 0.84	0.07 ± 0.106
$_{B}GH_{cc}$	59 ±0.00	28.19 ± 0.40	51.00 ± 2.52	22.31 ± 0.66	72.42 ± 0.96	0.36 ± 2.312
$_{B}GH_{enz}$	55.06 ± 6.83	36.95 ± 0.39	50.60 ± 1.59	20.45 ± 0.23	71.26 ± 1.36	0.37 ± 6.194
$_{C}GH$	49.91 ± 0.51	12.11 ± 0.57	2.56 ± 3.62	0.00 ± 0.00	16.81 ± 6.09	2.76 ± 1.563
$_{C}GH_{A.\ niger}$	25.14 ± 0.25	1± 56.69	46.66 ± 8.09	72.13 ± 3.34	1.93 ± 3.19	0.08 ± 1.294
$_{C}GH_{T.\ viride}$	3.75 ± 0.25	11.27 ± 0.57	8.89 ± 0.79	73.67 ± 4.05	10.38 ± 1.85	0.05 ± 5.926
$_{C}GH_{cc}$	64.97 ±91.88	16.91 ± 0.17	20.39 ± 0.45	25.3 ± 3.00	8.41 ± 0.93	2.12 ± 5.08
$_{C}GH_{enz}$	59.90 ± 0.50	15.87 ± 0.32	15.43 ± 5.67	13.58 ± 2.17	7.03 ± 2.45	0.21 ± 5.41

Values are presented as Mean ± SD. **Key**: $_{B}GH$- Biological pretreated GH; $_{C}GH$ - Chemical pretreated GH

$_{B}GH_{A.niger}$-Biological pretreated GH on which *A. niger* is inoculated;

$_{B}GH_{T.viride}$-Biological pretreated GH on which *T. viride* is inoculated;

$_{B}GH_{cc}$- Biological pretreated GH on which both organism were inoculated;

$_{B}GH_{enz}$ -Biological pretreated GH on which crude enzyme from both organism was inoculated;

$_{C}GH_{A.\ niger}$- Chemical pretreated GH on which *A. niger* is inoculated

$_{C}GH_{T.\ viride}$ - Chemical pretreated GH on which *T. viride* is inoculated

$_{C}GH_{cc}$- Chemical pretreated GH on which both organism were inoculated

$_{C}GH_{enz}$- Chemical pretreated GH on which crude enzyme from both organism was inoculated

APPENDIX VII

Percentage Change in Anti-nutrient of fermented GH with respect to Control

	Saponin (mg/100g)	Alkaloid (mg/100g)	Oxalate (mg/100g)	Phytate (mg/100g)	Tannin (mg/100g)
$_{B}$**GH**	212.35 ± 1.75	93.7 ± 0.52	0.30 ± 0.42	22.01 ± 2.57	147.38 ± 3.71
$_{B}$**GH**$_{A.\ niger}$	131.48 ± 0.88	62.14 ± 0.34	26.31 ± 2.49	4.17 ± 2.42	46.02 ± 5.63
$_{B}$**GH**$_{T.\ viride}$	29.34 ± 3.90	66.47 ± 2.98	0.42 ± 1.95	39.85 ± 1.88	74.10 ± 1.29
$_{B}$**GH**$_{cc}$	24.36 ± 2.23	65.20 ± 10.04	0.76 ± 1.05	19.06 ± 1.95	0.47 ± 0.67
$_{B}$**GH**$_{enz}$	23.16 ± 3.06	3.35 ± 3.34	1.45 ± 2.04	29.84 ± 1.75	52.94 ± 1.37
$_{C}$**GH**	416.27 ± 2.06	27.14 ± 3.37	26.70 ± 3.04	24.99 ± 2.09	149.27 ± 1.03
$_{C}$**GH**$_{A.\ niger}$	136.88 ± 0.23	9.29 ± 5.06	0.26 ± 0.36	10.14 ± 2.29	24.64 ± 0.52
$_{C}$**GH**$_{T.\ viride}$	79.40 ± 2.28	5.81 ± 1.22	48.50 ± 2.13	22.04 ± 2.11	25.58 ± 0.82
$_{C}$**GH**$_{cc}$	27.48 ± 2.05	74.39 ± 8.22	48.45 ± 2.19	4.09 ± 2.54	35.71 ± 3.39
$_{C}$**GH**$_{enz}$	25.02 ± 2.21	20.36 ± 2.19	37.72 ± 5.36	24.22 ±1.89	24.70 ± 0.27

Values are presented as Mean ± SD.

Key: $_{B}$GH- Biological pretreated GH;

$_{B}$GH$_{A.niger}$-Biological pretreated GH on which *A. niger* is inoculated;

$_{B}$GH$_{T.viride}$-Biological pretreated GH on which *T. viride* is inoculated;

$_{B}$GH$_{cc}$- Biological pretreated GH on which both organism were inoculated;

$_{B}$GH$_{enz}$ -Biological pretreated GH on which crude enzyme from both organism was inoculated;

$_{C}$GH - Chemical pretreated GH

$_{C}$GH$_{A.\ niger}$- Chemical pretreated GH on which *A. niger* is inoculated

$_{C}$GH$_{T.\ viride}$ - Chemical pretreated GH on which *T. viride* is inoculated

$_{C}$GH$_{cc}$- Chemical pretreated GH on which both organism were inoculated

$_{C}$GH$_{enz}$- Chemical pretreated GH on which crude enzyme from both organism was inoculated

