# A FEASIBILTY STUDY INTO THE BIOPROCESSING OF COFFEE RESIDUES


## Abstract

Coffee residues are generated in large quantities from the processing and consumption of coffee, and are generally disposed of as waste through non-sustainable practices. The capacity of coffee residues to support microbial growth was determined, using indigenous bacteria and fungal species- *Aspergillus awamori* and *Aspergillus oryzae* as model organisms. Solid and liquid state fermentation by fungal species and indigenous bacteria respectively, were carried out under basic conditions and the results showed that coffee residues contain chemical compounds which can be utilized by micro-organisms in bio-processes to produce value-added products. Increase in free amino nitrogen and total reducing sugars was obtained; and maximum protease activity of 92 U/g dry matter basis was obtained.

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## Acronyms

# Chapter 1: Introduction

Bioprocessing is a concept in biotechnology which has important application in a wide array of human related activities. It is based on the principle of using biological materials, such as enzymes and micro-organisms, to produce desired products under the right conditions. An area of increasing development is in waste management.

The issue of waste management has become one of global concern in recent times. Current waste management practices are being discouraged due to the severe impacts they have on the environment. A prevalent waste management practice is the dumping of waste in landfills, which results in environmental problems such as release of toxic gases, leachate, land use, as well as aesthetic issues. However, it has gradually come to realisation that materials considered as waste contain desirable chemical compounds and are potential raw materials for the generation of value added products. This realisation brought about the concept of transforming waste to products.

The biological conversion of agro-industrial and food processing residues has gained increasing interest in the area of valorisation, one of which is the coffee industry. A rapidly developing bioprocess employed is the solid state fermentation of these by-products for the generation of useful products such as industrial chemicals, enzymes, food additives and health care products (Ward, 1989).

Coffee is a popular beverage which is widely consumed for its refreshing and stimulatory effects in various parts of the world(Arya and Rao, 2007b); and is regarded as an important cash crop in most producing countries. It is produced in large quantities and its production involves a number of processes which results in the generation of large amounts of by-products.

Coffee pulp and husk, which are generated from the primary processing stage of coffee production, have gained considerably large attention in the area of bioprocessing. Nevertheless, large amounts of coffee residues are also produced from the secondary processing and consumption stages in the life cycle of coffee. These residues are currently being disposed of in landfills on a large scale, or used as compost on a smaller scale. The

quantity of coffee residues produced, as well as the high possibility of obtaining homogenous materials, presents the potential for valorising these by-products.

The purpose of this project is to determine if coffee residues can support microbial growth in bioprocessing, and to evaluate its potential in the generation of value-added products. Laboratory –scale experiments were carried out in the course of this project, incorporating the various stages of bioprocessing and investigating the application of solid and liquid state fermentation on coffee residues. Two different strains of fungi- *Aspergillus awamori* and *Aspergillus oryzae* were used for the solid state fermentation experiment, and indigenous bacteria isolated from the coffee grounds were used for liquid state fermentation experiment.

An overview of Solid state fermentation, in comparison with Liquid state fermentation is provided in chapter two of this work. It also contains an overview of coffee, including a description of coffee life cycle and its environmental impacts, its chemical characteristics, as well as the quantity of coffee produced and the by-products generated. Previous works done in the area of valorising coffee by-products are described.

Chapter three further describes the objectives, as well as the experimental design of this project. The materials and methods employed, including the micro-organisms are described in detail in chapter four.

The results obtained from the experiments and analyses performed are presented and discussed in chapter five.

The last chapter draws conclusions from the work carried out, whilst making comparisons with previous works done, and recommendations for further work are suggested.

# Chapter 2: Literature Review

## *Introduction*

## *2.2. Overview of Bioprocessing*

A bioprocess is described as any process which involves the transformation of raw materials into products of different commercial values by means of micro-organisms, animal or plant culture cells, or by materials, such as enzymes, derived from them (Chisti & Moo-Young, 1999).

Bioprocessing of raw materials into products basically involves fermentation of the raw materials and it has been widely applied in the food and beverage industries, in medicine and pharmaceuticals, energy production, as well as effluent and solid waste management. With modern biotechnology came the production of essential primary metabolites e.g. organic acids, amino acids, glycerol and vitamins; secondary metabolites e.g. Penicillin, streptomycin and gibberellins; industrially useful enzymes such as amylases, proteases and asparaginase; as well as production of vaccines and therapeutic proteins (Smith, 2004).

Bioprocessing is made up of three basic steps which are illustrated in Figure 2.1. These include the pre-treatment, bioreaction and downstream processing stages (Chisti & Moo-Young, 1999).

The pre-treatment stage involves the conversion of the raw materials into forms suitable for processing. This is done by any of a number of processes such as sorting, sieving, and sterilization, and it is an important part of bioprocessing which contributes to the success and efficiency of the process (Smith, 2004). This stage is followed by the bioreaction process, where the actual biotransformation occurs in a containment system, with the appropriate micro organisms, suitable carbon and nitrogen sources, as well as suitable physical conditions, resulting in metabolite biosynthesis and biomass production. The product of interest is further processed and obtained in the downstream processing stage through predominantly physical processes (Chisti & Moo-Young, 1999).

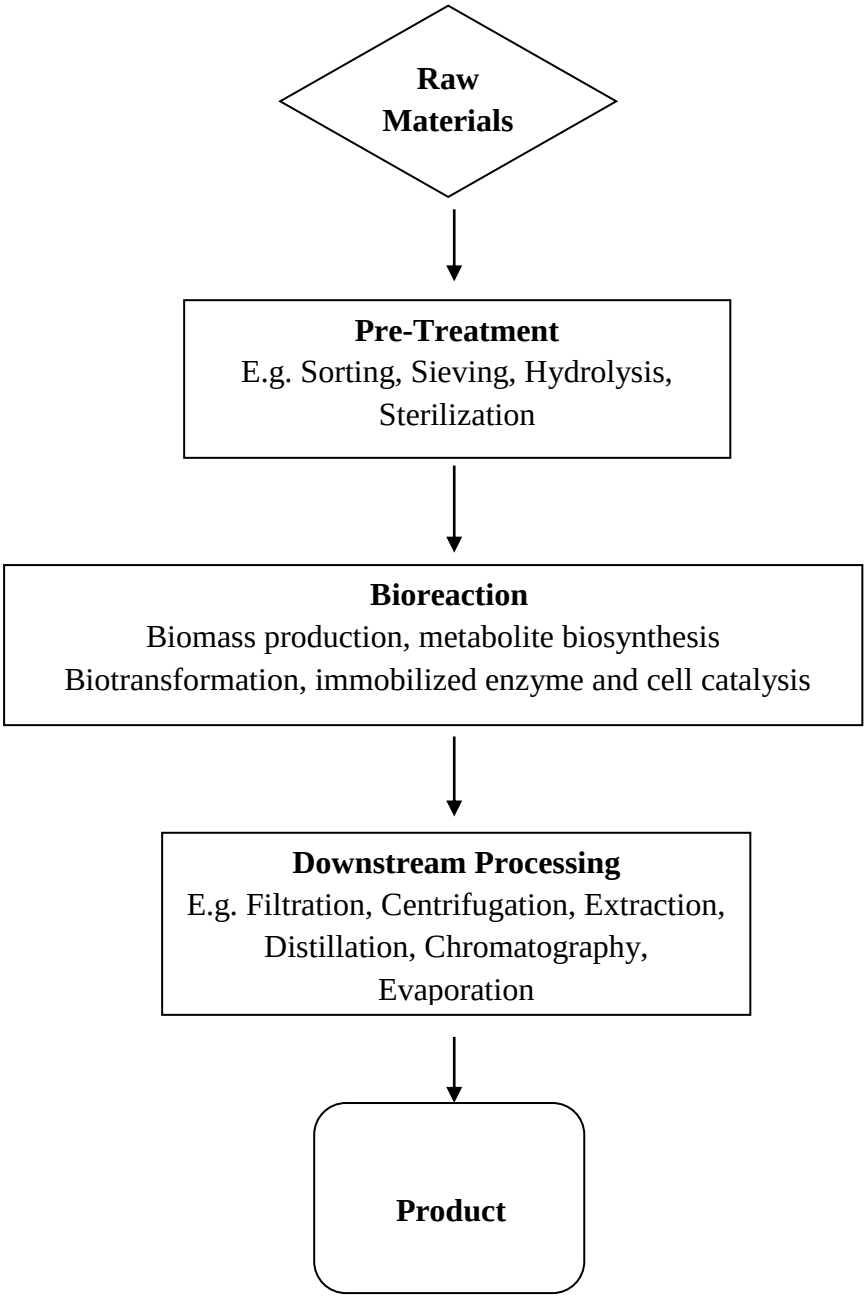



**Figure 2.1**. **Stages of Bioprocessing** (adapted from Chisti & Moo-Young, 1999)

## *2.3. Fermentation in Bioprocessing*

Fermentation can be described as the process of transforming organic materials into products by micro-organisms, in the presence or absence of oxygen (Ward, 1989).

The micro-organisms used in fermentation processes consist of a range of bacteria, yeasts and fungal species. Structural differences in these micro-organisms such as the lack of a well defined nucleus in bacteria, compared to the presence of a well defined nucleus as well as other functional organelles in yeast and fungi results in differences in the metabolism of these organisms (Ward, 1989). The fungi commonly employed in industrial fermentation processes are of the groups Zygomycotina such as the *Mucor* and *Rhizopus* genera; and Deuteromycotina such as *Aspergillus*, *penicillium* and *Trichoderma* genera. *Saccharomyces cerevisiae* is the common yeast employed.

There are two basic types of fermentation processes –

Liquid/Submerged State Fermentation (LSF) and Solid State Fermentation (SSF)

### 2.3.1. Liquid State Fermentation

Liquid state fermentation (LSF) involves the conversion of organic matter/substrate by micro-organisms in liquid medium, in an agitated environment. It has wider industrial application than solid state fermentation due to some advantages. These include the efficiency of process scale-up, as well as heat and mass transfer; continuous processes can also be obtained resulting in homogeneity of the process (Raimbult, 1998). However, this process requires large amounts of water which need to be disposed of after use, thus contributing to environmental problems. Water insoluble materials such as starch and lignin cannot be directly utilized, thus limiting the applicability of LSF to specific types of substrates (Raimbult, 1998).

### 2.3.2. Solid State Fermentation

Solid state fermentation (SSF) is defined as any fermentation process performed on a non-soluble material, which acts both as physical support and source of nutrients in absence of free flowing liquid (Couto and Sanroman, 2006). SSF has been traditionally applied in various parts of the world and it presents the potential for improved production of new and existing bio products (Biesebeke et al., 2002). The most commonly used substrates for SSF include cereal grains, wheat bran, lignocelluloses materials like straw and sawdust; and a

wide range of plant and animal materials. These substrates are usually cheap and easily obtainable, and provide the required nutrient sources for microbial growth (Smith, 2004). SSF possesses the potential for more efficient production of bio products than submerged state fermentation. Examples of this advantage are the higher yields of fungal enzymes obtained, and the more virulent and robust spores, as well as higher yields obtained in its application for bio pesticide production (Viccini et al., 2001). Also, Polymeric insoluble substrates such as starch, cellulose, and lignin which are the major components of agro-residues can be broken down in SSF for microbial growth; and the problem of water use and disposal does not occur (Raimbult, 1998). SSF also presents the potential for simpler downstream processing than LSF due to its limited water requirement.

However, large scale industrial application of this process is limited due to the lack of a systematic framework which guides the design and operation of large scale bio-reactors. The medium for SSF is static and heterogeneous, thus presents challenges such as non-uniform structure of the macromolecular components of the substrate, variability between batches of substrates which limits reproducibility, difficulty in obtaining uniform solid mass for fermentation, thus non uniform growth and gradients in pH, moisture and temperature (Raimbult, 1998). The problem of heat transfer in large scale processes also arises.

Recent advances have been made in developing the application of SSF in bioprocesses such as bioremediation and biodegradation of hazardous compounds, biological detoxification of agro-industrial residues, biotransformation of crops and crop-residues for nutritional enrichment, and production of value-added products, such as enzymes, bio fuel, organic acids and biologically active secondary metabolites including antibiotics, alkaloids and plant growth factors (Pandey et al., 2000a).

The use of bacteria, yeast and filamentous fungi in SSF processes have been investigated in various studies. Their growth is affected by factors such as nutrient availability, substrate particle size, temperature, pH and moisture (Pandey et al., 2000a)

A wide variety of filamentous fungi are used in SSF processes because of their relatively high tolerance of low moisture content, their ability to produce hydrolytic enzymes and their morphology. The choice of fungi depends on the chemical components of the substrate and the desired products.

In SSF, filamentous fungi grow by hyphal extension to form a mycelial mat on the solid substrate. The growth occurs in phases after inoculation, which include a lag phase for environmental adaptation, a log phase where nutrients are consumed for cell growth and

products excreted, a deceleration phase and  a stationary phase where growth ceases due to nutrient depletion or generation of a toxic product (Ward, 1989). To grow, these organisms utilize the nutrients from the carbon and nitrogen compounds in their environment to obtain the energy and low molecular weight compounds they require for cellular activity and for the synthesis of the macro molecular components of their cellular structure.

Simple metabolites (hexoses,C3 metabolites) are obtained from the breakdown of polysaccharides, proteins and lipids in their environment, and utilized in catabolic pathways such as Embden-Meyerhof-Parnas pathway or hexose monophosphate pathway to generate products further utilized for biosynthesis (Ward, 1989, Flipphia et al., 2009). Their aerobic/respiratory processes completely oxidize some of the substrates into carbon dioxide and water, releasing energy.

## *2.4. History of Coffee*

The word "Coffee" is reported to be derived from "quahweh" which is an Arabic poetic term for wine. Coffee has an origin dating as far back as AD 575 and various legendary tales of its origin and spread have been documented. A popular story is the discovery of its stimulatory effects on goats who chewed the berries from certain bushes, by a goat herder named Kaldi. A similar effect was also experienced by the neighbouring monks who roasted and brewed the berries which kept them more alert during prayers (Smith, 1985).

Coffee is accounted to have been cultivated first in the Arabian continent in the 15$^{th}$ century where it was guarded jealously to prevent cultivation in other parts of the world, but that intention was thwarted by an Indian pilgrim who smuggled coffee seeds capable of germination to other regions (Smith, 1985). Similar accounts exist for the introduction of coffee into Europe and the Americas in the 16$^{th}$ and 17$^{th}$ centuries, bringing about the evolution of the coffee industry in various parts of the world (Ref ICO).

The boost in coffee cultivation around the world brought about business growth through opening of coffee houses, which served as hubs for commercial, social and political activities (Ref ICO).With this also came gender, moral, social, political and religious controversies, such as the restriction of women from coffee houses, the perceived negative effects of coffee on people by some religious leaders as well as its prohibition and smuggling in some regions (Smith, 1985). Overtime, these controversies were resolved and some of the misconceptions were disproved. Some beneficial qualities of coffee have also been confirmed by scientific research and the surge in coffee consumption has been maintained.

## *2.5. Botany, Physiology and Ecology of Coffee*

Coffee is a perennial tropical plant and a member of the *Rubiaceae* botanical family. Its botanical genus is "*Coffea*". A wide variety of species have been identified, which include *arabica*, *canephora*, *liberica*, *stenophylla*, and *humilis* (Charrier and Berthaud, 1985). The most commercially important species are *C.arabica* and *C.canephora* which is popularly referred to as Robusta coffee, with *C.arabica* constituting over 60% of world production.

All species of coffee are woody with sizes which range from small shrubs to tall trees which are over 10m tall (Ref ICO). The plants possess evergreen leaves which produce flowers after 3-5 years of planting, from which coffee cherries/fruits are developed. The coffee cherries are drupes which contain the coffee beans that are eventually consumed (Griffin, 2006).

The different species vary in plants, seeds, growth conditions, chemical composition, yield, disease and drought resistance, etc. Hybrids of existing species -*C.arabusta* from *C.arabica* and *C.canephora*- have been developed to obtain optimum qualities of coffee plants (Charrier and Berthaud, 1985).

## 2.6. Structure of Coffee

The coffee fruit is made up different components, illustrated in Figure 2.2. these include a coloured exocarp (skin), fleshy yellowish-white mesocarp (pulp), a mucilage layer made up of mainly pectin and which covers the two beans joined together along the flat sides, and two coats- a thin fibrous textured parchment; and a fine membrane referred to as the silver skin (Chanakya and Alwis, 2004). The coffee beans makes up 50-55% of the dry matter of the coffee cherry (Saenger et al., 2001).

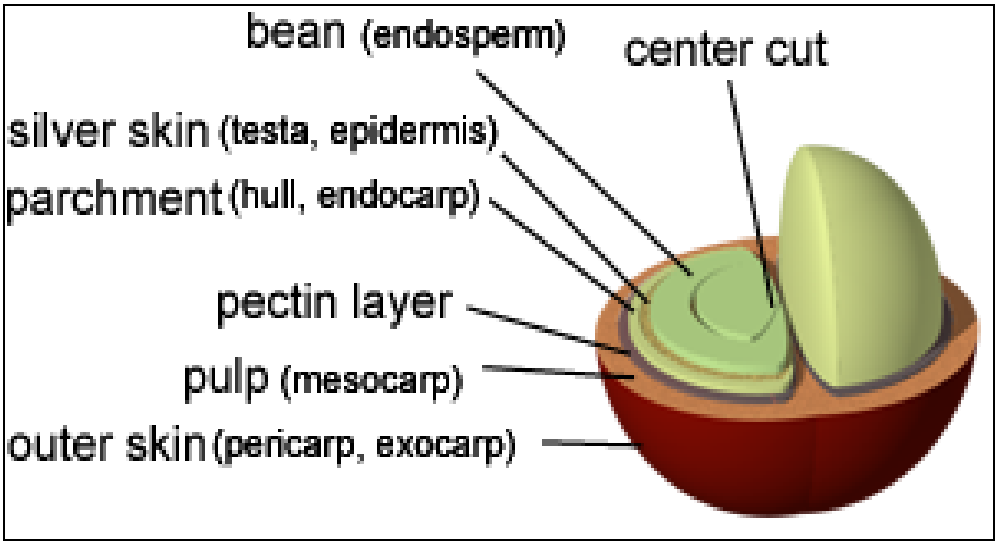



**Figure 2.2. Coffee bean structure**

## *2.7. Chemical composition of Coffee*

Hundreds of chemical compounds have been identified in coffee beans, with qualitative and quantitative variations occurring in the chemical composition of the various species of coffee. These variations are enhanced between green coffee beans and roasted coffee beans due to the reactions that occur during the roasting process, which contribute to the characteristic flavour of brewed coffee (Viani, 1993).

The basic compounds identified include carbohydrates, proteins, lipids, alkaloids and acids. These compounds and their quantities have been outlined in Table 2.1.

The chemical compounds present in roasted coffee beans have varying solubility in water, as illustrated in Table 2.2. This property determines the chemical composition of both brewed coffee beverage and the resulting coffee residues.