APPENDIX VIII

Percentage Change in Minerals of Fermented GH with Respect to Control

	Copper (ppm)	Zinc (ppm)	Manganese (ppm)	Magnesium (ppm)	Sodium (ppm)	Phosphorous (ppm)	Calcium (ppm)	Iron (ppm)
$_{B}$**GH**	12.771 ±8.31	720.30 ± 5.57	460.38 ± 14.20	42.60 ± 0.42	46.66 ± 4.55	72.27 ± 3.66	46.32 ± 0.11	1.20 ± 0.484
$_{B}$**GH**$_{A.\ niger}$	75.23 ±3.33	89.21 ± 15.86	86.74 ± 10.08	95.60 ± 0.05	92.05 ± 0.23	33.21 ±8.75	72.82 ± 0.33	89.30 ± 4.458
$_{B}$**GH**$_{T.\ viride}$	79.51 ±3.38	1348.50 ± 16.82	20.89 ±0.26	11.67 ± 3.04	195.09 ± 22.66	5.43 ± 9.66	27.66 ± 0.02	32.37 ± 0.767
$_{B}$**GH**$_{cc}$	81.22 ±3.38	1469.21 ±11.71	65.00 ±0.01	72.36 ± 6.64	365.90 ± 1.09	44.40 ± 3.38	140.83 ± 0.49	107.54 ± 0.51
$_{B}$**GH**$_{enz}$	1.71 ±0.02	1576.97 ± 27.07	11.00 ±64.00	35.10 ± 2.42	171.06 ± 103.78	33.21 ± 8.75	61.82 ± 0.04	13.83 ± 0.19
$_{C}$**GH**	82.91 ±0.21	114.68 ±19.84	43.28 ± 19.13	77.81 ± 0.69	23.96 ± 6.39	0.00 ±0.00	66.54 ±0.23	68.12 ±0.05
$_{C}$**GH**$_{A.\ niger}$	81.20 ±0.98	1359.16 ± 3.84	60.076 ± 0.45	22.95 ± 1.12	29.71 ± 10.11	0.00 ±0.00	112.96 ± 0.29	46.41 ± 0.03
$_{C}$**GH**$_{T.\ viride}$	79.52 ±4.59	45.57 ±1.08	0.00 ± 0.00	97.41 ± 0.03	96.60 ± 0.47	61.15 ± 2.84	94.91 ± 2.35	100.36 ±2.53
$_{C}$**GH**$_{cc}$	2.58 ±2.45	1448.49 ± 14.14	13.37 ±0.33	47.98 ± 6.65	1.29 ± 9.69	72.27 ± 3.66	430.18 ± 8.48	62.99 ± 0.23
$_{C}$**GH**$_{enz}$	70.92 ± 3.98	19.60 ±1.70	165.03 ± 6.05	75.36 ± 0.03	45.58 ± 7.57	5.64 ± 5.12	46.58 ± 0.11	90.50 ±0.07

Values are presented as Mean ± SD.

Key: $_{B}$GH- Biological pretreated GH;

$_{B}$GH$_{A.niger}$-Biological pretreated GH on which *A. niger* is inoculated;

$_{B}$GH$_{T.viride}$-Biological pretreated GH on which *T. viride* is inoculated;

$_{B}$GH$_{cc}$- Biological pretreated GH on which both organism were inoculated;

$_{B}$GH$_{enz}$ -Biological pretreated GH on which crude enzyme from both organism was inoculated;

$_{C}$GH - Chemical pretreated GH

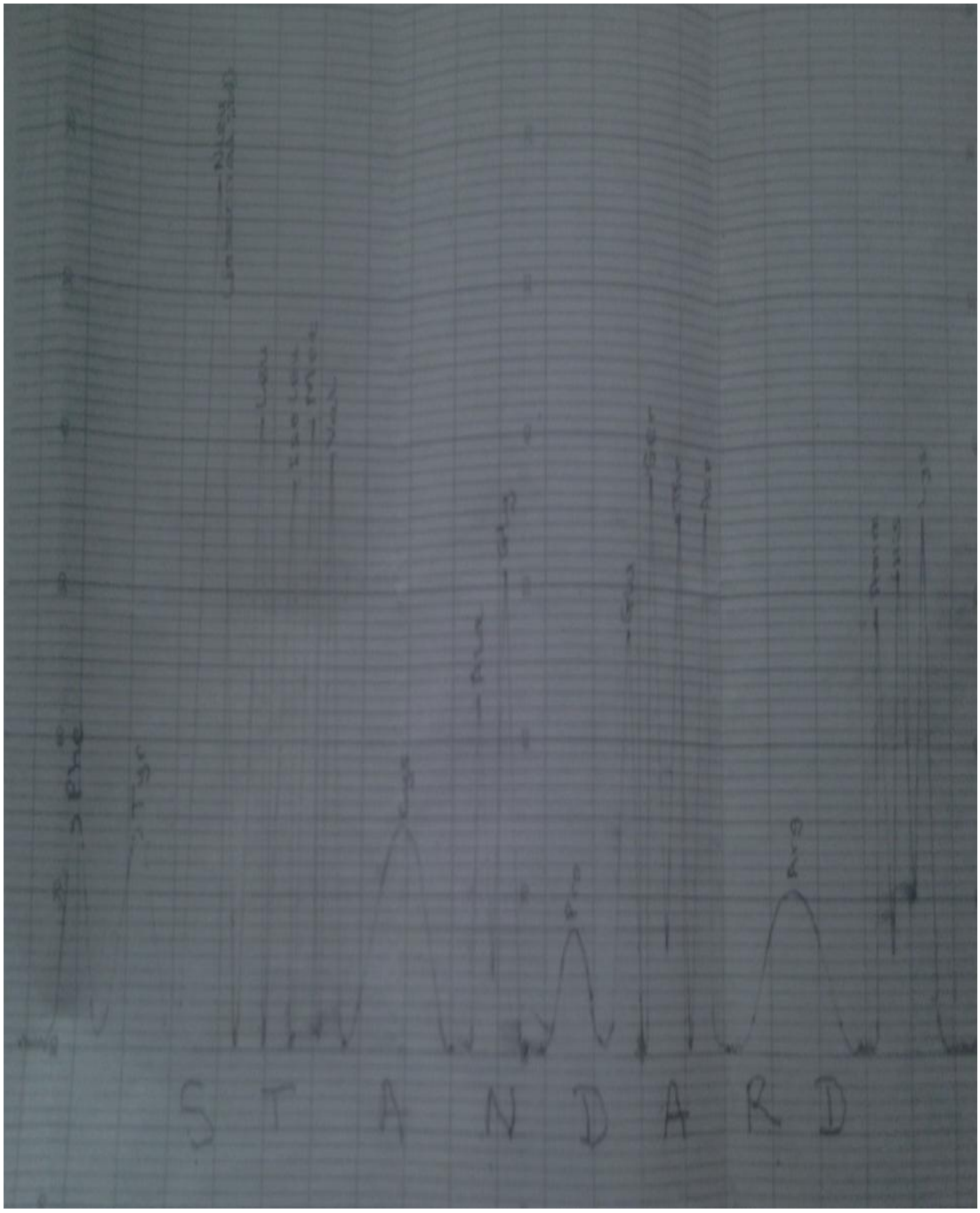
$_{C}$GH$_{A.\ niger}$- Chemical pretreated GH on which *A. niger* is inoculated

$_{C}$GH$_{T.\ viride}$ - Chemical pretreated GH on which *T. viride* is inoculated

$_{C}$GH$_{cc}$- Chemical pretreated GH on which both organism were inoculated

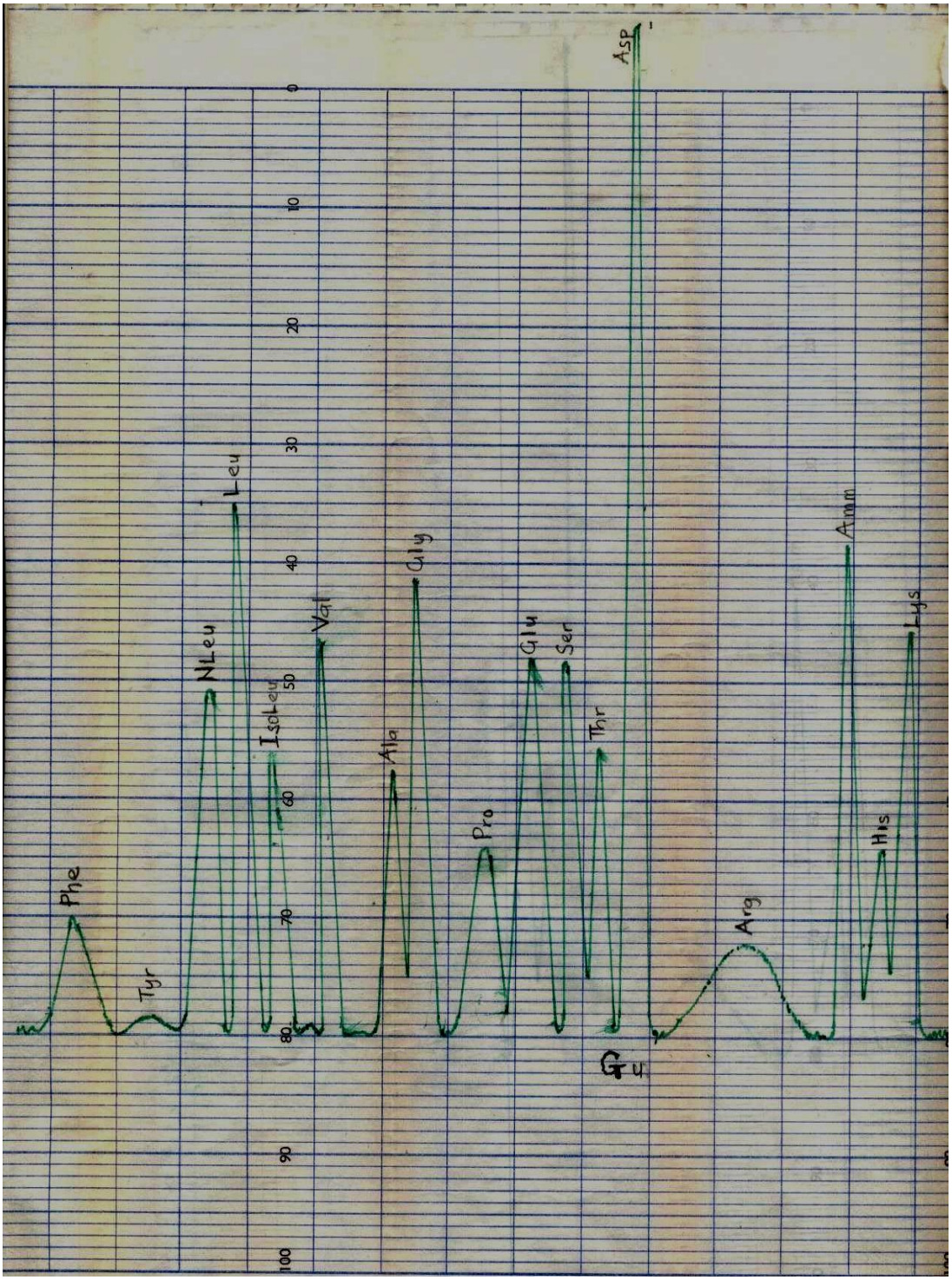
$_{C}$GH$_{enz}$- Chemical pretreated GH on which crude enzyme from both organism was inoculated