**Table 2.1: Chemical composition of green and roasted coffee beans**

| | **Green Coffee Beans(% dry basis)** | | **Roasted Coffee Beans (% dry basis)** | |
|---|---|---|---|---|
| **Constituent** | Arabica | Robusta | Arabica | Robusta |
| Carbohydrates | 58.0 | 59.5 | 38.0 | 41.5 |
| Lipids | 16.0 | 10.0 | 17.0 | 11.0 |
| Proteins, amino acids | 11.5 | 11.8 | 10.0 | 10.0 |
| Chlorogenic acids | 6.5 | 10.0 | 2.7 | 3.1 |
| Minerals(oxides) | 4.2 | 4.4 | 4.5 | 4.7 |
| Aliphatic acids | 1.4 | 1.4 | 2.4 | 2.4 |
| Caffeine | 1.2 | 2.2 | 1.3 | 2.4 |
| Trigonelline | 1.0 | 0.7 | 1.0 | 0.7 |
| Glycosides | 0.2 | tr. | - | - |
| Melanoidins | - | - | 23.0 | 23.0 |
| | | | | |

**Table 2.2: Soluble and insoluble constituents of roasted coffee beans(Arya and Rao, 2007a)**

| Constituent | Soluble (%) | Insoluble (%) |
|---|---|---|
| Carbohydrates | | |
| Reducing sugars | 1-2 | - |
| Caramelized sugars | 10-17 | 7-10 |
| Hemi-cellulose (hydrolysable) | 1 | 14 |
| Fiber (not hydrolysable) | - | 22 |
| Oils | - | 15 |
| Proteins | 1-2 | 11 |
| Ash (oxide) | 3 | 1 |
| Acids–non–volatile | | |
| Chlorogenic | 4.4 | - |
| Caffeic | 0.5 | - |
| Quinic | 0.5 | - |
| Oxalic, Malic, Citric, Tartaric | 1.0 | - |
| Volatile acids | 0.35 | - |
| Trigonelline | 1.0 | - |
| Caffeine | 1-2 | - |
| Phenolics | 2.0 | - |
| Volatiles | | |
| Carbon dioxide | Trace | 2.0 |
| Essence (Aroma) | 0.004 | - |

### 2.7.1. Carbohydrate Content of green coffee beans

Carbohydrates make up about half of the components of green coffee beans on a dry matter basis. These carbohydrates include soluble and insoluble oligosaccharides as well as polysaccharides.

Sucrose is the major soluble low molecular mass carbohydrate contained in green coffee beans and it is contained in a higher proportion in Arabica coffee (6-9% db) than Robusta coffee (3-7%db) (Viani, 1993).

Polysaccharides make up about 43-48% db of green coffee beans, containing soluble polymers of mannose, galactose, arabinose, glucose, xylose, rhamnose and galacturonic acid which have been extracted at increased temperatures; as well as water-insoluble pectin (Clifford, 1985, Viani, 1993). The insoluble polysaccharides include hollocelluloses, which

are described as fibrous residues made up of cellulose, β-1,4-mannan and a small amount of hemicellulose which is mainly arabinogalactan (Viani, 1993).

### 2.7.2. Carbohydrate Content of roasted coffee and coffee products

Roasting of green coffee beans brings about quantitative and qualitative changes in the chemical composition. Studies on the effect of roasting on the components of green coffee report a rapid destruction of sucrose during roasting, directly proportional to the degree of roast (Feldman et al., 1969). The arabinose and galactose components of the degradable polysaccharides are reported to also decrease, while a proportionate increase in mannose occurs. A slight decrease in the mannan component of hollocellulose occurs and an increase in cellulose proportionate to roasting is reported (Feldman et al., 1969).

### 2.7.3. Amino acids, peptides and Proteins

The free and bound amino acids in free coffee beans are estimated to be 8.7-12.2% of the chemical compounds present (Viani, 1993). About 0.15-0.25% of this are believed to be free amino acids, all of which are lost during roasting and their nitrogen incorporated into the heterocyclic aroma volatiles (Clifford, 1985, Viani, 1993). Studies report comparable amounts of amino acids in green and roasted coffee beans indicating that roasting may not have an effect on bound amino acids (Feldman et al., 1969). Nevertheless, about half of the proteins present is reported to be water soluble (Viani, 1993).

### 2.7.4. Lipids

15-18% of Arabica green coffee beans and 8-12% of Robusta green coffee beans are made up of lipids, which are mostly coffee oil in the endosperm; and 0.2-0.3% wax in the outer layer of the bean (Viani, 1993).

The coffee oil is composed mainly of triglycerides of fatty acids, as well as a relatively large unsaponifiable fraction of free and esterified diterpenes (Viani, 1993). Normal roasting may have little chemical effect, however, the coffee oil is expelled to the surface and gives a characteristic appearance to the beans (Clifford, 1985).

Most of the lipids are insoluble and remain in spent coffee grounds after brewing or extraction (Viani, 1993).

### 2.7.5. Caffeine

Caffeine is the major purine alkaloid present in green and roasted coffee, in higher proportion in Robusta coffee(15-26g/kg dry basis(db)) than Arabica coffee(9-14g/kg db) (Viani, 1993). It is believed to contribute to the bitter taste of coffee.

Other purine alkaloids include theobromine, theophylline and paraxanthine, which are present in smaller amounts than caffeine. These alkaloids are sources of non- protein nitrogen in green and roasted coffee beans (Clifford, 1985).

About 10% of the green beans caffeine content is volatilised during roasting and the remaining content in roasted beans is transferred to soluble coffee powder as well as coffee brew (Clifford, 1985).

Decaffeination of coffee is a common practice to meet the preference of some consumers.

### 2.7.6. Acids

Apart from amino acids, other acids which are important to the flavour of coffee beverage are present in coffee beans (Feldman et al., 1969). These acids can be classified into Volatile acids; Non volatile(non-phenolic ) acids; and Phenolic acids(Feldman et al., 1969). The non volatile and phenolic acids are thought to be responsible for the acidity of the beverage (Feldman et al., 1969).

The volatile and non volatile acids include aliphatic acids such as acetic acid, formic acid, citric acid, lactic acid, malic acid, amongst others. The quantity of some of these acids, e.g. acetic, fumaric and formic acids are increased during roasting while others like citric and malic acids are reduced (Viani, 1993).

Phenolic acids make up 84-87% of the total acids present in green coffee beans and include chlorogenic acid, neochlorogenic acid, quinic acid, ferulic acid and caffeic acid.

Chlorogenic and neochlorogenic acids constitute 67-76% of the phenolic acids present in coffee and their concentration decrease with roasting (Feldman et al., 1969).

### 2.7.7. Minerals

About 4% of the chemical composition of coffee is made up of minerals (Viani, 1993). The major minerals include Potassium, Calcium, Magnesium, Phosphate and Sulphate.

Potassium constitutes the largest proportion of minerals, making up about 40% (Viani, 1993).

The processing method of the coffee beans contributes to the mineral content, with wet processed beans containing lower amounts than dry processed beans (Clifford, 1985).

No significant change in quality or quantity of minerals is observed with roasting and most of the minerals are extracted during brewing of Coffee beverage (Clifford, 1985).

## 2.8. The Coffee market

Coffee is produced by some fifty or more countries, most of which are developing countries, and exported to consuming countries, constituting an important source of foreign income in major producing countries(Ref C.F Marshall). From the International Coffee Organization statistics, a total of about 725 million 60-kg bags of coffee were produced between 2004 and 2009, with an annual average of about 121 million 60-kg bags (ICO, 2010). Brazil and Colombia are the two largest world producers of coffee and contribute about 40-50% of world supply (Ref C.F Marshall).

About 60-70 million 60-kg bags of coffee were imported and consumed annually between 2000 and 2009 by the importing countries, with a total of about 677 million 60-kg bags imported (ICO, 2010). As illustrated in Figure 2.3, the United States of America is the largest single importing country, making up about 30% of the total quantity imported. The European community imports about 52% of the total quantity imported, with the UK making up about 7% of this amount (ICO, 2010).

The economic importance of the coffee market resulted in the establishment of International Agreement in 1962, with the Governments of producing and consuming countries as members. The International Agreement is negotiated and controlled by the International Coffee Organisation which was established in 1963, with their headquarters located in London. The organization controls the flow of coffee to the market to secure price stability, and their objectives have recently included the development of a sustainable coffee industry, in line with internationally agreed development goals such as the Millennium Development Goals (ICO, 2010).

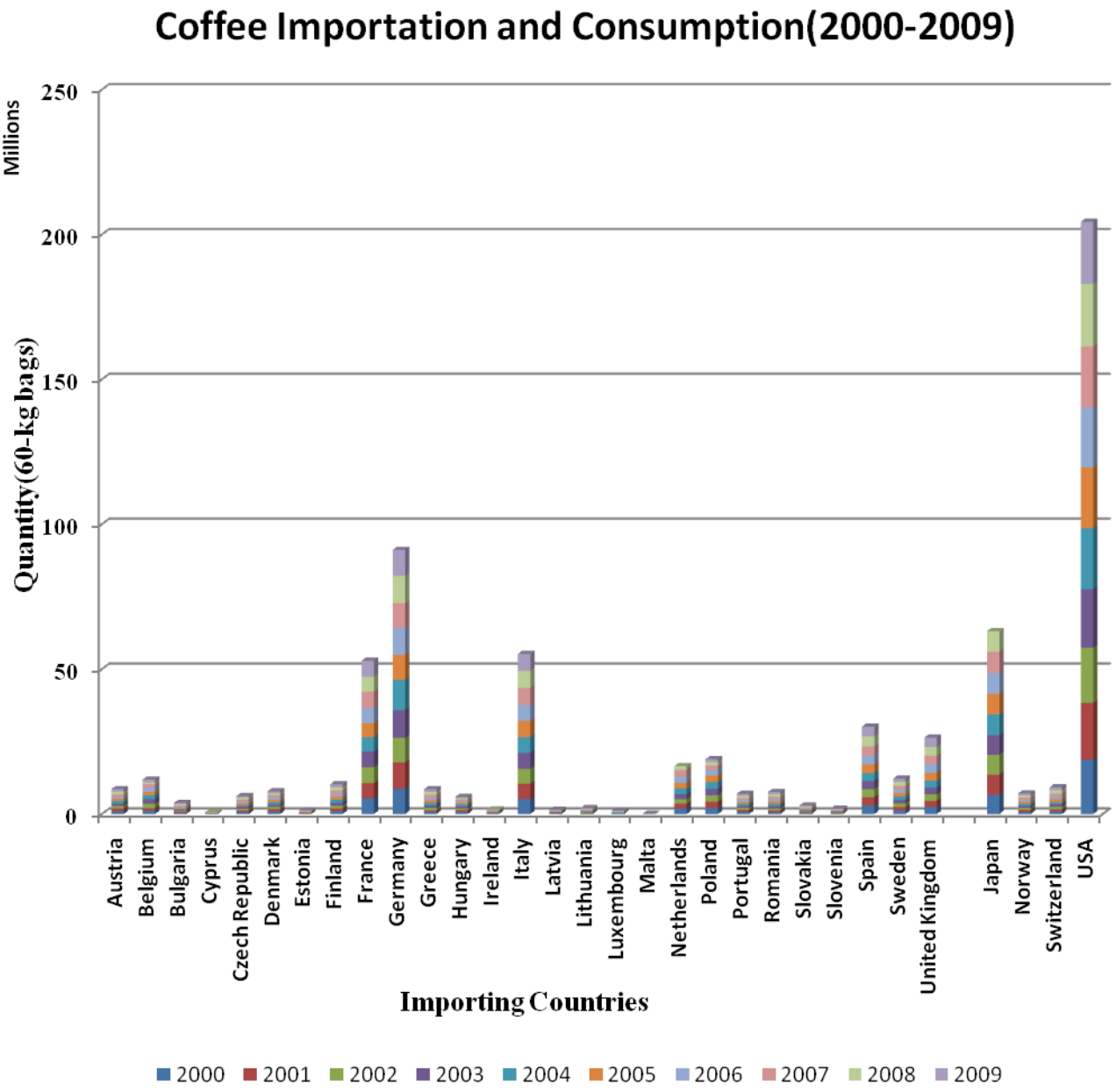



**Figure 2.3. Quantity of coffee consumption by importing countries (ICO, 2010)**

## *2.9. 'Cradle to Grave' of Coffee*

Several stages are involved in the production of coffee for end users consumption. These include – the Planting stage, the Harvesting stage, the Field processing stage, Roasting& Extraction, packaging and consumption, as well as waste disposal stages.

### 2.9.1. The Planting Stage

This involves the cultivation of coffee cherries' seeds. They are first propagated in nursery beds or poly bags until the seedlings are about 20-40cm, before they are transferred to the coffee fields. The plants begin to bear fruits after 3-5 years and have a productive life time of 20 or more years.

### 2.9.2. Harvesting

Coffee cherries have a bright deep red colour when they are ready for harvesting. The cherries are harvested either manually or mechanically, depending on the topography of the plantation. Coffee grown on mountainous areas can only be effectively harvested by hand picking (ICO, 2010). Harvesting is performed either by strip-picking where all the cherries are removed at the same time by hand or mechanically; or by selective picking where only the ripe cherries are removed by hand and resulting in better quality Coffee beans (ICO, 2010).

### 2.9.3. Field processing

This can also be referred to as the primary processing stage and it involves the extraction of green coffee beans from the coffee berries. This process is carried out entirely in the producing countries before export of the processed green coffee.

Field processing is performed using either of two methods- the dry Processing method; or the wet processing method.

#### 2.9.3.1. The Dry Processing method

This is the oldest and simplest coffee processing method. It is performed in three basic stages- Classification, Drying and dehusking.

Classification involves the separation of sub-standard cherries, dirt and any physical contaminants from the collection, especially if the strip-picking method of harvesting was used. This can be achieved by sieves/screens, and floatation/separation on flowing water (Ref. R.J Clark).

Drying is achieved either by sun-drying or mechanically to obtain a required moisture content of 13% w/w or less. Sun drying takes about 8-10 days of good weather with regular turning, and it involves the risk of undesirable microbial growth and intermittent weather conditions. After drying, the coffee cherries are dehusked to release the green coffee beans. Traditional

African methods involve the use of mortars and pestles, which have been replaced by the use of machines. Further polishing is required to remove the silverskin of the coffee.

### 2.9.3.2. The Wet Processing method

The wet process method is more sophisticated than the dry method and is believed to result in better quality and more expensive coffee. The stages of this process include Classification, pulping fermentation, drying and hulling (Ref. R.J Clark).

As in the dry process method, undesirable materials are separated in the classification stage by handpicking and/or floatation.

Classification is immediately followed by the pulping stage where the skin and soft pulpy part of the cherry are removed with the use of pulping machines.

The mucilage and any remaining pulp are removed in the fermentation stage. The enzymes contained in the mucilage are reported to play a role in the fermentation process. The process is carefully monitored and carried out in rectangular concrete tanks and occurs for a location and temperature dependent period, from 6-80hrs (Ref. R.J Clark).

The coffee is immediately washed with water at the end of the fermentation process and dried by sun-drying, mechanically or a combination of both.

The parchment shell of the dried coffee and some silverskin are removed by the hulling process with the use of any of the hulling machines available. The processed green coffee is then packaged, usually in 60kg sacks/bags, for shipment to the consuming countries (Ref. R.J Clark).

### 2.9.4. Roasting and Extraction

This can be referred to as the secondary processing stage in the life cycle of coffee and it occurs in the importing countries. The green coffee beans are roasted to the desired degree at temperature above 200$^{o}$C for a period of between a few to 30 minutes with the use of various large scale roasting plants commercially available (Ref. R.J Clark).

Roasted coffee beans are packaged and sold; or grinded and extracted for the manufacture of soluble instant coffee.

The Extraction process for the manufacture of soluble instant coffee is carried out using liquid water. One method of achieving this involves the use of percolated batteries at elevated temperature and pressure to ensure extraction of substantial soluble materials and obtain satisfactory instant coffee without supplementing with external chemical components (Ref.

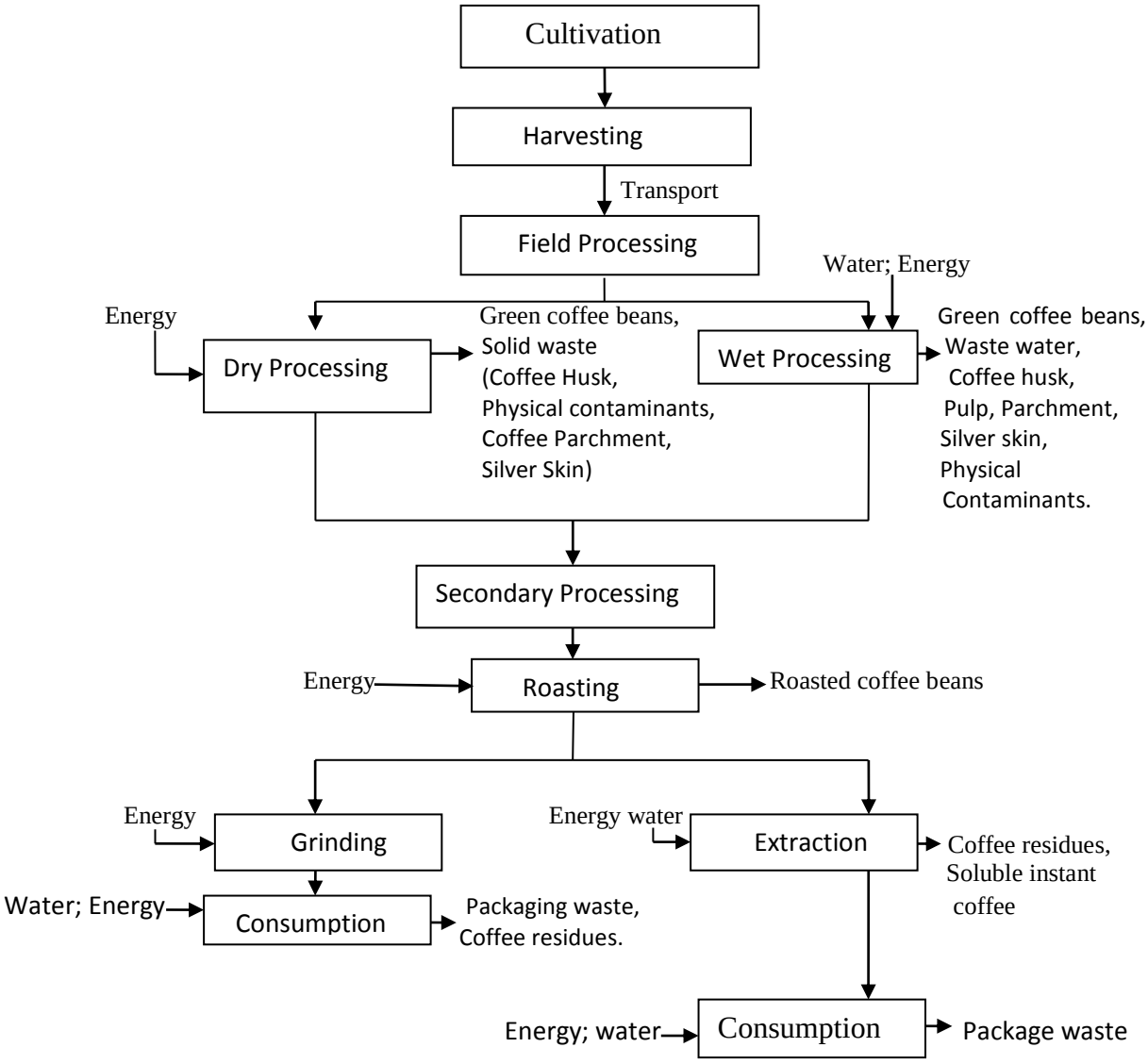
R.J Clark). After the extraction process, the coffee extract obtained is dried to obtain a recommended moisture content of not more than 4% using methods such as spray drying or freeze drying (Ref. R.J Clark).