APPENDIX IX



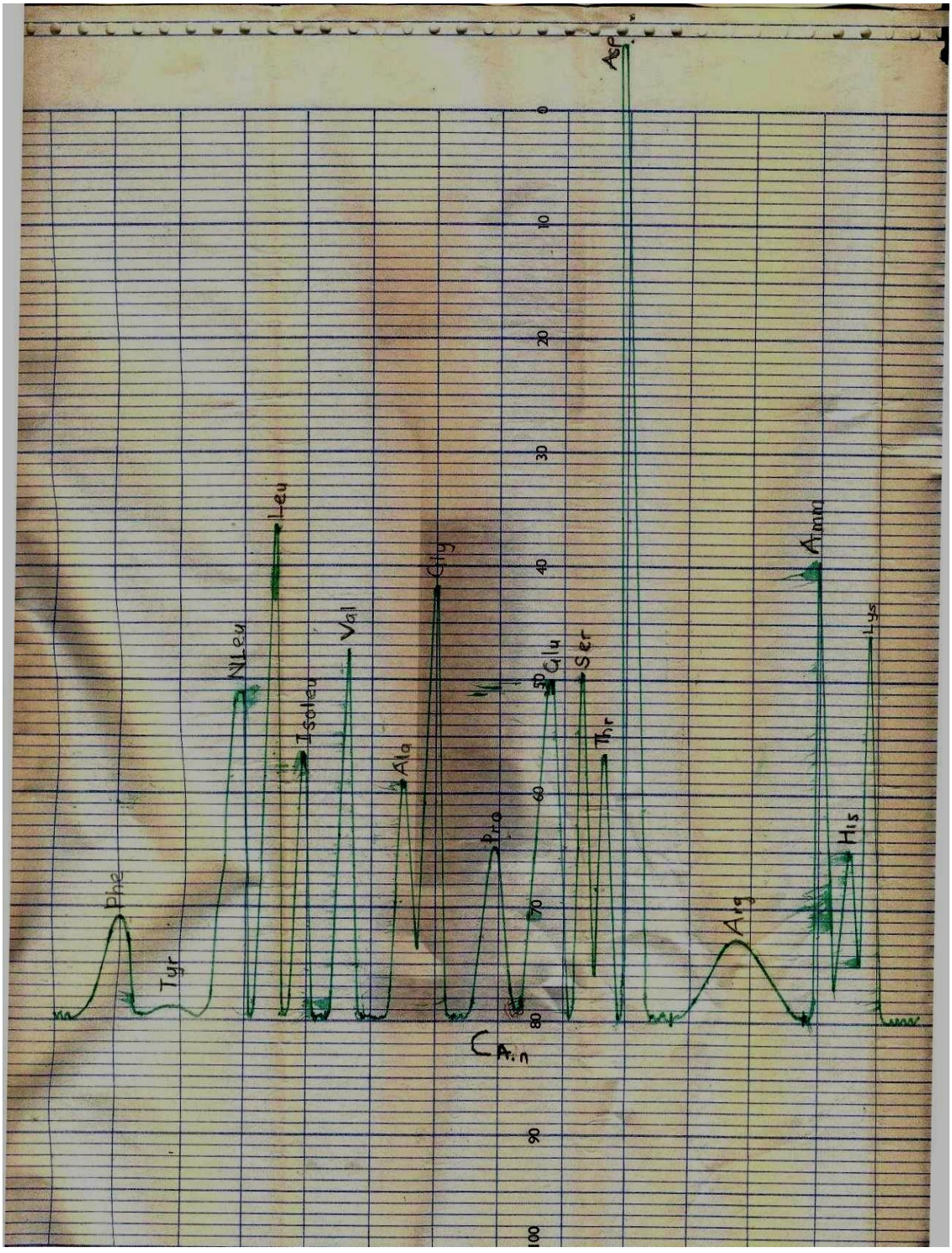
Standard Amino Acid Profile

APPENDIX X



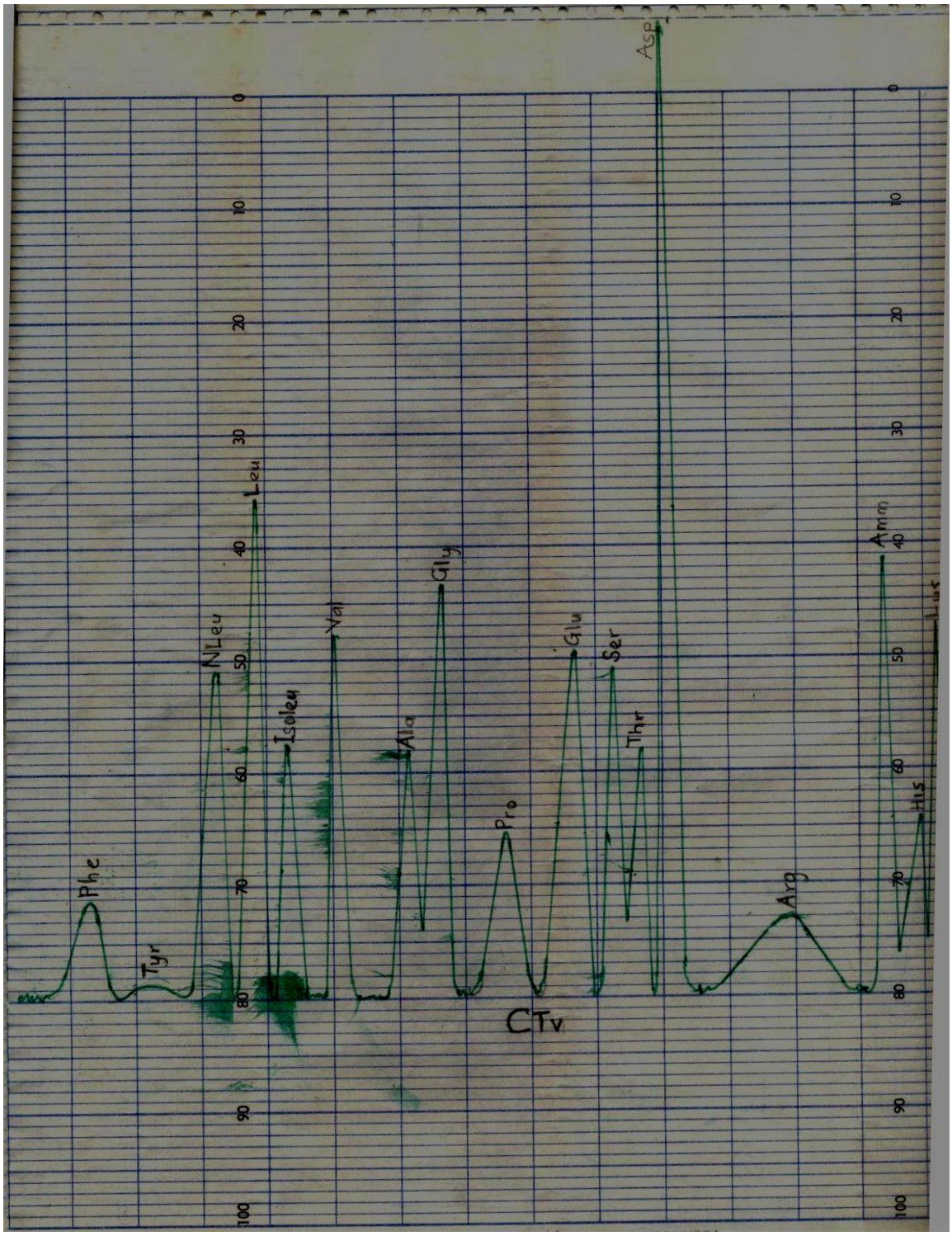
Amino Acid Profile of untreated GH (G_u) (Control)

APPENDIX XI



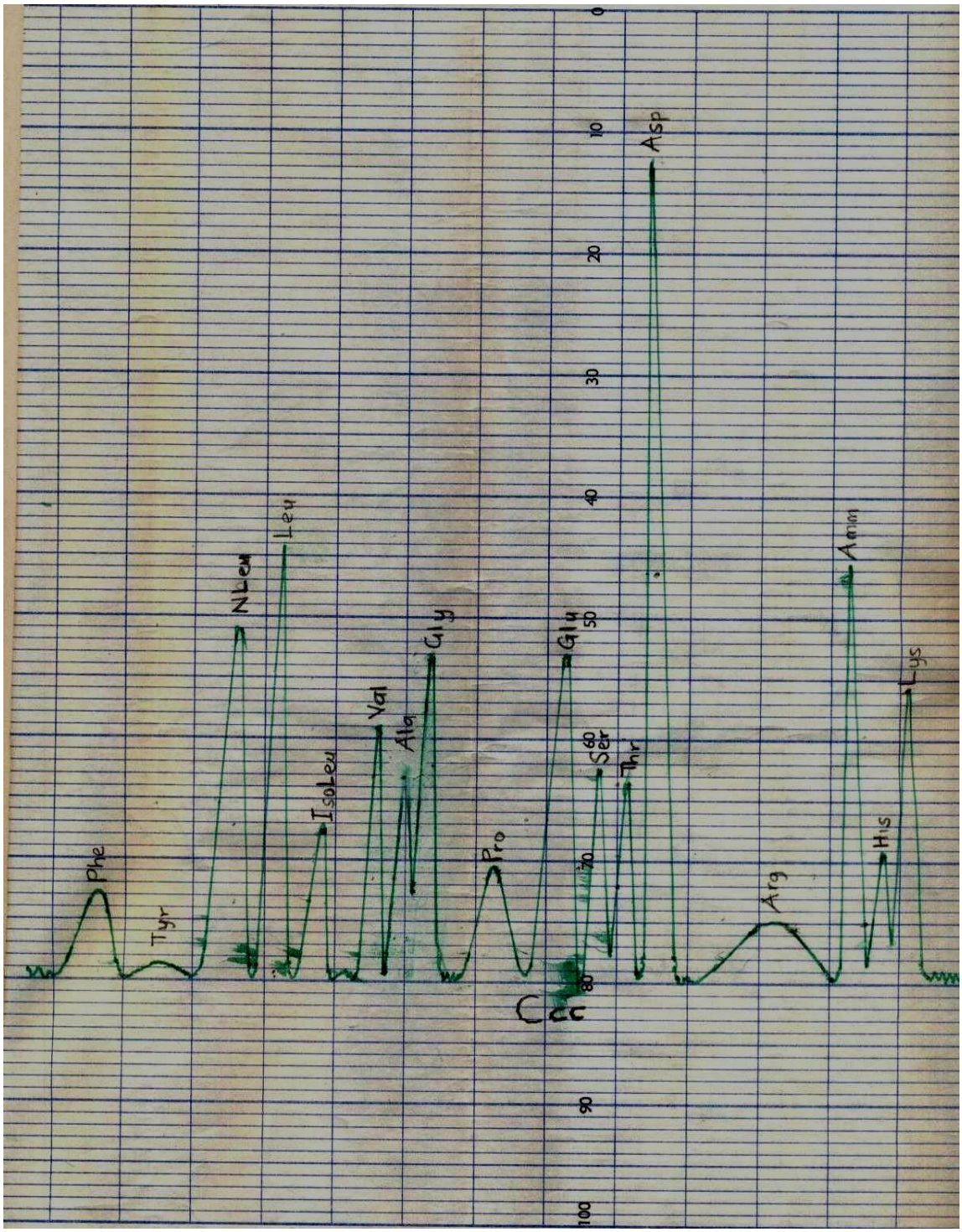
Amino Acid Profile of cGH*A. niger*

APPENDIX XII



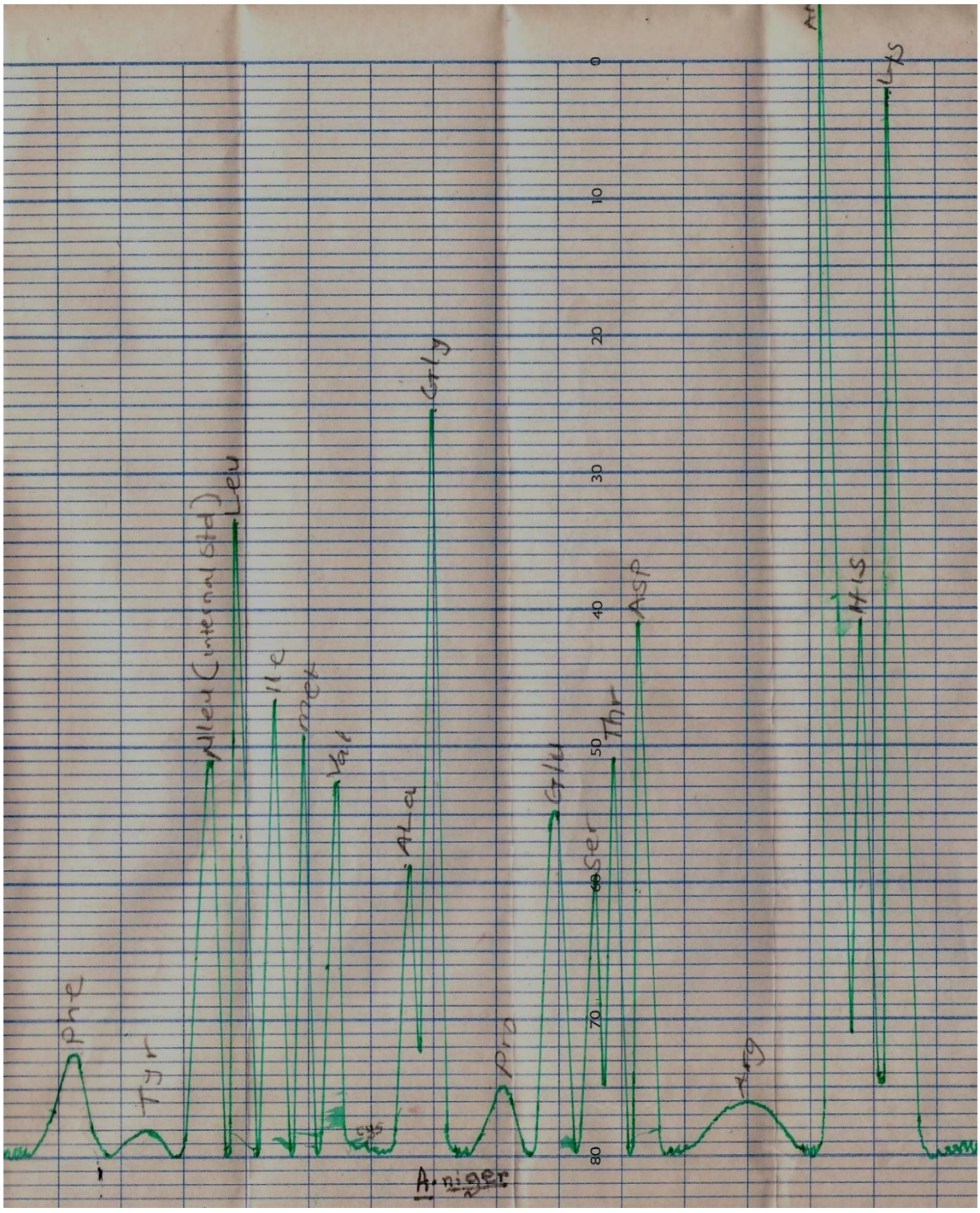