The roasted beans and instant soluble coffee obtained from the above mentioned stages are packaged and distributed for sale to the final consumers.

### 2.9.5. Coffee Brewing

A variety of classical and indigenous methods are available for brewing the coffee beverage enjoyed by 'coffee drinkers', depending on tradition and purpose of brew. These include the Filter method, the Plunger method, the espresso/Cappuccino method which requires high pressure to extract the flavour; the percolator method, amongst others. The filter method is a very popular commercial method of brewing coffee. The Espresso and cappuccino methods are also fast growing methods for brewing coffee. The average extraction rate for a cup of coffee is 20% of the soluble solids. Higher extraction increases the bitterness of the brew, while lower extraction produces a light cup (Wintgens, 2009).

### 2.9.6. Coffee waste disposal




## *2.10. Coffee Life Cycle Assessment and Environmental Impacts*

Life cycle assessment is a tool which is used to evaluate the environmental loads and potential impacts of products or services through their lifetime (Salomone, 2003). This tool has been employed by researchers to assess the input, output and environmental impact of the various stages involved in coffee production described above.

The boundaries of the life cycle assessment included the cultivation, distribution, consumption and disposal of coffee and its by-products. The inventory analysis of coffee life cycle reports input of energy in the form of electricity and natural gas used to power equipment and roast beans respectively, water for cleaning coffee cherries, pesticides, fertilizers, packaging materials and land use. The outputs include packaged coffee product, emissions to air from electricity use and waste production (Salomone, 2003). Transportation of coffee products, as well as transport of fertilizers and pesticides are also considered in the assesment(Salomone, 2003).

A coffee life cycle analysis carried out by Coltro et.al in Brazil using a functional unit of 1,000kg green coffee reports the input of 11,400 kg of water for cleaning and wet processing method, 10,670MJ energy, 900 kg of total fertilizers, 620 kg of correctives, 10 kg of pesticides and 0.05 hectare of annual land use (Coltro et al., 2006).

The environmental impacts of coffee production identified include Eutrophication from discharge of organic-rich waste water, terrestial Eco-toxicity, air acidification from transport emissions and energy consumption, human toxicity, aquatic eco-toxicity, waste management, green house gas effect as well as ozone depleting effect. The major contributing stages to the impacts are the cultivation and consumption stages (Salomone, 2003).

This project is focused on coffee residues, which are the waste products from consumption of roasted coffee beans as well as from the extraction of soluble instant coffee powder. The global rising concerns about the issues of waste management have resulted in various researches into generation of value added products from waste resulting from various products and services.

## *2.11. Transforming waste to products ($TW_2P$)*

Waste can be described as a partially exploited resource which the current owner is directly or indirectly unable to exploit further; an attribute of a resource which may be obtained by further exploitation (Martin and Holmes, 2010).

Waste is usually classified either by its physical state- Solid, Liquid and Gaseous waste; by its origin e.g. Household, Packaging, Construction and Agricultural waste; or by its characteristics e.g. Biodegradable waste, hazardous waste and inert waste (Pongrácz and Pohjola, 2004).

According to an OECD report, over 4 billion tonnes of waste are generated annually by its 32 member countries which include municipal waste and hazardous waste(OECD, 2008).

Household waste and similar waste from offices, institutions and small businesses, yard and garden waste, street sweeping, markets, etc. are classified as municipal solid waste, which are collected and treated by municipalities. The degradable fraction of municipal solid waste are referred to as bio-degradable municipal solid waste (Martin and Holmes, 2010).

Land filling and incineration are the major disposal routes for solid waste generated. These practices have negative impacts on the environment, thus resulting in intensive research into sustainable waste management practices.

Waste generation is inevitable in most cases. However, materials referred to as waste, which are not useful for a particular process, have inherent properties which may be useful for other processes if exploited, thus the concept of transforming waste to value added products (Martin and Holmes, 2010) .

Waste from food processing industry is generally bio-degradable municipal waste and it constitutes a significant portion of the total quantity of waste generated annually. The characterization of waste from the food industry includes waste from the agricultural processes, food processing and from end users' consumption (Kroyer, 1995).

These waste materials contain high amounts of organic materials such as carbohydrates, proteins, lipids as well as other materials which present the potential for generation of bio resources from bioprocesses (Kroyer, 1995).

Work has been carried out on obtaining value from a large variety of waste from the food industry. These include Composting by micro-organisms under the right physical and chemical conditions to produce stable soil-like materials for improved plant growth (Schaub and Leonard, 1996); anaerobic digestion by micro-organisms to produce biogas (Zhang et al., 2007); use of by- products like maize bran and rice husk as bio sorbents to remove heavy metals from aqueous solutions (Hasan et al., 2008, Bansal et al., 2009); application of microbial solid state fermentation to produce important enzymes such as L-Asparaginase which is an anti-cancer agent from some agro wastes ((Mishra, 2006), cellulolytic and hemicellulolytic enzymes which can break down polymeric carbohydrates into fermentable sugars for further processing into liquid fuels and other chemical bio products (Leite et al., 2007)(ref. Rodrigo); use as animal feeds; amongst others.

An estimated 8.3 million tonnes of household food waste is reported to be produced annually in the United Kingdom (WRAP, 2010), while 43.6 million tons is reported for food waste disposed of in America each year (Zhang et al., 2007).

## 2.12. Coffee by-products

A variety of by-products, which may be regarded as waste, are generated from the various processes involved in the production of coffee for consumption. The processing of coffee beans is reported to yield about 36.4% solid wastes, 45.4% liquid wastes and only 18.2% clean finished product (Kostenberg and Marchaim, 1993).

### 2.12.1. By-products of Primary Processing

The by-products from the dry processing method of coffee cherry is referred to as coffee husk (Shankaranand and Lonsane, 1994). Solid skin and pulp as well as pulp waste water are generated from the pulping stage in coffee cherry wet processing method. Water used and discharged is reported to range from 1.5 $m^3$-23 $m^3$ per tonne of coffee fruit processed (Chanakya and Alwis, 2004). The resultant green coffee beans are covered by the mucilage layer which is removed by a series of processes which involve natural or enzymatic fermentation or chemical methods; and washing/attrition. The parchment layer and silver skin are subsequently removed after drying the coffee beans by the hulling process (Chanakya and Alwis, 2004). According to Saenger et al. in a study carried out in Kenya, for every one ton of clean coffee produced, an average of 1 ton of husks are generated during dry processing, whereas, for wet processing, 0.28 tons of parchment husks, 2 tons of pulp and 22.73 tons of wastewater would be generated (Saenger et al., 2001).

### 2.12.2. By-products of Secondary Processing and Consumption

Coffee is consumed in either of two forms- as soluble instant coffee; or ground roasted coffee beans.

Production of soluble instant coffee from ground roasted coffee beans results in the integration of only 33.3% of the raw material in the final product (Kostenberg and Marchaim, 1993). This process results in high amounts of coffee residues. Similarly, brewing of ground roasted coffee beans, which is common practice in coffee consuming parts of the world, especially in the numerous retail coffee shops available, results in large amounts of coffee residues daily.

Thus, the generation of these by-products, which are generally regarded as waste, is inevitable.

According to Lane (1983), the commercial extraction of 1 tonne (dry weight) roasted coffee beans generates approximately 1.65 tonnes of moist coffee residues. Calculating from the 2000-2009 statistics provided by the International Coffee Organization for the quantity of

coffee imported, a total annual average of about 6.5 million tonnes of moist coffee residues were produced in the importing countries, with the U.S generating about 2 million tonnes and the U.K generating about 250 thousand tonnes annually. The quantity calculated for coffee residues generated world wide corresponds with the annual quantity of 6 million tons reported by Tokimoto et.al. (Tokimoto et al., 2005).

## 2.13. Generation of Value-Added products from coffee by-products

The major solid by-products from the coffee industry include coffee pulp, coffee husks and coffee residues. The feasibility of valorising these by-products is determined by their physical and chemical composition. homogeneity of coffee residues improves the ease of obtaining these materials for further processing, compared to generic food waste produced from end-users consumption. Majority of the work recorded in literature for the valorisation of coffee by-products is focused on coffee pulp and husk which are products of the primary processing of coffee cherries in coffee producing countries. Various approaches have been taken in valorising coffee by-products in different parts of the world, and some of the works done are described below.

### Energy Production

Coffee by-products have been exploited as sources of renewable energy.

In Kenya, 70% of coffee husks from dry processing have been converted into briquettes and domestically burned as fuel with better burning characteristic than wood charcoal since the 1970s. Laboratory and pilot scale experiments on these husks showed that the low moisture content (10-11%) is advantageous in its combustion and its relatively low ash content also reduces the problem of particle emission and control. However, issues such as its high volatile content, small size and low bulk density present some challenges(Saenger et al., 2001).Coffee pulp on the other hand is not considered as an ideal fuel source for combustion due to its high moisture content (Thiagalingam and Sriskandarajah, 1987).

Anaerobic methanogenic thermophilic digestion of spent coffee residues to produce biogas as well as fertilizer has been reported by Kostenberg and Marchaim (1993). Their aim was to improve the biogas generation of 335ml/g containing 58% methane, which was obtained in previous work, by optimizing fermentation conditions. Production of biogas from conversion of the organic matter was inferred from the decrease in organic matter observed during fermentation. pH was reported to play an important role in fermentation stability and optimum of 7.7-8.2 was obtained, resulting in biogas production of 1.5-1.6 l/l.d. The use of

digested coffee grounds as fertilizer resulted in improved growth of some plant species(Kostenberg and Marchaim, 1993).

The production of biodiesel from spent coffee grounds has been investigated by Kondamudi et. al (2008). According to their report, the oil content of spent coffee grounds is approximately 15%, and can be extracted and completely tranesterified into biodiesel. They estimated a world wide production of 340 million gallons of biodiesel if all coffee residues generated were utilized, arguing that these residues can be used for composting or used as fuel pellets afterwards (Kondamudi et al., 2008).

Also, Nestle`, which is one of the largest producers of soluble instant coffee, report the use of about 800, 000 tonnes of coffee residues generated from their factories as supplemental fuel annually(2000).

## Waste water Treatment

The use of spent ground coffee residues in removal of heavy metals from aqueous solutions has been investigated. Fiol et al (2008) reported the successful biosorption and reduction of hexavalent Chromium from the effluent of various industrial processes, preventing the environmental and public health consequences of its disposal.

Biosorption is achieved by the metal binding functional groups of some chemical components of coffee residues, such as tannins, providing a low cost process for waste water treatment. Other heavy metals which can be removed by this process include mercury, lead and copper. The metals are then desorbed with NaOH or HCl and the spent coffee residues can be re-used (ref. Macchi).

## Composting/Vermi composting

Coffee residues have traditionally been used as material for compost (Pandey et al., 2000b). Composting is a well established natural aerobic biochemical process which, under specific conditions, uses thermophilic microorganisms for the conversion of organic materials into a stable soil-like product (Schaub and Leonard, 1996). It is not considered as a very efficient method and has been improved on by vermicomposting, which utilizes certain earthworms to enhance waste conversion and yield better product (Adi and Noor, 2009).

A vermi composting study was carried out by Adi and Noor (2009) on a mixture of cow dung, spent coffee grounds and kitchen waste in varying proportions, as substrates for 49 days, using earthworms- *Lumbricus rubellus*. The results show that coffee grounds provide a suitable environment for the growth of earthworms and the volatile compounds contained in

the coffee grounds are believed to prevent the attraction of vermin which is obtainable in composting processes. The coffee grounds also stabilized kitchen waste to result in enhanced vermicomposting, thus presenting an alternative use for coffee grounds rather than disposal (Adi and Noor, 2009).

Direct application of these residues as animal feed is considered to be limited due to issues of palatability and digestibility related to the presence of compounds such as tannins, polyphenols and caffeine (ref. Pandey).

## Solid State Fermentation of coffee by products

Biotechnological approaches have also been extended to the valorisation of coffee by-products. The application of solid state fermentation to achieve a number of purposes has been investigated.

### Biotransformation

Detoxification to remove/degradation the tannin and caffeine content of coffee husk and pulp, in order to improve their nutritional value as animal feed as well use as fertilizer, has been reported (ref. Pandey). Fungal strains- *Rhizopus, Aspergillus and Phanerochaete sp* were observed to degrade the caffeine and tannin content of coffee husk at optimized pH and temperature conditions without nutrients supplementation. The best efficiency was exhibited by *Aspergillus sp* with caffeine degradation of 92% and tannin degradation of 70%. This discovery presents a less expensive method for detoxification of these by-products, compared to existing chemical methods employed (ref. Brand).

Bacteria strains- *Streptomyces sp* – have also being used in solid state fermentation of coffee pulp for the degradation of the anti-physiological and anti- nutritional compounds. Orozco et.al reports a decrease in the polyphenol content of coffee pulp, which was detected by pyrolysis-Gas chromatography-mass spectrophotometer assay, in residues treated with these bacteria strains compared to raw coffee pulp residues (ref. Orozco) .
Work on biotransformation of spent coffee grounds was carried out by Wong and Wang (1991). Fungal strain- *Pleurotus sajor-caju* - was used for solid state fermentation of these grounds at 25$^{o}$C for 32 days and87% tannin degradation was obtained (Wong and Wang, 1991).

## Enzyme Production

Enzymes such as pectinase, protease, tannase, caffeinase, cellulases, and α-amylases have been produced from solid state fermentation of coffee pulp and husk (ref. A. Pandey).

Solid state fermentation of coffee husk by bacteria strain-*Lactobacillus sp*- was reported to produce tannase with maximum activity of 0.85U/gds, enhanced by supplementation with tannic acid (Sabu et al., 2006). This enzyme is commercially important and is used to remove the undesirable effects of tannins in the food and beverage industry, produce substrates such as gallic acid and propylgallate used in the pharmaceutical and food industries as well as degradation of tannins present in effluents from tanneries which may result in environmental pollution (Belmares et al., 2004).

Protease production from solid state fermentation of coffee by-products by fungal strain-*Aspergillus oryzae* was reported by Murthy and Naidu (2010). This enzyme accounts for 60% of world sale in the enzyme market and it has wide application in meat processing, dairy processing, detergents, silk industry amongst others (Murthy and Naidu, 2010). The study was carried out using Coffee cherry husk, coffee parchment husk, silver skin and spent coffee. Emphasis was laid on coffee cherry husk due to the relatively significant enzyme activity of 10,236 U/gds obtained (Murthy and Naidu, 2010).

Murthy et.al (2009) also studied the production of α-Amylase from solid state fermentation of the above mentioned coffee residues as well as a mixture of all of them using fungal strain-*Neurospora crassa* , and obtained maximum enzyme activity of 7084U/g with coffee pulp under optimized conditions. This enzyme presently has important biotechnological application in food, textile, fermentation and paper industries (Murthy et al., 2009).

# GHG Emissions Analysis — Summary Report

(Version 10, 11/09)

Analysis of GHG Emissions from Waste Management

| | |
|---|---|
| GHG Emissions from Baseline Waste Management Scenario (MTCE): | 27,039 |
| GHG Emissions from Alternative Waste Management Scenario (MTCE): | 5,937 |
| **Total Change in GHG Emissions (MTCE):** | **-21,102** |

| **Baseline Scenario** | **Alternative Scenario** | |
|---|---|---|

| Material | Tons Recycled | Tons Landfilled | Tons Combusted | Tons Composted | Total MTCE | Tons Source Reduced | Tons Recycled | Tons Landfilled | Tons Combusted | Tons Composted | Total MTCE | Change (Alt - Base) MTCE |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Food Scraps | N/A | 165,346 | 82,673 | 27,558 | 27,039 | 0 | N/A | 82,673 | 27,558 | 165,346 | 5,937 | **-21,102** |

Note: A negative value indicates an emission reduction; a positive value indicates an emission increase.

a) For an explanation of the methodology used to develop emission factors, see EPA report: Greenhouse Gas Emissions from Management of Selected Materials in Municipal Solid Waste (EPA530-R-98-013) — available on the Internet at http://www.epa.gov/climatechange/wycd/waste/reports.html Please note that some of the emission factors used to generate these results do not

# Chapter 3: Project Description and Objectives

This project is a laboratory scale work, aimed at determining if spent coffee residues can be utilized in bioprocesses and to evaluate the possibility of obtaining value-added products from these residues.

From the literature reviewed, it is evident that not much work has been done on the valorisation of spent coffee grounds compared to coffee pulp and husk.

Studies such as work carried out by Murthy and Naidu (2010) focused on coffee husks because of the significance of the results obtained, compared to spent coffee grounds. However, differences occur between the origins of the different coffee by-products which must not be overlooked.

Spent coffee grounds are produced from entirely different processes than other coffee by-products. These residues are the principal by- products obtained in large quantities from secondary processing of green coffee beans in the importing countries, thus does not allow for a general approach in dealing with the by-products of the coffee Industry. Also, strict comparisons between the different coffee by- products and their performance in obtaining value added products may not be environmentally relevant because the aim of valorisation is to further process these by- products to the 'irreducible minimum' before final disposal . The use of a mixture of all the by- products can only be applicable in producing countries which are also relatively large consumers, e.g. Brazil.

Availability and quantity needs to be considered in any attempt at valorisation of waste/ by-products of any processes. Large amounts of these spent coffee residues are generated from the generation of instant soluble coffee, as well as the numerous commercial coffee shops available. These residues can be easily collected separately, providing homogenous raw materials for valorisation purposes.

## *Project Design*

The principal bioprocess carried out is Solid state fermentation by fungal strains. The effect of fermentation on the chemical constituents of spent coffee residues is analysed, and from the results obtained, conclusions on the feasibility of valorising spent coffee grounds through bioprocesses are drawn.

Chemical analysis of the spent coffee grounds is performed before solid state fermentation, to provide information about the nutrient sources available for growth of the micro-organisms during fermentation, as well as the prevailing conditions. These characteristics are Moisture Content, Total solids, Volatile solids & Ash content; pH; Total nitrogen content; Total carbohydrate content; free amino nitrogen (FAN) and total reducing sugars present. The chemical changes occurring due to fermentation are determined by comparing the results obtained with the initial parameters.

A preliminary solid state fermentation experiment is carried out to determine the ability of the fungal strains to utilize the available nutrient sources for growth on the spent coffee grounds, thus providing a basis for the continuation of this study.

Solid state fermentation of raw roasted coffee beans is also performed, using the two strains of fungi. The objective of this experiment is to determine the presence of any chemical compounds in raw roasted coffee beans and spent coffee grounds, which may promote or inhibit the growth of the micro-organisms.