Amino Acid Profile of cGH*T.viride*

APPENDIX XIII



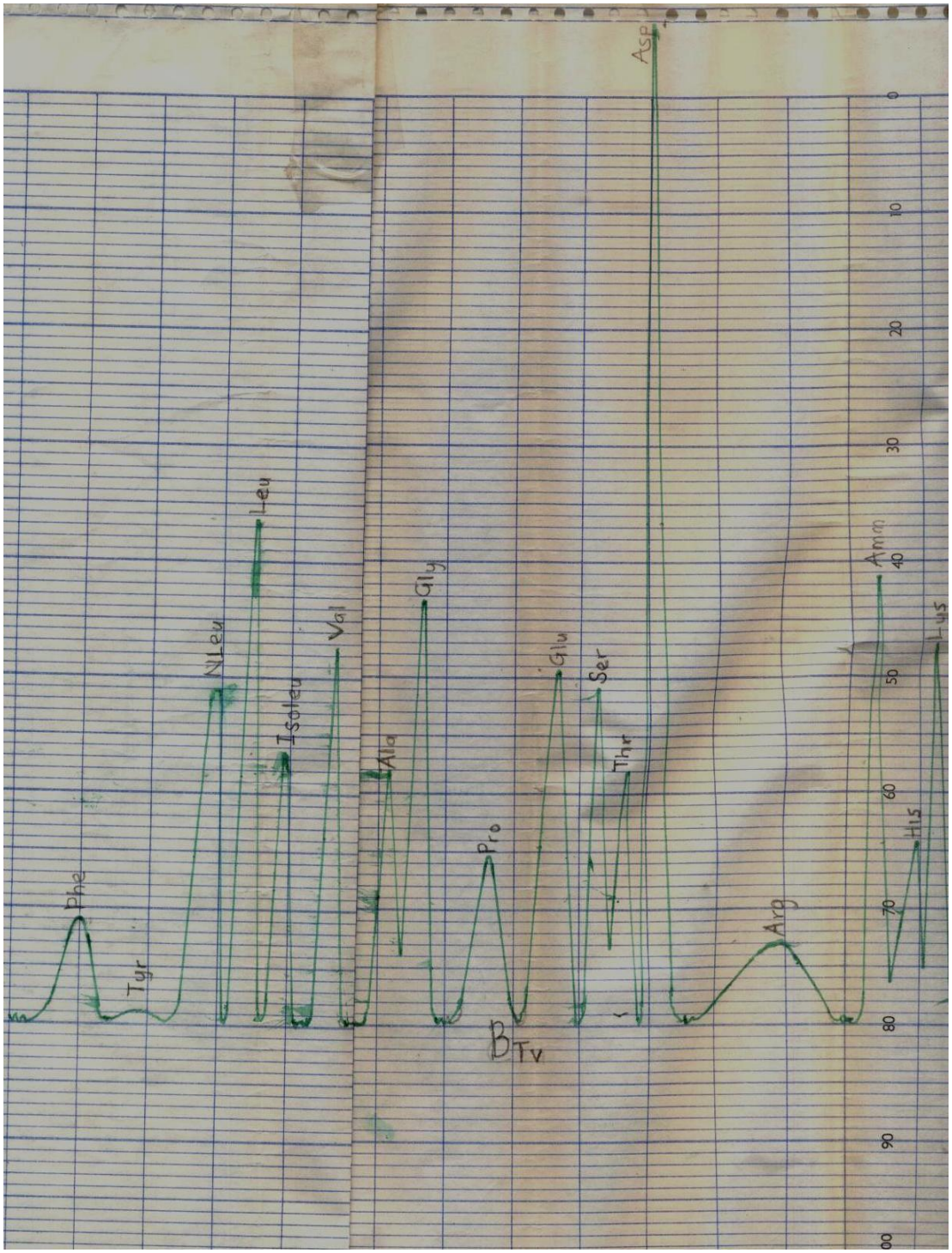
Amino Acid Profile of $cGH_{co\text{-}culture}$

APPENDIX XIV



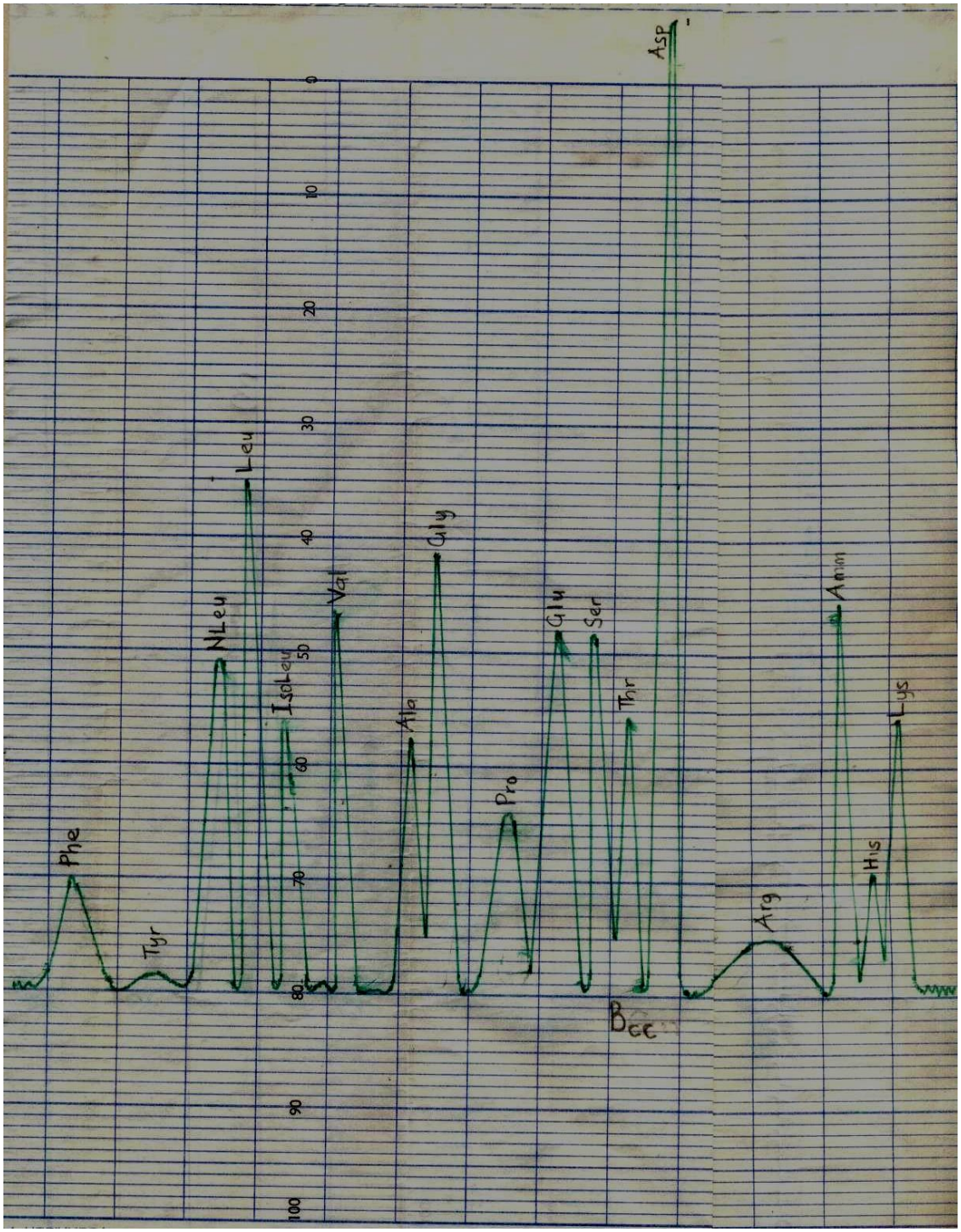
Amino Acid Profile of $_{B}GH_{A.niger}$