Presence of any Indigenous bacteria in spent coffee residues is determined by microscopy and agar plating experiments. This serves as a confirmation of the ability of spent coffee residues to support microbial growth thus a potential raw material for bio processes. Liquid state fermentation with the isolated bacteria is performed and analyses of the chemical characteristics are carried out, in order to make a rough comparison with solid state fermentation of the residues.

Solid state fermentation is performed by two strains of fungi – *A.awamori* and *A. Oryzae*, in order to compare their growth capability in coffee grounds, and the resulting changes in chemical components which occur in the coffee residues. *A.oryzae* is used because it has been widely applied in SSF processes to produce enzymes, and has been qualified as safe in food industry. Protease is the model hydrolytic enzyme analysed.
Fermentation is performed under basic conditions for 4-6 days. No factorial process optimization or nutrient supplementation was performed, because the principal aim of the study is to determine the ability of the coffee residues to support microbial growth. The experiment was performed in triplicates, with an accompanying control experiment, in Erlenmeyer flasks under aseptic conditions to minimize contamination and to facilitate the calculation of inoculum size required. Sampling was done randomly at intervals over the

total fermentation time by sacrificing three fermentation flasks and one control flask per sampling time. The purpose of performing the experiments in separate flasks and sacrificing whole flasks for analysis was to obtain homogenous samples representative of the whole flask. This would have been impossible if the fermentation was performed in a single medium and the experiment disrupted at intervals to take sample, because one sample cannot be representative of the substrate medium, and the microbial activities would be disrupted (Viccini et al., 2001).

The effect of Liquid state fermentation of coffee residues by the isolated indigenous bacteria is also investigated in order to make a comparison between the chemical changes occurring in solid and liquid state fermentation. This experiment is performed for 48 hours, with sampling at intervals.

**Autolysis**

Autolysis defined in the American Heritage dictionary as the destruction of tissues or cells of an organism by the action of substances, such as enzymes, that are produced within the organism. This experiment was performed after 72 hours of solid state fermentation and analysed to investigate the increase in desirable chemical constituents with autolysis, thus the possibility of increasing the yield of value-added products.

# Chapter 4: Materials and Methods

## *4.1. Introduction*

This chapter presents a detailed description of all the materials and experimental procedures used in this project. The micro-organisms employed in Solid state fermentation are described, as well as all experiments carried out, the operating conditions and the analytical methods used.

## *4.2. Materials*

The following sections provide information of the chemical reagents and analytical materials used for the laboratory experiments performed, in the course of this project. Details of the micro-organisms as well as the raw materials used are also provided below.

### 4.2.1. Chemical Reagents

### 4.2.2. Analytical Instruments

UV-VIS Spectrophotometer, Microscope, Analytical measuring scale, Fume cupboard, Bench pH/mV meter, Magnetic stirrer, Microflow cabinet, Micro- centrifuge, Water bath, Oven, Incubator, Eppendorf centrifuge, Autoclave, COD reactor, Dessicator, orbital shaker, Erlenmeyer flasks, Measuring cylinder, Round bottom flask, Thermometer, Duran bottles, Aluminium dishes, Pippete, Pippete tips, Non-absorbent cotton, Aluminium foil

### 4.2.3. Micro-organisms

Two strains of filamentous fungi were used for solid state fermentation of coffee residues. These micro-organisms are *Aspergillus awamori* and *Aspergillus oryzae.*

*Aspergillus oryzae* is an obligate aerobic filamentous fungi, which has been widely used traditionally for over 1,000 years for fermentation in the food and beverage industry and it is listed as Generally recognised as safe (GRAS) by the Food and Drug Administration in the USA. It was first isolated from *Koji*, a fermented Japanese meal ,in 1876 and it reproduces asexually by the means of vegetative spores known as conidiospores or mycelia fragmentation (Machida et al., 2008).Genetic studies reveal that *A.oryzae* is made up of a genome rich in metabolic genes such as secretory hydrolases, transporters, primary metabolism genes as well as secondary metabolism genes which are most prominent, and this

property is believed to be important in the wide application of *A.oryzae* in biotechnology(Machida et al., 2008).

Aspergillus awamori is a strictly aerobic filamentous fungi which is well known in the food industry (Koutinas et al., 2003). The strain employed for solid state fermentation was *A. awamori* 2B. 361 U2/1

### 4.2.4. Substrate for Fermentation

Coffee residues were the principal substrate used for solid state fermentation by *A.awamori* and *A.oryzae*; as well as liquid state fermentation by indigenous bacteria. These residues were collected in from a local Starbucks coffee shop at Manchester City centre, and were obtained from brewing of *Arabica* coffee beans by the espresso method. The spent residues were preserved in plastic bags at -30$^{o}$C before use.

### 4.2.6. Substrate for toxicity/inhibition test

Packaged roasted coffee beans were purchased from a local supermarket in Manchester to carry out a comparative study between coffee residues and roasted coffee beans. The packaged coffee beans were stored at room temperature prior to use.

## *4.3. Experimental Methods*

### 4.3.1. Raw material preparation

In preparation for fermentation experiments, frozen coffee residues and roasted coffee beans were homogenized with a domestic blender and sieved through a …..mm mesh to obtain uniform particle size. The moisture content was adjusted to the desired values, after which the residues were sterilized by autoclaving.

### 4.3.2. Inoculum Preparation

The methods of preparing fungal and bacterial cultures for solid state fermentation and liquid state fermentation respectively are described below.

### 4.3.2.1. Preparation of Fungi inoculum

Spores of *A.awamori* and *A.oryzae* were aseptically cultured in media containing 20% milled wheat flour and agar for seven days at 30$^{o}$C. For each fungus, 500ml of the medium was prepared and 50ml was poured into five 250ml Erlenmeyer flasks which were covered with non-adsorbent cotton wool and foil; and sterilized in an autoclave for 15 minutes at 121$^{o}$C.

The medium was allowed to cool and solidify in a laminar flow cupboard, and then inoculated with fungal spores.

The cultivated fungal spores were harvested by manual shaking with sterile 5mm glass beads and 0.9% sodium chloride solution. 1 ml of spore suspension was transferred into vials, using a 1000µl pipette; and the spores produced were counted under the microscope, using a haemocytometer. The fungal spores were stored at 0$^{o}$C in vials prior to use in Solid state fermentation.

#### 4.3.2.2. Preparation of Bacteria inoculum

Indigenous bacteria, which were isolated by the method described in section 4.6 below, were cultured in liquid medium to obtain inoculum for Liquid state fermentation(Rao, 2010). 5ml nutrient broth was sterilized in bottles, and then inoculated aseptically with isolated bacteria. The bottles were incubated on rotor shakers at 30$^{o}$C for 48 hours. Control bottles were also prepared without bacteria inoculation.

### *4.3.3 Test for Indigenous Micro-organisms*

The presence of indigenous bacteria and fungi in coffee residues was determined in the course of this project, using methods described below. The determination influenced the need to sterilize coffee residues before fermentation experiments were performed.

#### 4.3.3.1. Test for indigenous bacteria

The presence of indigenous bacteria in coffee residues was determined by Nutrient-Agar plating experiment, using a solution of unsterilized coffee residues in sterilized distilled water. Nutrient –Agar plates were prepared with 13g/L nutrient broth and 20% agar. Sterilized coffee residues and distilled water were used for control experiments.

Bacteria found in spent coffee residues were isolated aseptically on nutrient- agar plates by the streak plate method (Microbeid, 2010). A sterile loop was used to streak the indigenous bacteria obtained over the surface of nutrient- agar plates to isolate colonies. The plates were incubated at 30$^{o}$C for 48 hours.

#### 4.3.3.2. Test for indigenous fungi

The presence of indigenous fungi was determined by isolating coffee residues in Petri dishes for 10 days, at room temperature outside the Morton laboratory. The moisture content of coffee residues was carefully adjusted to …, and the petri dishes were kept covered to prevent contamination by atmospheric fungi as well as loss of moisture.

### *4.3.4. Preliminary growth experiment*

This experiment was carried out to determine the ability of the fungal strains to grow on coffee residues. Unsterilized coffee slurry was prepared and inoculated with 1ml each of *A.awamori* and *A.oryzae* in Petri dishes. The experiment was performed in triplicate with accompanying control experiments, and incubated at 30$^{o}$C. The Petri dishes were monitored over time for the emergence of fungal hyphae.

### 4.3.5. Test for toxicity/Inhibition

This experiment was performed to determine the presence or absence of any toxic or inhibitory compounds in coffee residues, relative to roasted coffee beans.

The experiment was performed in Petri dishes, containing agar and various forms of coffee, namely roasted coffee beans, coffee beans liquid extract and coffee residues. The media used were Coffee extract + 2% Agar; 5g milled coffee beans + 2% Agar; and 5g spent coffee+ 2% Agar. Coffee extract was obtained by boiling 5g of milled coffee beans in 200ml distilled water for 20 minutes and then filtering the suspension. The media were prepared in 250 ml Duran bottles and autoclaved, after which they were allowed to solidify in Petri dishes for 30 minutes.

The substrates were inoculated with *A.awamori* and *A.oryzae* respectively and fermentation was allowed to occur for 4 days.

### *4.3.6. Fermentation experiment*

The fermentation experiments performed in this study were solid state fermentation and liquid state fermentation. The prepared substrate (coffee residues) was inoculated with the respective micro-organisms and the experiments were run for 2 to 6 days with sampling, depending on the micro-organism used.

#### 4.3.6.1. Solid state fermentation

Solid state fermentation of coffee residues was performed by A*spergillus awamori* and *Aspergillus oryzae.* 10 g of coffee residues was weighed in twenty-eight 250 ml Erlenmeyer flasks for each fungal strain, and the moisture content was adjusted to 73% by adding 6ml of distilled water in each flask. The prepared substrates were each aseptically inoculated with 50 µL of *A. oryzae,* to obtain a spore concentration of 4.8 x $10^{6}$ spores/ g dry substrate; and 500 µL *of A. awamori,* to obtain a spore concentration of 33.5 x $10^{6}$ spores/ g dry substrate. The experiment was performed at 30$^{o}$C, which is within the 20-40$^{o}$C temperature range for the

growth of filamentous fungi (ref. R.L Greasham book).

Seven samples were taken in triplicates at intervals, by sacrificing 3 inoculated flasks and one control (un-inoculated) flask for each sampling time. Solid state fermentation by *A. oryzae* was allowed to run for 4 days with samples taken at 0, 6$^{th}$, 24$^{th}$, 30$^{th}$, 52$^{nd}$, 70$^{th}$ and 94$^{th}$ hours of fermentation; while SSF by *A. awamori* was run for 6 days, with samples taken at 0, 6$^{th}$, 24$^{th}$, 30$^{th}$, 50$^{th}$, 76$^{th}$, and 124$^{th}$ hours of fermentation.

### 4.3.6.2. Liquid state fermentation

Liquid state fermentation was performed with a medium consisting of 20 g coffee residue and 100 ml tap water in 250 ml Erlenmeyer flasks. The medium was autoclaved and cooled, then inoculated aseptically with 2 ml of the prepared bacteria inoculum. The experiment was performed in duplicate with an accompanying control flask, which were incubated at 30$^{o}$C on rotor shakers at 200 rpm. Fermentation was allowed to occur for 48 hours and samples were taken at intervals by sacrificing two inoculated flasks and one control flask per sampling time.

### 4.3.7. Autolysis experiment

In this experiment, solid state fermentation of coffee residues by *A. oryzae*, using the method described above, was performed in duplicate for 72 hours. The fermented coffee residues and mycelia obtained were weighed into 250 ml Erlenmeyer flasks and the moisture content was adjusted to obtain the optimum solid concentration (55 g/L db) required for hydrolysis. The solution was homogenized with a domestic blender and transferred into 100 ml Duran bottles which were incubated in a water bath at 55$^{o}$C. Autolysis was allowed to occur for 48 hours and 3 samples of the autolysate were taken at intervals.

### 4.3.8. Sampling and extraction

Samples of solid state fermentation were obtained by sacrificing a whole flask per given sample. The content of the flask was mixed with a spatula, and a portion of the solid material was weighed for moisture content analysis, after which 6 g of the remaining material was weighed in 100 ml Erlenmeyer flasks and dissolved with 30 ml of distilled water. Extraction was done by shaking the solution at 200 rpm for 15 minutes, and the supernatant was obtained by centrifuging at 11,000 rpm for 5 minutes. The pH of the supernatants was taken immediately after extraction and the supernatants were stored in sampling bottles at 0$^{o}$C for further chemical analyses. The extraction process was also employed for liquid state

fermentation samples obtained. Samples from autolysis experiment were taken with the use of a 5 ml automatic pipette, whilst stirring the solution on a magnetic stirrer.

## *4.4. Analytical procedures*

The chemical constituents of coffee residues were analysed before and after fermentation, to characterize the carbon and nitrogen sources available for fermentation; and to evaluate the chemical changes that occurred during fermentation. The analysis performed are Moisture content, total solids, volatile solids & ash content analysis; Total Carbohydrate content analysis; Total Nitrogen content assay; Glucose content analysis, pH analysis; Total reducing sugar analysis, Free amino nitrogen assay, as well as Protease activity determination. The following sub-sections describe the analytical procedures employed in this project.

### 4.4.1. Moisture content, total solids, volatile solids & Ash content analysis

The classic laboratory 'Loss on Drying' method which is based on evaporation of moisture was used to determine the moisture content of spent coffee residues. Aluminium dishes were placed in a furnace at 550$^{o}$C before use, to eliminate any inherent moisture. The empty dishes were weighed before and after introduction of the coffee residues, and left overnight in an oven at 100$^{o}$C. The aluminium dish and residues were cooled in a desiccator for 30 minutes and weighed after drying. Further burning was carried out at 550$^{o}$C for 1 hour to determine the ash content of coffee residues. Values obtained were used to calculate the moisture content, total solids and volatile solids.

### 4.4.2. pH analysis

pH analysis was performed by the use of a bench pH metre, to determine the change in acidity or alkalinity of coffee residues occurring during fermentation. The principle of electromagnetic pH measurement is based on determination of the activity of the hydrogen ions by potentiometric measurement, using a standard hydrogen electrode and a reference electrode(Hasan et al.). The pH meter was calibrated with buffer solution of pH 4 and 7 prior to use.

### 4.4.3. Total Carbohydrate content

The total carbohydrate content of coffee residues was determined using the phenol-sulphuric acid procedure by Dubois et.al (1956).This method is based on the absorbance at 485nm of a colour aromatic complex formed between phenol and the carbohydrate. It has the advantage of simplicity and reproducibility, and can be used to determine soluble and insoluble

carbohydrates (Safarik and Santruckova, 1992). Care was taken when using phenol and concentrated sulphuric acid, and the fume cupboard was appropriately utilized for safety. Details of the procedure, analysis and calculation are provided in Appendix A.

### 4.4.4. Total Nitrogen Assay

The total nitrogen content of coffee residues was analysed by the Hach persulphate digestion method. This method is based the principle of the conversion of all forms of nitrogen to nitrate by alkaline persulphate digestion, which reacts with chromotropic acid under strongly acidic conditions to form a yellow complex whose absorbance is read at 410nm. The total nitrogen content provides an estimate of the total protein content by multiplying with a factor of 6.25 which was obtained from the estimated 16% average nitrogen content of protein (FAO, 2003). Details of the procedure, analysis and calculation are provided in Appendix A.

### 4.4.5. Glucose Analysis

The glucose concentration of coffee residues was determined with the use of a glucose analyser – Analox GL 6 Analyser. This method is based on the oxidation of glucose by glucose oxidase enzyme in a rapid reaction, and the measurement of the residual oxygen by an oxygen sensor. The chemical equation for the reaction is-

$$\text{B-D Glucose} + O_2 \xrightarrow[\text{oxidase}]{\text{Glucose}} \text{Gluconic acid} + H_2O_2$$

4.5 g/L standard glucose was used for calibration, after which 10 µL of coffee residue sample was introduced into the sample port with a micropipette, to obtain the glucose concentration.

### 4.4.6. Total reducing sugars assay

The total reducing sugars content of coffee residues and fermented samples was determined by the Miller dinitrosalicyclic acid method. The reaction is based on the reduction of 3,5-dinitrosalicyclic acid by the aldehyde group of sugar, resulting in colour change which is measured at an absorbance of 540nm (Miller, 1959). Known concentrations of glucose solution were used to obtain a standard glucose curve, from which the total reducing sugar content of the samples analysed were calculated. The procedure for sample preparation, analysis and total reducing sugars calculations are provided in Appendix A.

### 4.4.7. Free amino nitrogen assay

The free amino acid content of coffee residues and fermentation samples was determined by the ninhydrin colorimetric method recommended by the European Brewery Convention (Chandrashekar, 2002). It is based on the reaction of ninhydrin with the free α-amino group of amino acids to yield a purple-coloured complex , whose intensity is read at 570nm using a spectrophotometer. Known concentrations of glycine solution were used to obtain standard free amino nitrogen curve, from which the free amino nitrogen content of the samples were calculated. The procedure for sample preparation, analysis and total reducing sugars calculations are provided in Appendix A.

### 4.4.8. Neutral protease activity assay

Test for protease activity was performed to determine the production of protease during solid state fermentation of coffee residues by *A. oryzae.* Protease activity in solid state fermentation samples was determined using a caseinolytic assay. The method is based on the hydrolysis of casein by the protease contained in the samples at 55$^{o}$C for 30 minutes. The reaction was terminated using 10% Trichloroacetic acid and the amino acids produced were analysed by the Ninhydrin colorimetric method for free amino nitrogen analysis. One unit of protease activity was defined as the amount of enzyme which generated 1µg FAN from casein per minute, under the reaction conditions. The procedure for sample preparation, analysis and total reducing sugars calculations are provided in Appendix A.

## *4.5. Summary*

Extensive experimental and analytical procedures were performed in the course of this study, to investigate the application of bio-processing to coffee residues. The results obtained from these procedures are presented and discussed in the next chapter.

# Chapter 5: Results and Discussion

## *5.1. Introduction*

As described in chapter three, the major experiments carried out were the initial characterization of the chemical constituents of coffee residues; preliminary growth experiment on Petri dishes; isolation of indigenous micro-organisms; solid state fermentation in Erlenmeyer flasks, liquid state fermentation; as well as autolysis. The results of these experiments and analyses are presented in separate sections below, followed by a general comparative discussion of the results obtained.

## 5.2. Chemical Characterization of Coffee residues

The chemical constituents of coffee residues were characterized by determining the moisture, total solids, volatile solids and ash content; the carbohydrate and total reducing sugars content; and the protein and free amino nitrogen content. Knowledge of the initial chemical composition provided baseline information for solid and liquid state fermentation. The results obtained for each parameter are presented below in Table 5.1.

### 5.2.1. Moisture Content, Total solids, Volatile solids & Ash content

The initial moisture content of the coffee residues obtained was in the range 52.6%-59.9%. Coffee residues were collected in batches, thus the range in initial moisture content observed is likely due to discrepancies in coffee brewing procedure. The results obtained correspond with the 50-60% moisture content reported by Kondamudi et al. (2008) in their report. Moisture content of the substrate is an important factor in solid state fermentation as it plays a role in nutrient diffusion, microbial growth as well as enzyme stability (Krishna, 2005). The optimum moisture content for SSF varies amongst fungal strains and substrates, and lies between 30-80% w/w, thus moisture content is not an entirely efficient parameter for determining fungal growth (Chisti, 2010). Water activity, which is the ratio of the vapor pressure of water in the substrate to the ratio of pure water at the temperature of the substrate, provides a better prediction of the optimum water requirement for microbial growth (Chisti, 2010).