APPENDIX XV



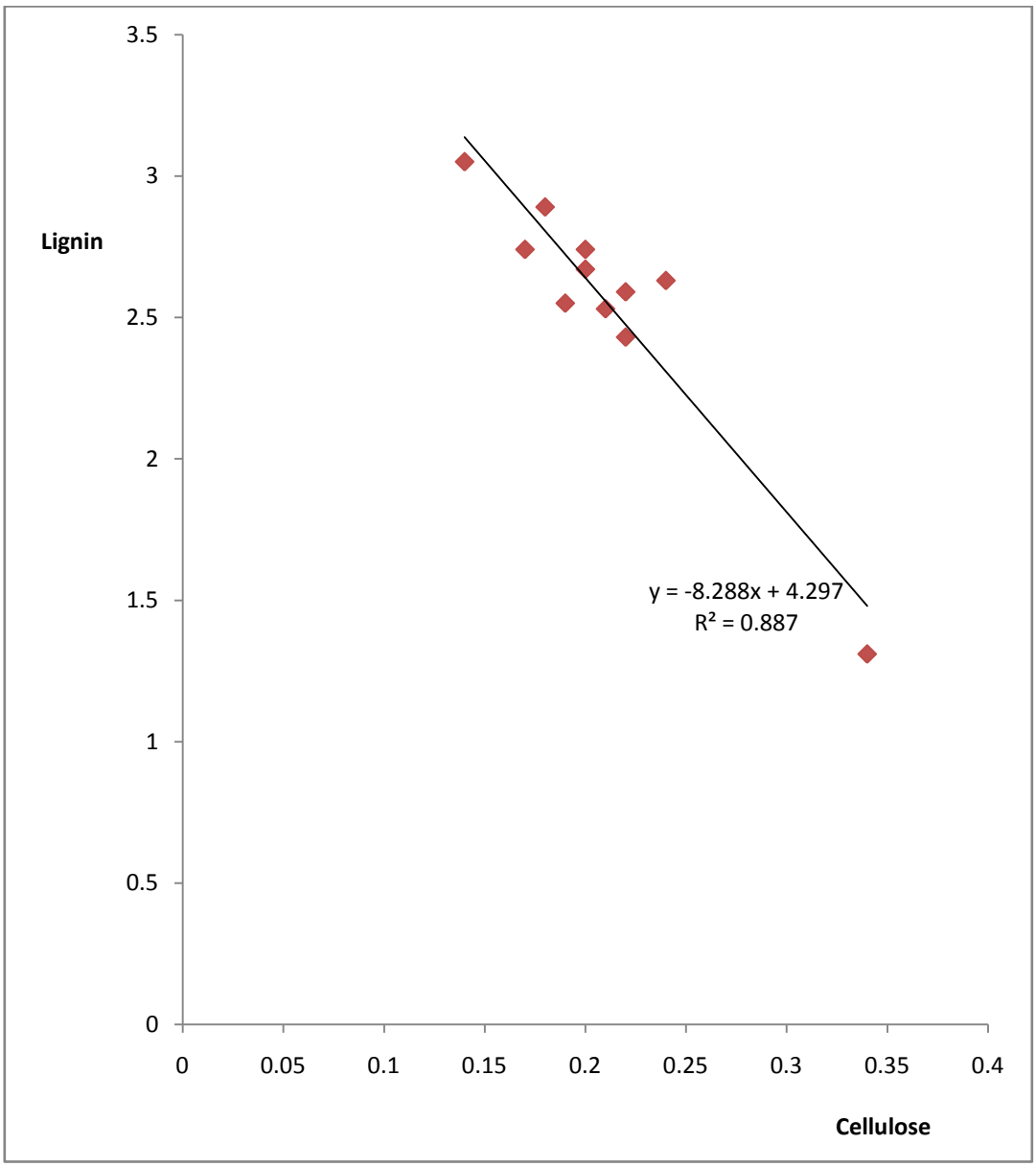
Amino Acid Profile of $_{B}GH_{T.\ viride}$

APPENDIX XVI



Amino Acid Profile of $_{B}GH_{co\text{-}culture}$

APPENDIX XVII



Correlation Chart of Lignin and Cellulose.