Nevertheless, a moisture content of 60-70% is employed in most SSF processes, thus, considerably small quantity of water is required for moisture content adjustment when using coffee residues as substrate for solid state fermentation.

The coffee residues analyzed showed low ash content of about 1%. Low ash content in substrates is desirable for bio conversion processes because it is suitable for growth of microbial cultures (Pandey et al., 2000c, Bakker and Elbersen, 2005); thus coffee residues are suitable for bioprocesses in that regard.

### 5.2.2. Total reducing sugars & Carbohydrate content

Considerably high amounts of carbohydrate were obtained in coffee residues, making up about 35-41% of the total solids on a dry basis. Only about 1% of the carbohydrate content obtained was available as simple sugars. Acid digestion of the total carbohydrates resulted in an increase in total reducing sugars from 1% to 38%.

The result for total carbohydrate content corresponds with the quantity reported for roasted coffee beans in Table 2.1 from the literature reviewed. This indicates that brewing has little or no effect on the carbohydrate content of roasted coffee beans; and infers that these carbohydrates are water insoluble polysaccharides. Also, the increase in total reducing sugars after acid digestion confirms that the carbohydrates available for microbial growth are mainly polysaccharides, which need to be hydrolyzed to obtain carbon and energy source. From the literature reviewed, these polysaccharides are believed to be majorly celluloses, β-1,4-mannan and hemicelluloses (Viani, 1993).

### 5.2.3. Free amino nitrogen and Protein content

Very small amount of free amino nitrogen was measured in the coffee residues, constituting about 0.08 mg/g of coffee residue on a dry basis.

The protein content of coffee residues was estimated from the total nitrogen content obtained, using the 6.25 multiplication factor discussed in chapter 4, under the method for total nitrogen content assay. Protein content was found to constitute 0.8-1.6% of the total solids present, on a dry weight basis. This value may not be accurate for the protein content of coffee residues, considering the presence of non-protein nitrogen in roasted coffee beans as reported in literature. However, most of the compounds containing non-protein nitrogen are reported to be water soluble by Arya and Rao (2007) as presented on Table 2.2, thus, a valid estimate of the total protein content may have been obtained.

**Table 5.1: Chemical composition of Coffee residues**

| Parameter | Quantity |
|---|---|
| Moisture Content (%) | 52.6-59.9 |
| Total Solids (%) | 47.45 |
| Volatile Solids (%) | 46.56 |
| Ash content (%) | 1.06 |
| Carbohydrate( mg/g CR db) | 350-410 |
| Free Amino Nitrogen(FAN)( mg/g CR db) | 0.08 |
| Total Reducing sugar (mg/g CR db) | 10 |
| Total Reducing sugar(Digested) (mg/g CR db) | 380 |
| Protein Content (mg/g CR db) | 8-16 |

## *5.3. Preliminary Growth Experiment*

Preliminary fermentation experiment was carried out in Petri dishes and the aim was to determine the growth of *Aspergillus awamori* and *Aspergillus oryzae* on coffee residues in solid state.

Growth of fungi is usually indicated by the observation of hyphae on the substrate**.** Fungal hyphae play an important role in the metabolism of these micro organisms. They effectively colonize and penetrate the substrate, resulting in contact with available nutrients e.g polysaccharides, which are broken down by hydrolytic enzymes into simple carbon and energy sources utilized by the fungi. These enzymes are released from the hyphal tips into the substrate medium.

The stoichiometric equation for microbial growth is presented as-

**Carbon Source + Water + oxygen + Phosphorous**

Biomass +$CO_2$ + Metabolite + Heat

Growth of *A.awamori* and *A.oryzae* hypha was observed in the preliminary experiment, as illustrated in Figure 5.1. However, *A.oryzae* hyphae emerged faster than *A. awamori.* Greenish *A.oryzae* hyphae were observed on the coffee residues after 24 hours of fermentation while grayish *A.awamori* hyphae were observed after 72 hours. Observation of

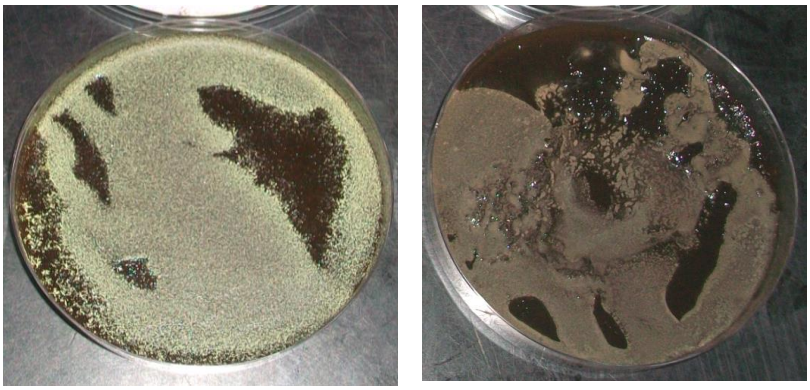
hyphal growth indicated the presence of available nutrients in coffee residues; thus, facilitated the performance of solid state fermentation in flasks, with sampling and chemical analysis performed.

The slower hyphal growth observed for *A. awamori* influenced the use of larger inoculum size for *A.awamori* SSF, but this had no effect on the growth pattern. *A.awamori* may thus be considered to have a longer lag phase than *A.oryzae*.

**Figure 5.1: Preliminary solid state fermentation with *A. oryzae, A. awamori* and un-inoculated control (left to right).**

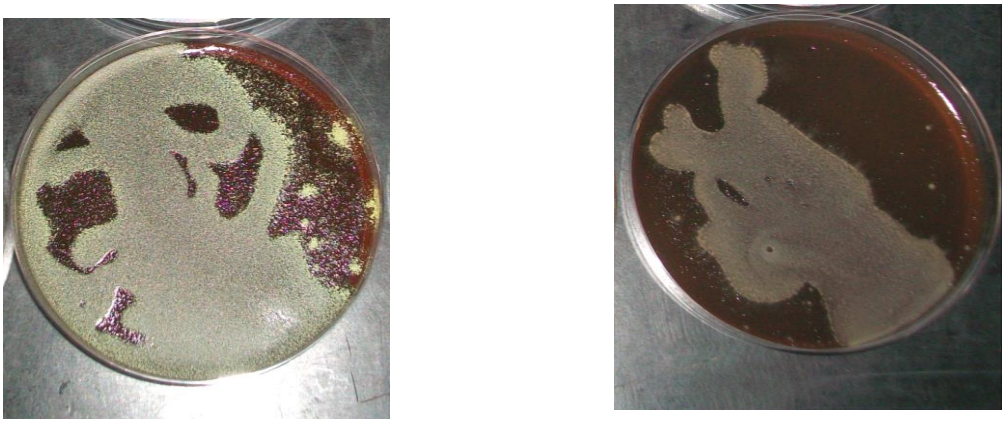
## *5.4. Toxicity/ Inhibition of various forms of coffee*

These experiments were performed on Petri dishes to determine the presence of any compounds which are toxic or inhibitory to the growth of *A. awamori* and/or *A. oryzae* in coffee residues, as well as roasted coffee beans. Similar growth patterns of *A. awamori* and *A. oryzae* were observed for coffee residues, milled roasted coffee beans and coffee extract; as illustrated in Figure 5.2. This suggests the absence of any compounds which are inhibitory or toxic to the growth of the fungal strains used, in any of the coffee forms. The results also suggest that coffee brewing process does not result in any change to the chemical composition of coffee residues.



**a).*A. oryzae* SSF on milled roasted coffee beans and agar**

**b). *A. awamori* SSF on milled roasted coffee beans and agar**



**c).*A.oryzae* SSF on coffee extract and agar**

**e). *A.awamori* SSF on Coffee extract and agar**

**Figure 5.2: Inhibition/Toxicity test on various forms of coffee**

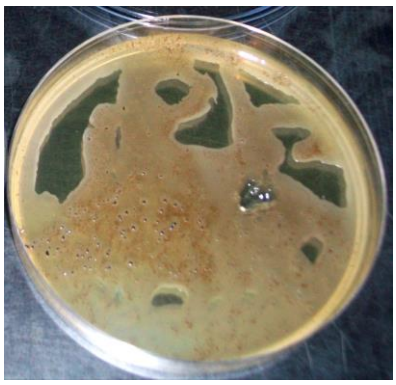
## *5.5. Isolation of indigenous micro-organisms*

Agar plating experiments were performed in Petri dishes to determine the presence of indigenous fungi and bacteria in the coffee residues. Presence of bacteria and fungi confirmed the ability of coffee residues to support microbial growth. It also resulted in the need to sterilize the coffee residues before performing solid state fermentation, in order to prevent interference with the experiment. Presence of bacteria was further investigated by liquid state fermentation to determine the effect of indigenous bacteria on the chemical constituent of coffee residues.

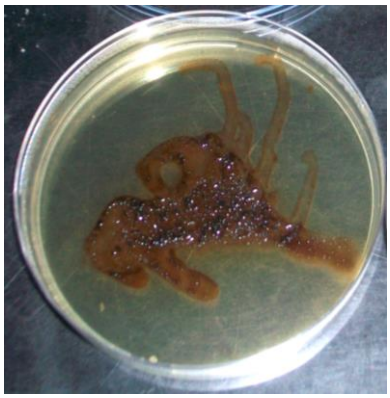
### 5.5.1. Bacteria Isolation

The presence of bacteria in unsterilized coffee residues was indicated by the colour change observed in Figure 2.3a below, in comparison to Figure 2.3b which was performed with sterilized coffee residues. The bacteria obtained were re-isolated, as shown in Figure 2.3c, to obtain homogenous colonies. Observation under the microscope showed rod-shaped bacteria, shown in Figure 2.3d.

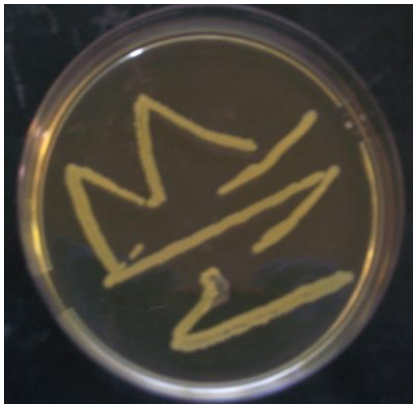
Inoculation of sterilized coffee residues-agar medium with the isolated bacteria resulted in bacteria growth, as illustrated in Figure 2.3e, indicating the presence of available nutrients in the coffee residues. Performance of a similar experiment on glucose-agar medium also resulted in bacteria growth, shown in Figure 2.3(g), suggesting the presence of sugars which can be utilized by bacteria in coffee residues.



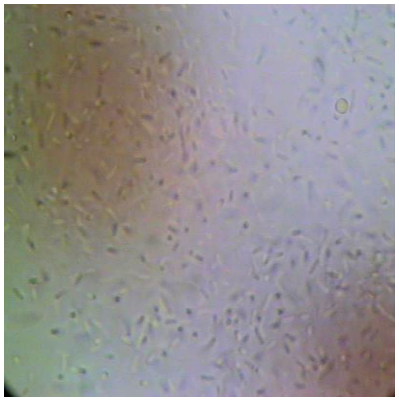
a).Bacterial growth in unsterilized coffee grounds



b).Experiment with sterilized coffee grounds



c). Isolated indigenous bacteria on nutrient agar medium



d).Microscopic picture of rod-shaped indigenous bacteria

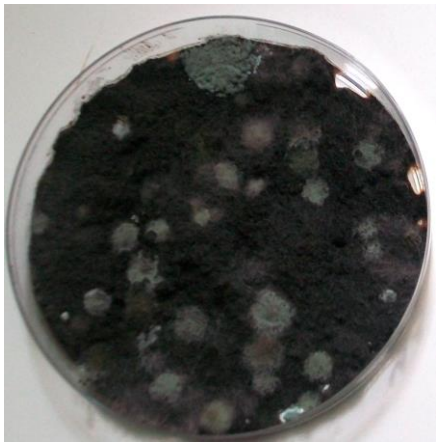
e).Growth of bacteria on sterilized coffee residue and agar medium

f). Growth of bacteria on sterilized glucose and agar medium

**Figure 5.3: Test for indigenous bacteria**

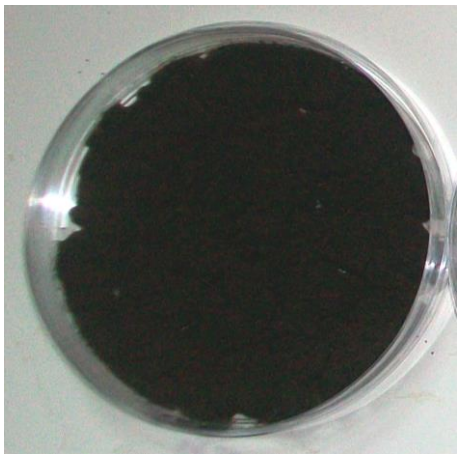
### 5.5.2. Fungal Isolation

Indigenous fungi in unsterilized coffee residues were observed on covered Petri dishes after seven days at room temperature. This experiment was performed away from the Morton laboratory to prevent interference by the fungal species present in the laboratory. As shown in Figures 5.4 below, different fungal species were found in the coffee residues. Figure 5.4a has a fungal growth pattern with different defined colonies, compared to Figure 5.4b. This is likely due to more suitable growth condition resulting from moisture content adjustment of coffee residues in Figure 5.4a, unlike Figure 5.4b which was unadjusted. Figure 5.4c was uncovered for the period of this experiment, and it contained unsterilized coffee residues with the same adjusted moisture content as Figure 5.4a, but no indigenous or atmospheric fungal growth was observed. This observation was likely due to an unsuitable moisture condition, which may have resulted from evaporation of moisture from the exposed surface; thus further confirms the importance of moisture to fungal growth in solid state fermentation.



**a). Fungal isolation on unsterilized coffee residues with adjusted moistue**

**b). Fungal isolation on unsterilized coffee residues with unadjusted moisture**



**c). Unsterilized coffee residues in opened Petri dish**

**Figure 5.4: Test for indigenous fungi**

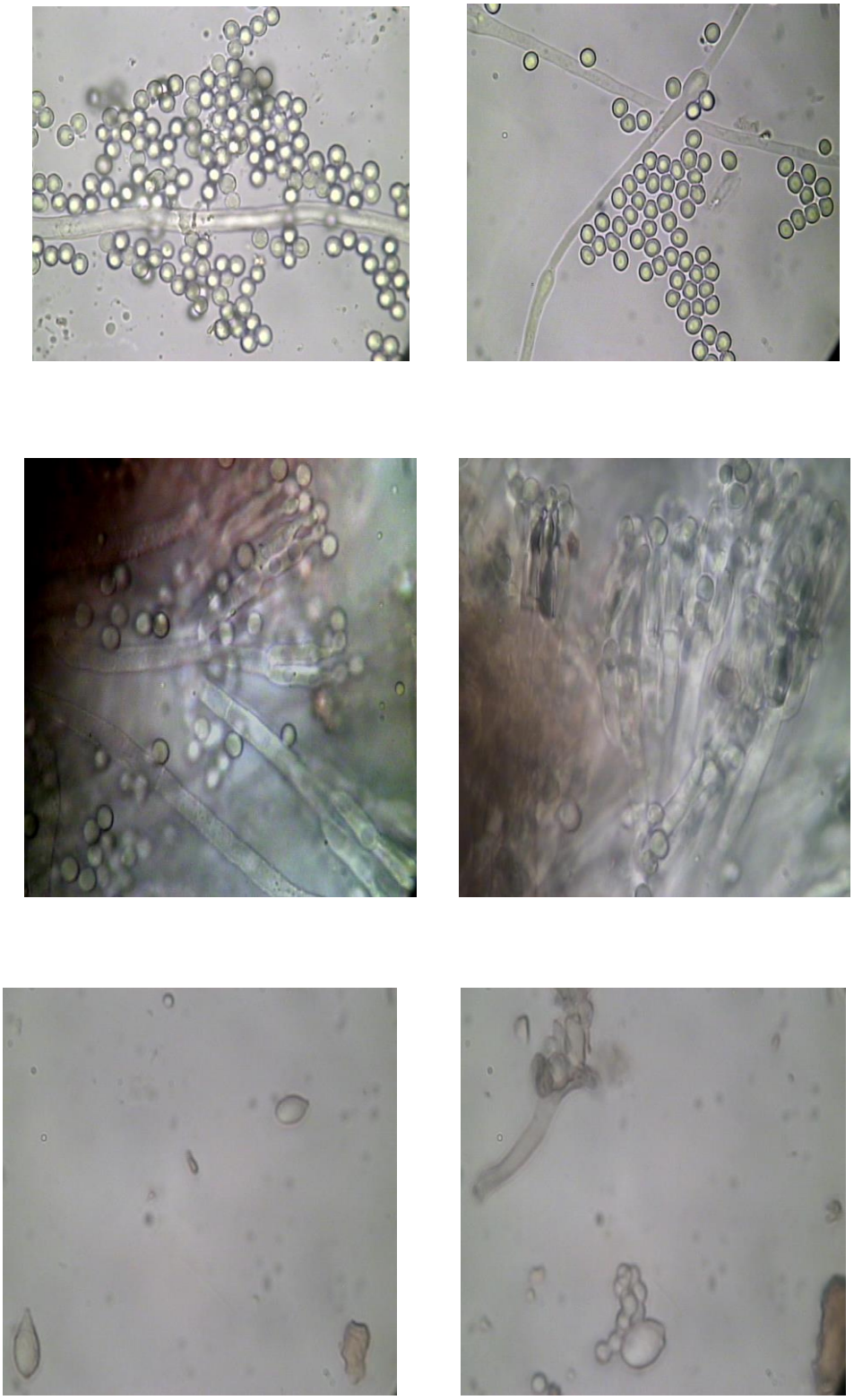


**Figure 5.5: Microscopic pictures of indigenous fungal spores and hypha**

## *5.6. Solid state fermentation*

Solid state fermentation by *A. awamori* and *A. oryzae* was carried out in 200ml Erlenmeyer flasks, in order to monitor the chemical changes occurring over the fermentation time. The parameters analysed for every sample include- moisture content, pH, total reducing sugars and free amino nitrogen. Protease activity was determined only for samples from *A. oryzae* SSF because *A. oryzae* is known to synthesize extracellular protease with ease of downstream enzyme separation (Murthy and Naidu, 2010). The results obtained for each fungus are presented below, using both column and scatter plots. The scatter plots present an average of the triplicate SSF experiment performed in separate flasks for each sampling time. However, variations occur in the separate flasks, as is typical of solid state fermentation, and so a column chart may give a clearer representation of the results obtained

### 5.6.1. Solid state fermentation by *Aspergillus awamori*

As obtained in the preliminary growth experiment, emergence of *A. awamori* hyphae was observed after 48 hours of inoculation. Samples were taken at intervals for chemical analysis. Figure 5.5 below shows the presence of grey mycelia in the SSF experiment, compared to the control experiment.


Figure 5.5: Solid state fermentation of coffee residues by *Aspergillus awamori*

### 5.6.1.1. Moisture content analysis

The initial moisture content of coffee residues was adjusted to 73% in each flask before sterilization in the autoclave; after which 1ml of *A. awamori* spores was inoculated. This adjustment was to enable as much mixing between the inoculum and the coffee residues as possible. As presented in Figure 5.5a below, the trend for moisture content shows decrease and increase as fermentation proceeds, whereas, the trend for the control experiment shows a marked decrease with time. The difference in initial moisture content observed between the SSF sample and the control experiment sample is very likely due to the addition of inoculum to the SSF flasks. The observed decrease in initial moisture content from the adjusted value of 73% may be as a result of moisture evaporation during sterilization.

The results have been represented on a column chart in Figure 5.5b below. This chart shows that there are variations in the results obtained in the separate fermentation flasks, which confirms the challenge of non-homogeneity encountered in solid state fermentation systems.

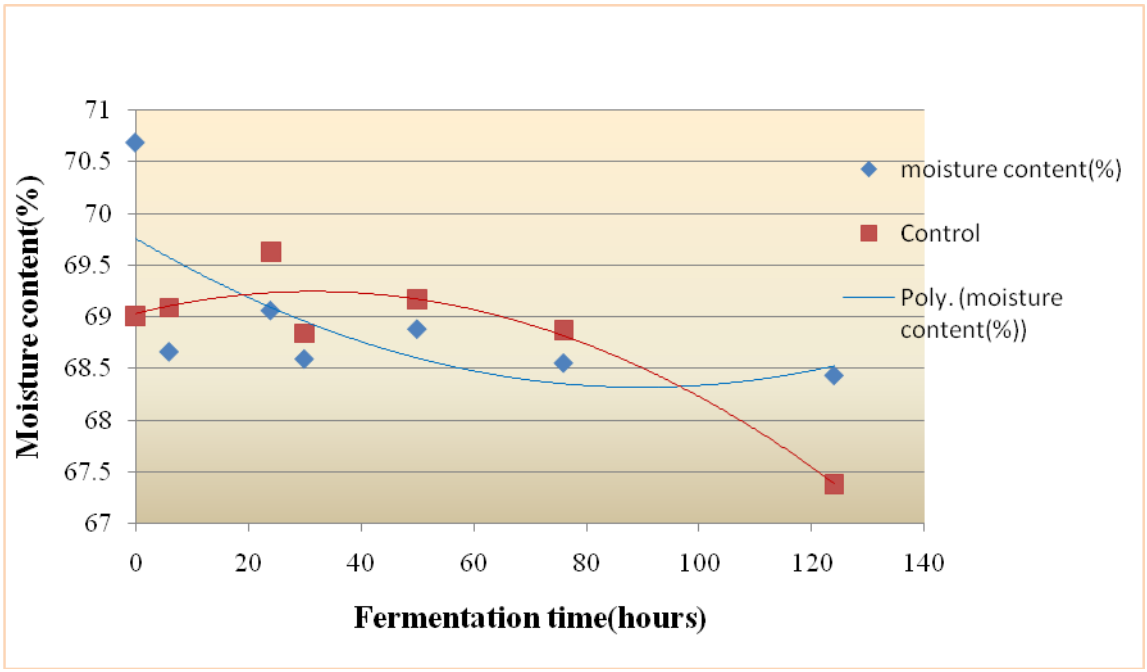



Figure 5.6a: Scatter plot for change in moisture content during solid state fermentation by *Aspergillus awamori*

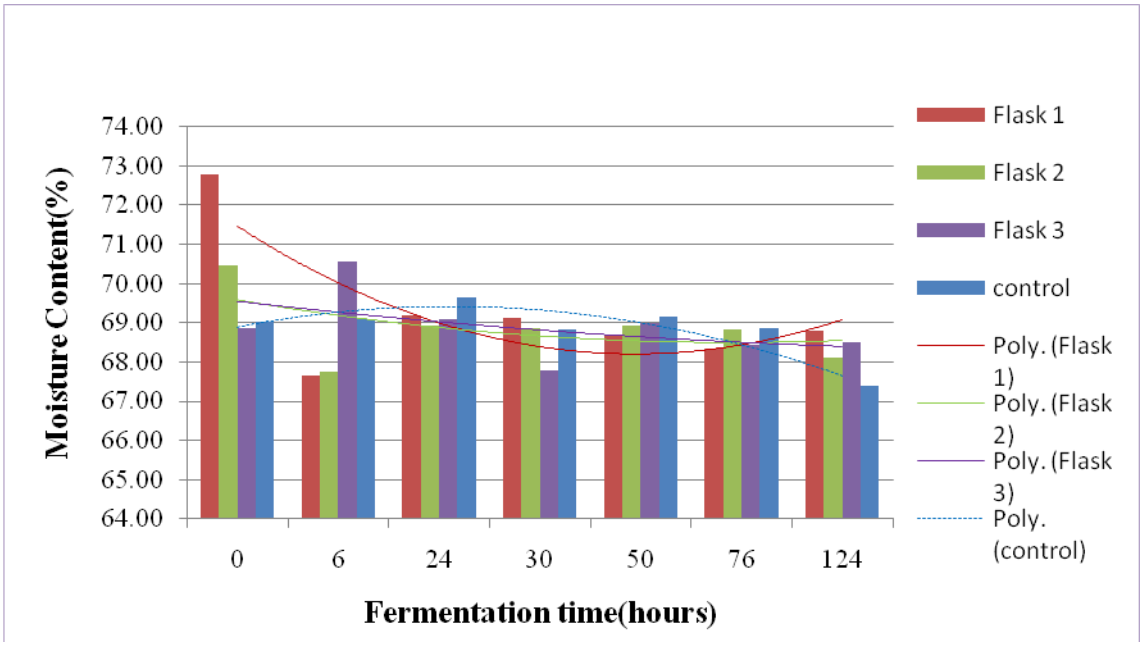



**Figure 5.6b:** Column plot for change in moisture content during solid state fermentation by *Aspergillus awamori*

### 5.6.1.2. pH analysis

The pH of each sample was taken to detect any change in acidity or alkalinity occuring with fermentation. As provided in Fig 5.6 below, solid state fermentation by *A. awamori* occurred in an acidic environment, with pH between 5.1 and 5.7. The slightly higher pH of the initial fermentation sample is likely due to the inoculum which was preserved in Sodium chloride solution.

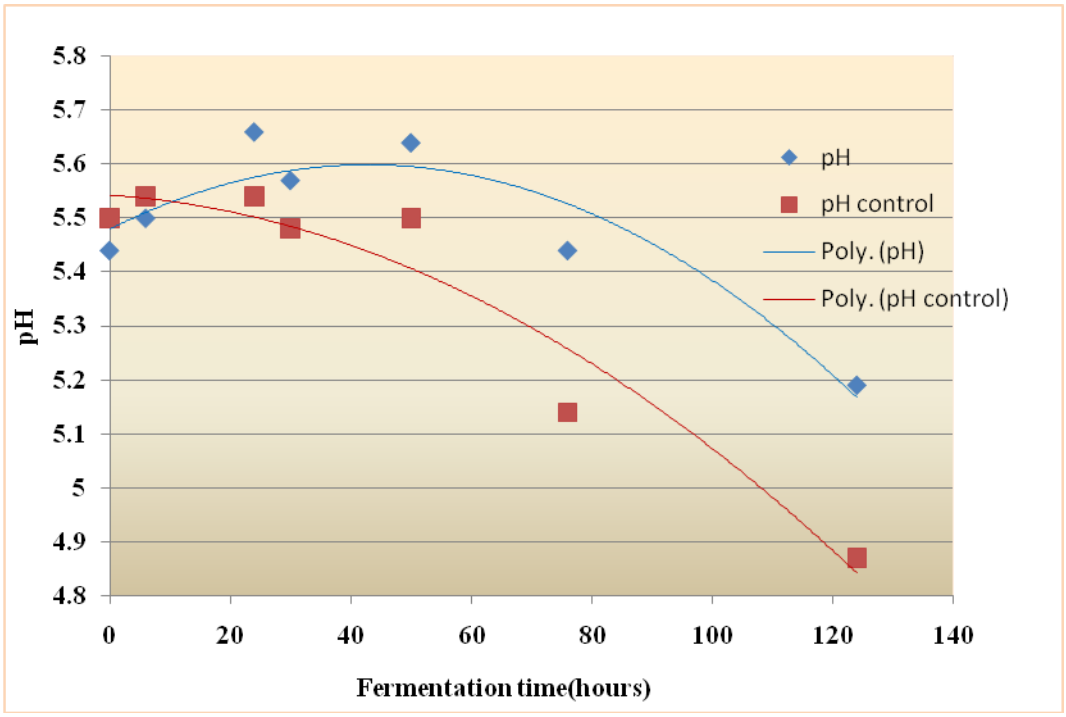



Figure 5.7: Scatter plot for change in pH during solid state fermentation by *Aspergillus awamori*.

### 5.6.1.3. Total reducing sugar analysis

The total reducing sugar was analysed in the samples taken to determine the ability of *A. awamori* to hydrolyze the polysaccharides present in coffee residues into simple sugars to obtain carbon source. From the trend depicted in Figure 5.7 below, a decrease in the concentration of total reducing sugar is observed until the 76$^{th}$ hour, after which an increase is observed. The decrease in concentration observed between 0hr and 70$^{th}$ hour is likely due to consumption of the available total reducing sugars. The increase in total reducing sugars observed from the 76$^{th}$ hour corresponds with the time of hyphae emergence, as discussed in section 5.3 above, suggesting the release of hydrolytic enzymes from the hyphal tips from the 76$^{th}$ hour.

A difference of 10mg/g is observed between the initial reducing sugar content of the coffee residues inoculated with *A. awamori* and the coffee residues characterised in Figure 5.1 above. According to Wilson and Lilly (1958), changes occur in certain sugars when autoclaved. This may thus be responsible for this difference observed.

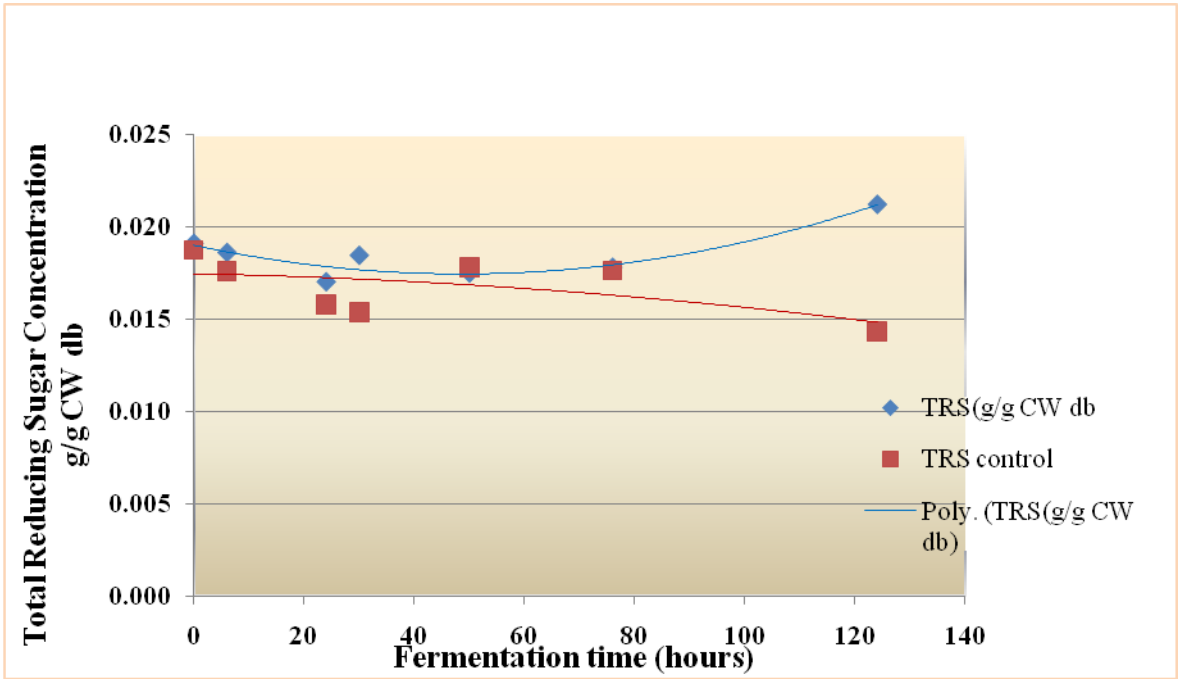



Figure 5.8a: Scatter plot for change in total reducing sugars during solid state fermentation by *Aspergillus awamori*

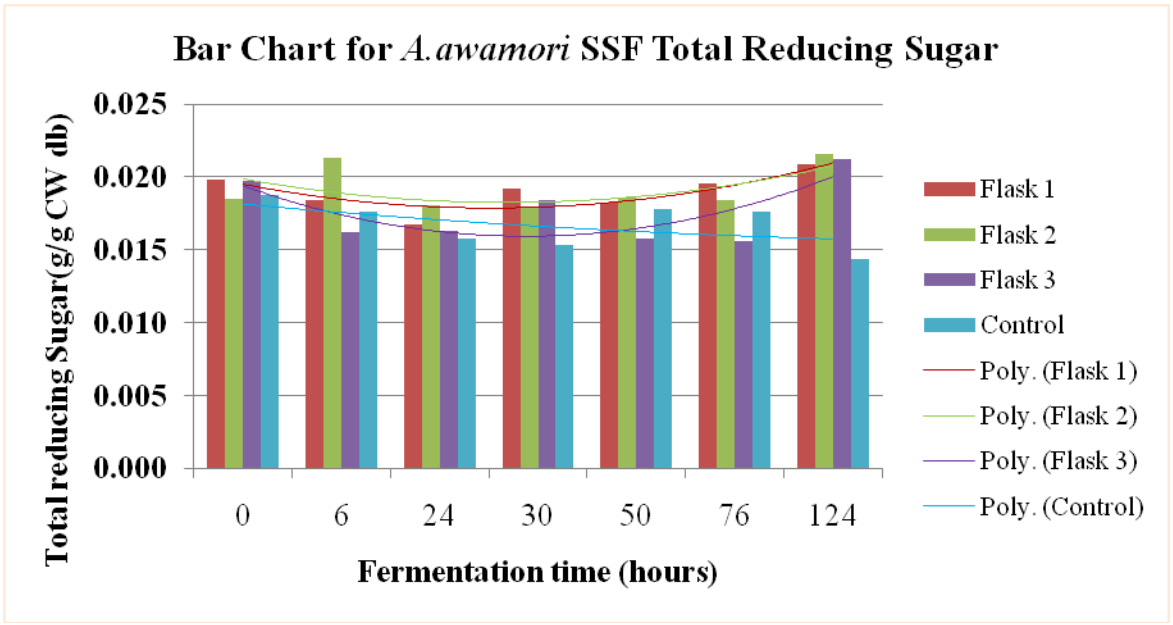



Figure 5.8b: Column plot for change in total reducing sugars during solid state fermentation by *Aspergillus awamori*

#### 5.6.1.4. Free amino nitrogen analysis

Similar to the trend for reducing sugar concentration, a decrease in free amino nitrogen concentration occurs during SSF by *A. awamori.* This further confirms the role of hyphae in the hydrolysis of macromolecular nutrient sources.

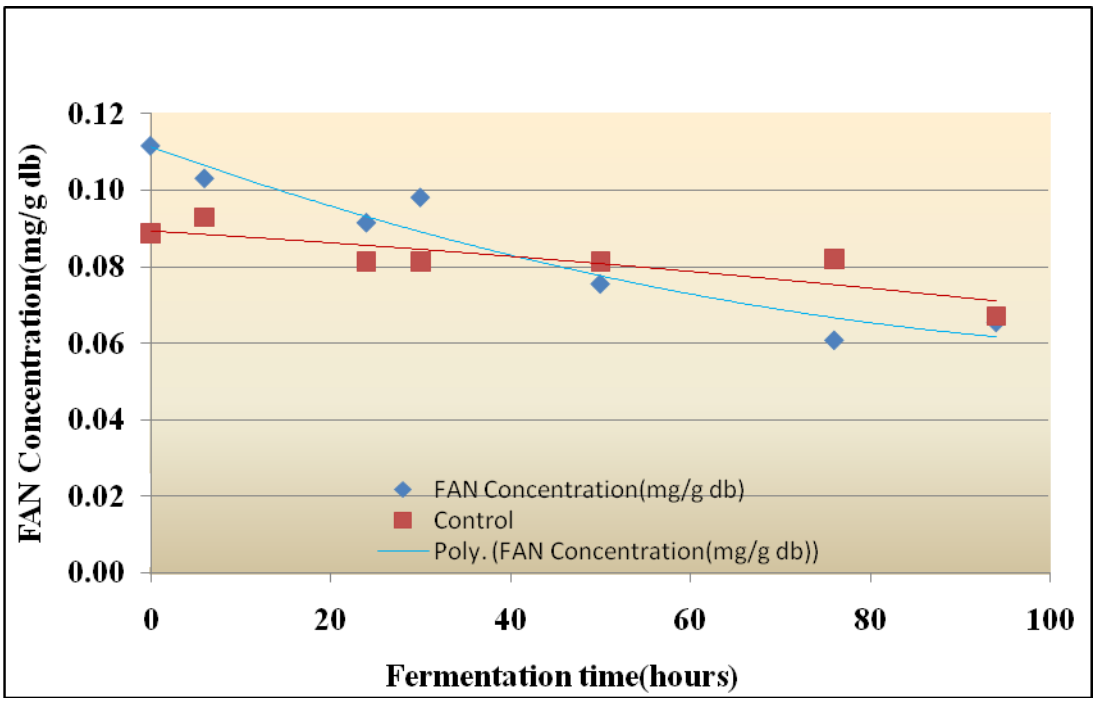



Figure 5.9: Scatter plot for change in free amino nitrogen during solid state fermentation by *Aspergillus awamori*

### 5.6.2. Solid state fermentation by *Aspergillus oryzae*

SSF by *A. oryzae* was performed for 4 days and samples were taken at 0, 6, 24, 30, 52, 70 and 94 hours of fermentation. Growth of *A. oryzae* hyphae in fermentation flasks was observed after 24 hours, as observed in the preliminary growth experiment described in section 5.3 above. The results obtained from the analysis performed are presented below.

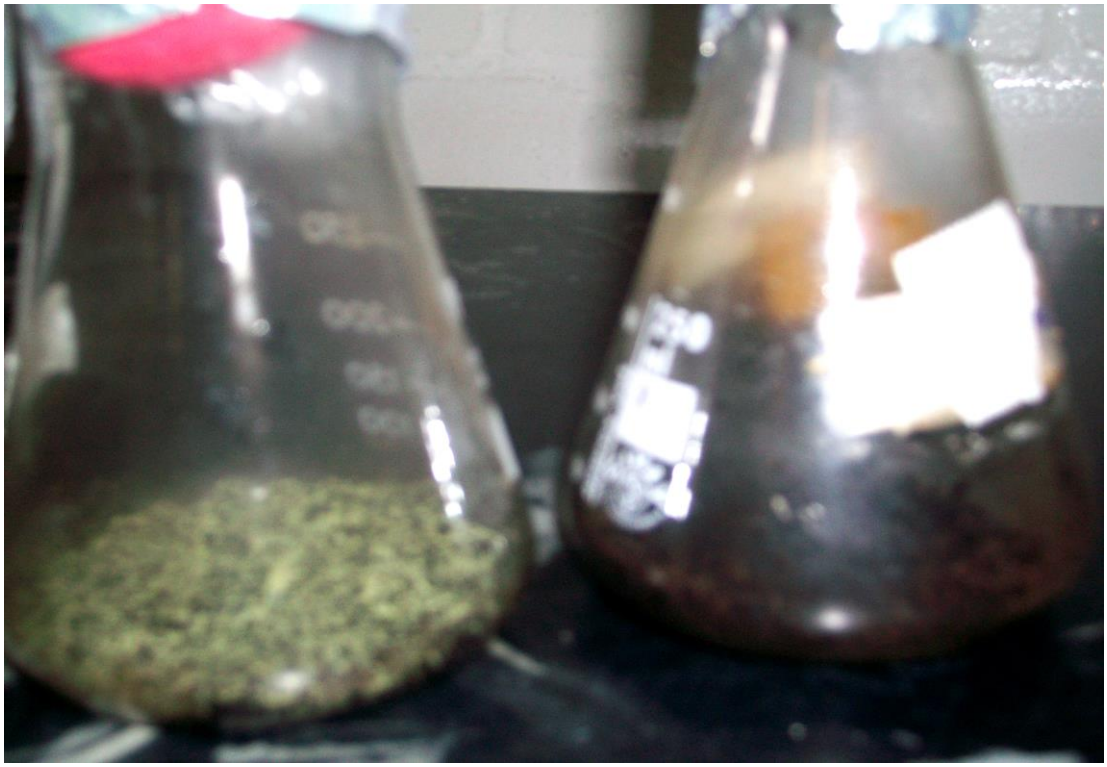


Figure 5.10: Solid state fermentation of coffee residues by *Aspergillus oryzae*

**5.6.2.1. Moisture content analysis**

As performed for SSF with *A. awamori*, the moisture content of coffee residues was adjusted to 73%, then autoclaved and inoculated with 50µl *A. oryzae* spores. As depicted in Figure 5.9 below, the trend obtained shows a slight increase and decrease in moisture content over fermentation time for the SSF samples taken, while the control experiment shows progressive decrease in moisture content. The difference between the initial moisture content of the SSF sample and the control experiment may be due to variations in the separate fermentation flasks or due to experimental errors. The initial moisture content of both fermentation and control flasks are lower than the adjusted value, which further suggests that autoclaving may result in evaporation of moisture.

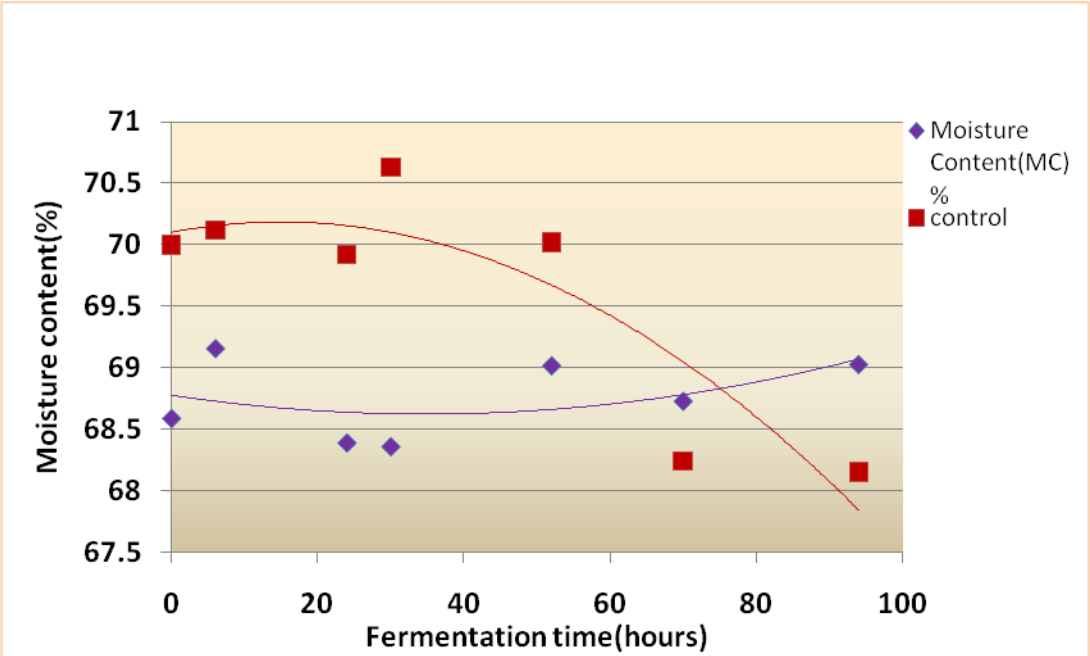



Figure 5.11: Scatter plot for change in moisture content during solid state fermentation by *Aspergillus oryzae.*

**5.6.2.2. pH analysis**

SSF by *A. oryzae* occurred in an acidic environment with pH between 5.0 and 5.5, as shown in Figure 5.10. below. The trend shows an increase in pH until the 52$^{nd}$ hour of fermentation after which a decrease is observed, while the trend obtained for the control experiment is fairly constant

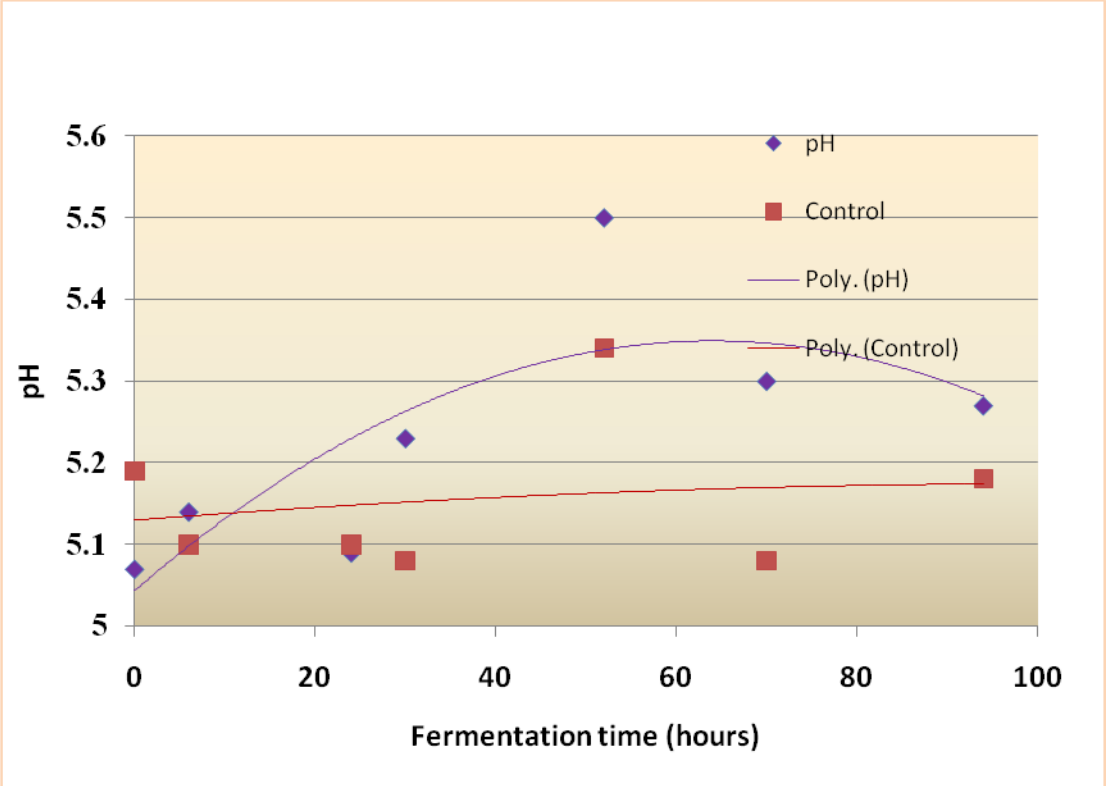



Figure 5.12: Scatter plot for change in pH during solid state fermentation by *Aspergillus oryzae.*

### 5.6.2.3. Total reducing sugar analysis

The results obtained for total reducing sugar analysis for SSF by A. oryzae are illustrated in Figure 5.11a and b below. The trend shows gradual increase and decrease with fermentation, with highest concentration observed between the 30$^{th}$ and 60$^{th}$ hour of fermentation. This corresponds with the time of hyphae emergence and indicates the release of polysaccharide hydrolytic enzymes by *A.oryzae* hyphal tips after 24 hours of fermentation. The decrease observed is likely due to consumption of reducing sugars by the fungus for carbon and energy source.

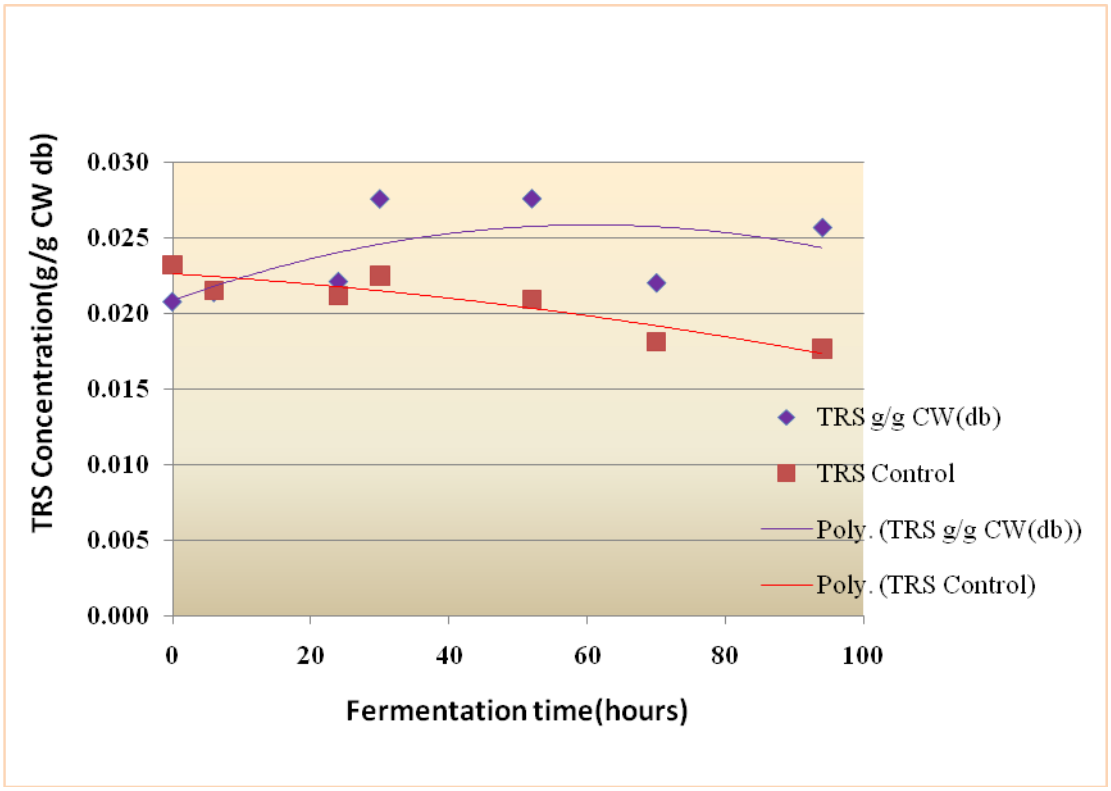



Figure 5.13a: Scatter plot for change in total reducing sugars during solid state fermentation by *Aspergillus oryzae.*

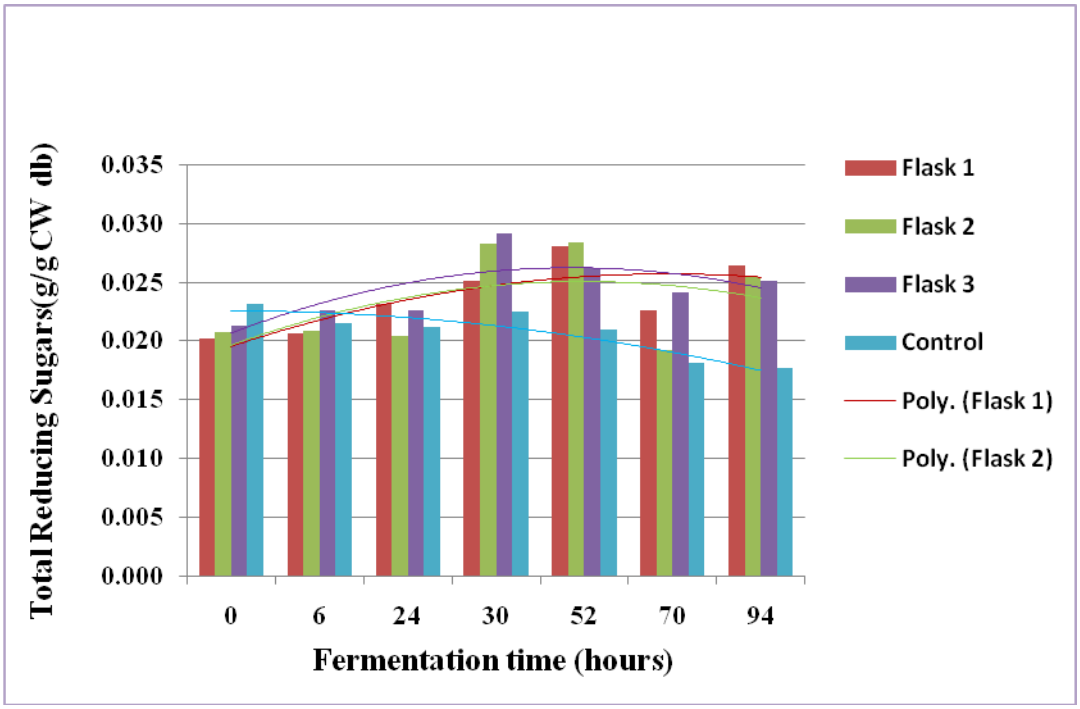



Figure 5.13b: Column plot for change in total reducing sugars during solid state fermentation by *Aspergillus oryzae*

### 5.6.2.4. Free amino nitrogen analysis

Analysis of the change in free amino nitrogen concentration showed an increase from the 30$^{th}$ hour of fermentation depicted in Figure 5.12, which also corresponds with the emergence of hyphae and indicates the release of proteolytic enzymes by *A. oryzae*.

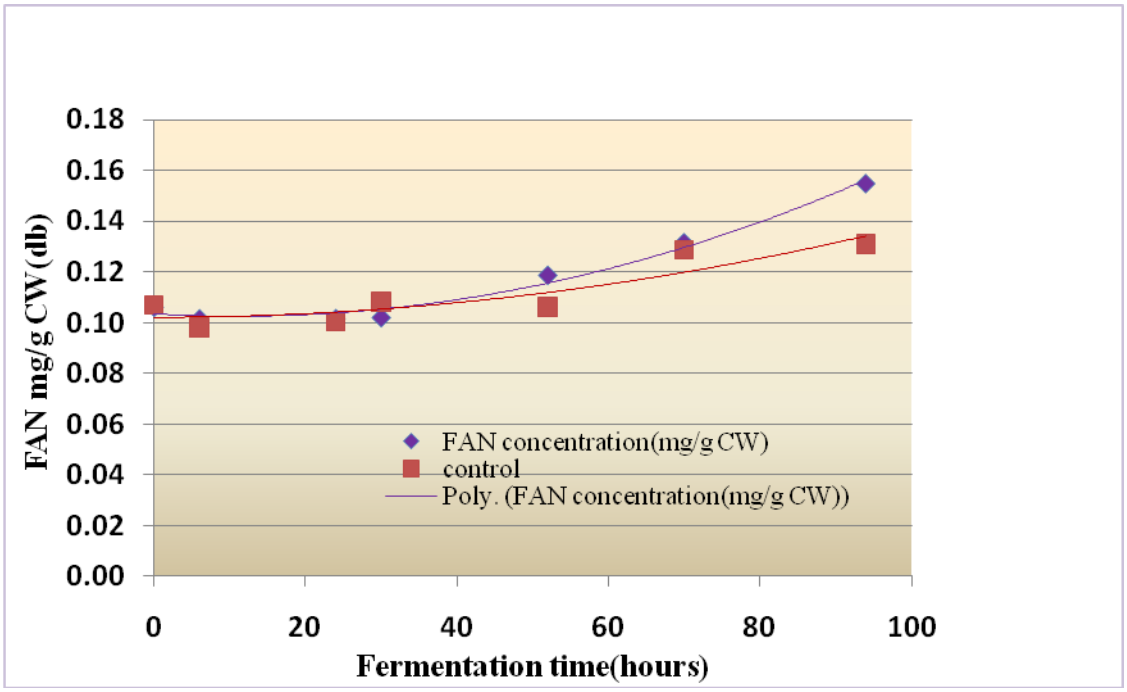



Figure 5.14a: Scatter plot for change in free amino nitrogen during solid state fermentation by *Aspergillus oryzae*

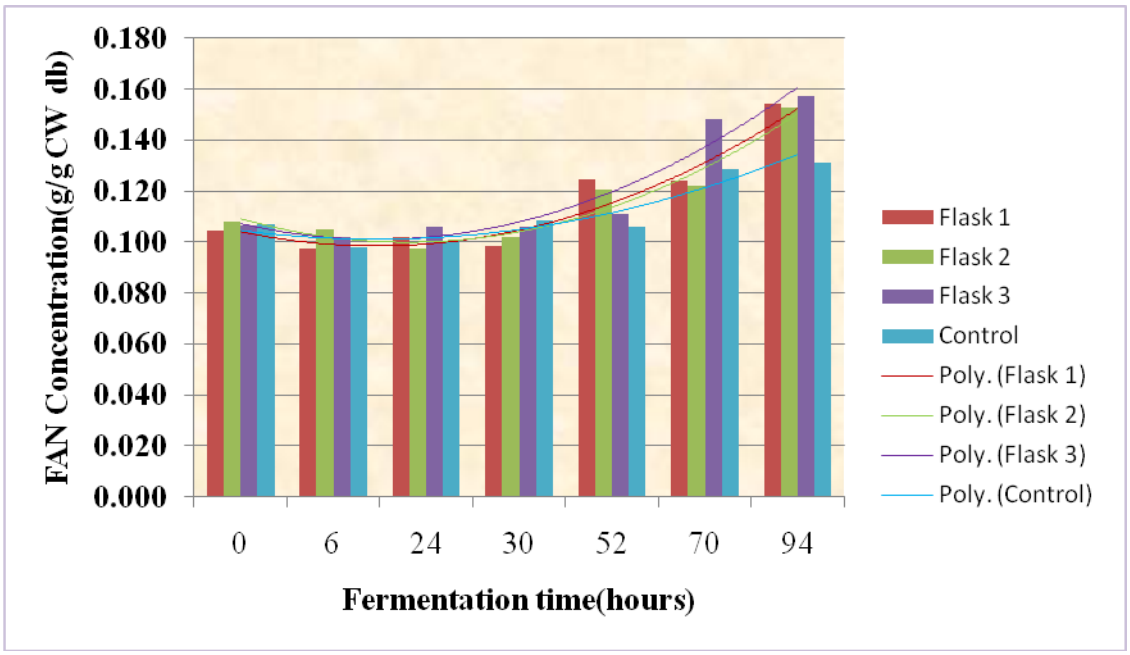



Figure 5.14b: Column plot for change in free amino nitrogen during solid state fermentation by *Aspergillus oryzae*

### 5.6.2.5. Protease activity analysis

Protease activity was determined to confirm the synthesis of this enzyme during SSF by *A. oryzae*. As shown in Figure 5.13 below, no protease activity was obtained until after 30 hours of fermentation, after which an increase in activity with fermentation was obtained, with maximum activity of 92U/g db at the 94$^{th}$ hour. This result corresponds with the emergence of *A. oryzae* hyphae, as well as the increase in free amino nitrogen concentration observed; indicating the hydrolysis of the proteins present in coffee residues to amino acids by protease released.

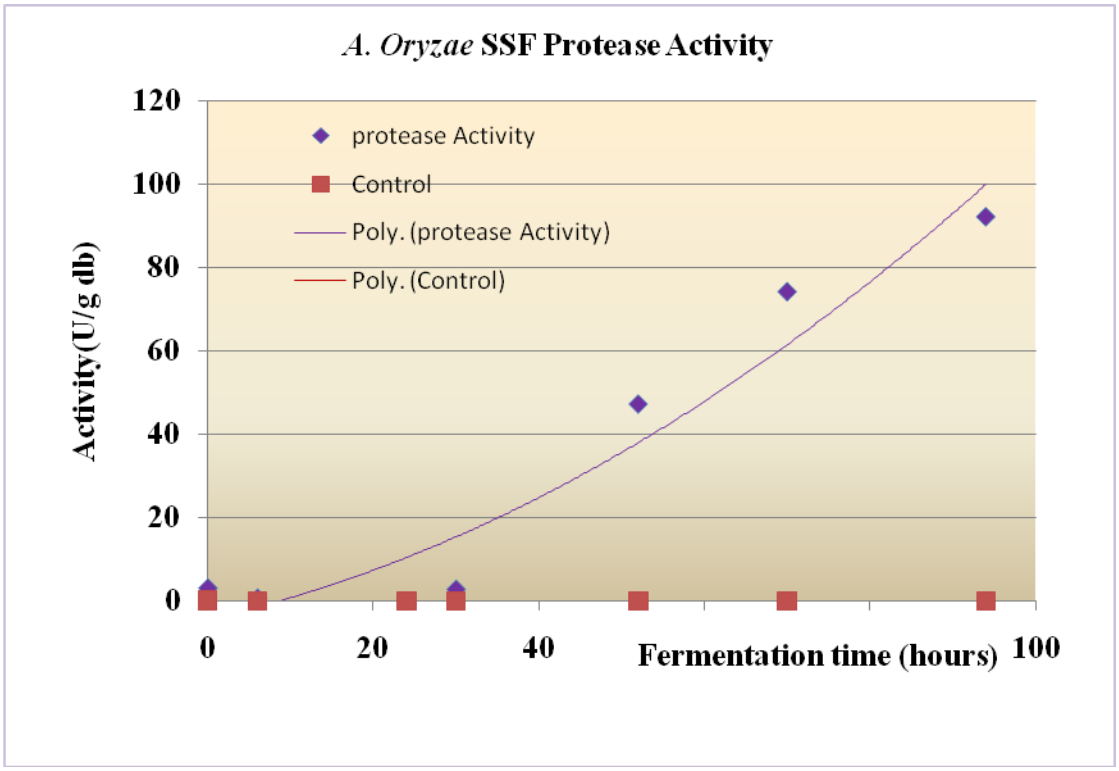



Figure 5.12b: Scatter plot for change in protease activity during solid state fermentation by *Aspergillus oryzae*

### 5.6.3. Autolysis Experiment

The samples obtained from the autolysis experiment carried out were analysed for the change in free amino nitrogen, as well as change in protease activity occurring during autolysis.

## 5.6.3.1. Effect of Autolysis on Free amino nitrogen content

An increase in free amino nitrogen was obtained during autolysis. As shown on Figure 5.12 below, the FAN concentration increased by over 90% of the initial concentration at the 16$^{th}$ hour of autolysis. A slight decrease is obtained by the 48$^{th}$ hour. The increase in FAN concentration is likely due to the breakdown of cellular proteins of *A. oryzae* and other proteins generated during fermentation. This indicates that solid state fermentation of coffee residues may be applied to increase the protein content of the residues. Also evident in the figure is the variation which occurred in the two SSF flasks which were prepared under the same conditions. This further confirms the challenge of variation and reproducibility encountered in SSF systems, however a similar trend was followed by the results obtained from both flasks, thus a conclusion may be drawn from these results.

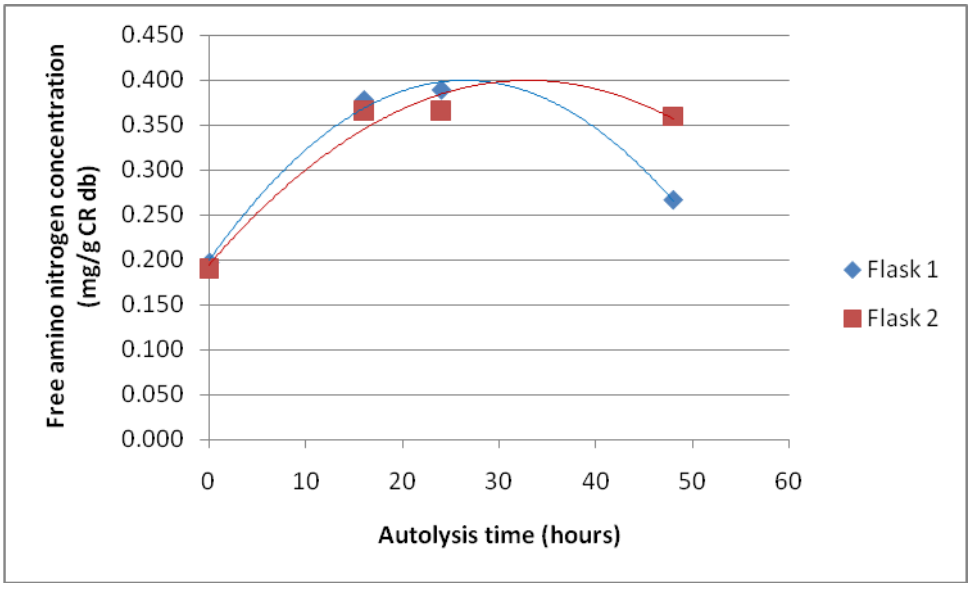



Figure 5.12: Plot for change in free amino nitrogen content during autolysis

### 5.6.3.2. Effect of Autolysis on protease activity

The change in protease activity with autolysis was also analysed. From Figure 5.13 below, it is apparent that a significant decrease in enzyme activity occurred with autolysis of the *A. oryzae* SSF product. Protease activity of between 100-120 U/g Coffee residue (db) was obtained for the 0 hour autolysis sample, which was reduced to about 24 U/g db by the 16$^{th}$ hour of autolysis and reducing to about zero activity by the 48$^{th}$ hour of autolysis. This reduction is likely due to the destruction of the enzymes, which are proteins, during autolysis, thus contributing to the increase in free amino nitrogen obtained.

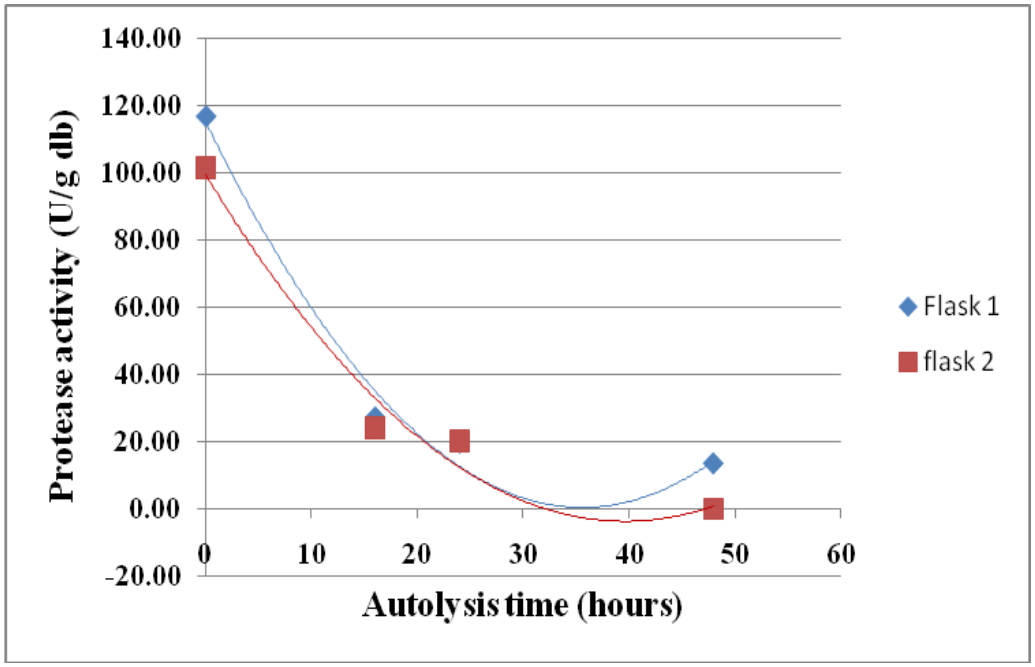



Figure 5.13: Plot for change in Protease activity during autolysis

## *5.7. Liquid state fermentation*

Liquid state fermentation in flasks by isolated indigenous bacteria was performed for 2 days. The aim was to determine the effect of the indigenous bacteria isolated on the chemical constituents of coffee residues, and to investigate the possibility of obtaining value- added products through this means. Samples were taken after 24 and 48 hours of fermentation and analysed for total reducing sugars as well as free amino nitrogen. The results obtained are presented below.

### 5.71. Total reducing sugar analysis

From Figure 5.13 below, it is apparent that an increase in total reducing sugars concentration occurred within 24 hours of fermentation, which levelled out by the 48$^{th}$ hour of fermentation.

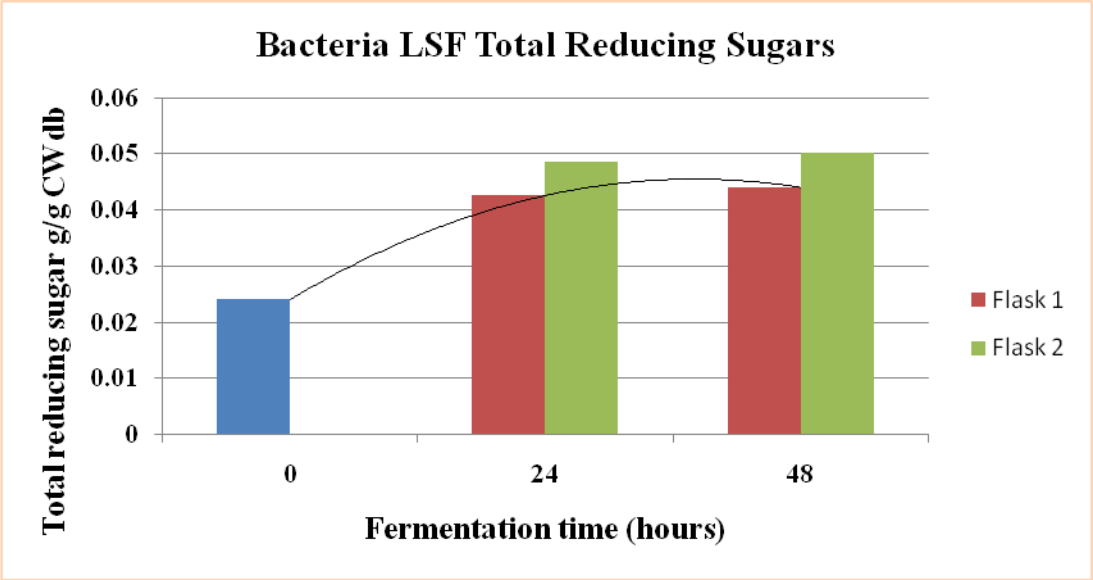



Figure 5.13: Column plot for change in total reducing sugars during liquid state fermentation by indigenous bacteria

A similar trend as obtained for the total reducing sugars concentration during bacterial liquid state fermentation was obtained for the free amino nitrogen concentration.

## *5.8. General and comparative discussion*

The results obtained have been presented for each experiment performed in the above sections. This section provides a general discussion of the results obtained, and makes comparisons between the trends observed for the different experiments.

### 5.8.1. Moisture content

Solid state fermentation by *Aspergillus awamori* and *Aspergillus oryzae* gave similar trend for moisture content, as depicted in Figure 5.14 below. A slight decrease and increase is observed for both fungi, while both control experiments show progressive decrease in moisture content. The trend observed for both strains of fungi can be explained by the metabolic activities of these organisms. Water is used by fungi for the metabolism of available nutrient sources, as illustrated in the stoichiometric microbial growth equation, to produce metabolites, carbon dioxide, biomass as well as heat (Biesebeke et al., 2002). Mass

and heat transfer occur slowly by diffusion and exchange of gases; and by conduction, free convection and evaporation of water (Mitchell et al., 1999b). These mechanisms are likely to play a role in the fluctuation of moisture content observed. The higher volume of *A.awamori* inoculum used explains the higher initial moisture content obtained.

The decrease in moisture content observed in the control experiments may be due to evaporation and condensation effects. Sterilization of the medium at 121$^{o}$C resulted in evaporation of moisture from the solid substrates into the air layer and walls of the flask. Condensation of moisture onto the solid may be responsible for the increase in moisture content observed between 0hr and 24hr of fermentation. Physical mechanisms such as capillary forces and temperature gradients may also contribute to the trend observed. As discussed above, variations in moisture content are obtained in the separate fermentation flasks, which may be due to physical dynamics or experimental errors. This confirms the challenge of homogeneity encountered with solid state fermentation and limits the ability to make definite conclusions about the moisture changes during solid state fermentation. Nevertheless, it is suggested that the fungal metabolic activities provide a counter-balancing effect on moisture content (Larroche et al., 1992), which explains the difference in the trend obtained between the control and fermentation experiments.

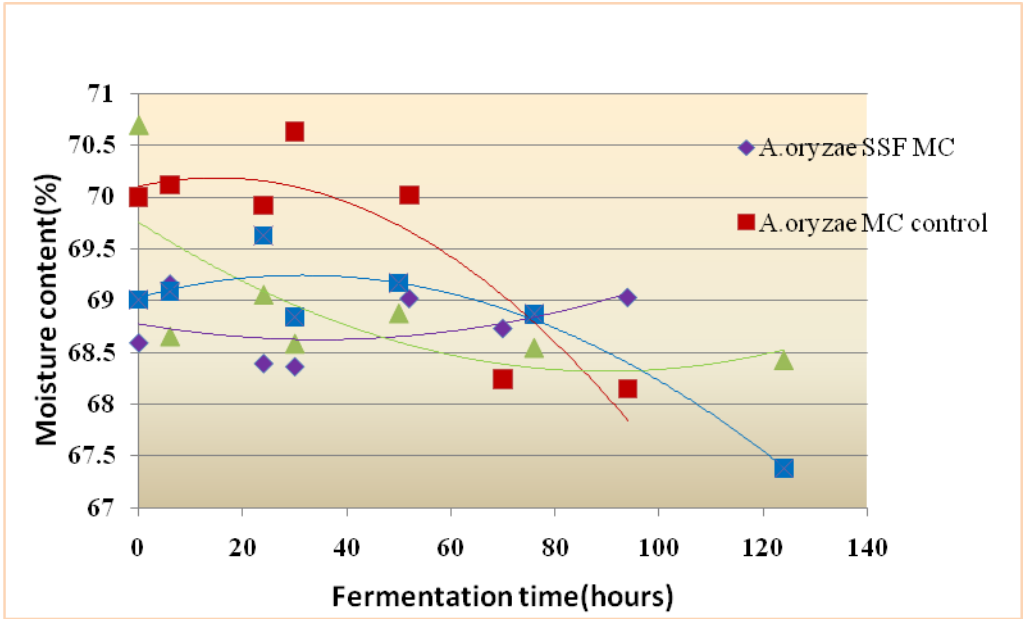



Figure 5.14: Scatter plot for comparison of change in moisture content during solid state fermentation by *A. awamori* and *A. oryzae*

### 5.8.2. Total reducing sugars

As presented in Figure 5.15 below, an increase in total reducing sugar concentration is observed between the 30$^{th}$ and 60$^{th}$ hour of fermentation by A. oryzae, while a decrease is observed for SSF by A. awamori until the 76$^{th}$ hour, after which an increase is observed. The increase in total reducing sugar concentration corresponds with the emergence of hyphae for both fungi, suggesting that *A. oryzae* adapted faster to the prevailing coffee residues environment than *A. awamori*.

The growth of hyphal tips depends on local nutrient concentration and the hyphae penetrate the substrate and release hydrolytic enzymes for the synthesis of carbon and energy sources (Mitchell et al., 1999b) . This theory may thus be used to explain the relationship between hyphal growth and the increase in total reducing sugar concentration. Polysaccharide hydrolytic enzymes are probably being released by *A.oryzae* after 24 hours of fermentation, and uptake of carbon by the fungi then results in decrease in reducing sugar concentration. The decrease observed between 0hr and 70$^{th}$ hour for *A.awamori* SSF is likely due to consumption of the available reducing sugars in coffee residues, which were characterized in section 5.2 above.

Comparing the results obtained from SSF with liquid state fermentation by indigenous bacteria, a marked difference in the trend for total reducing sugars concentration is observed. An increase of 89.5% was observed to occur within 24hours of liquid state fermentation, which apparently remained unchanged by the 40$^{th}$ hour. Sampling at more frequent intervals within 24 hours of LSF may have provided more definite information about the different growth phases of the bacteria inoculated, however, it is suggested that the lag and exponential phases occurred within 24 hours and the stationary phase may have been approached by the 48$^{th}$ hour of fermentation.

The results indicate the ability of the indigenous bacteria to better hydrolyse the polysaccharides contained in coffee residues than the fungal strains used, releasing relatively larger amount of reducing sugars. The difference in cell structure between bacteria and fungi may also contribute to the release and availability of hydrolytic enzymes. The more complex cell structure of fungi may result in slower production and release of these enzymes, compared to bacteria which have a simpler cell structure.

Also apparent from Figure 5.15 below, the initial TRS concentration at 0hr of fermentation is

the same for both fungal experiments, but a slight difference is observed for the LSF experiment. This is likely due to the nutrients contained in the culture medium used to grow bacteria for liquid state fermentation.

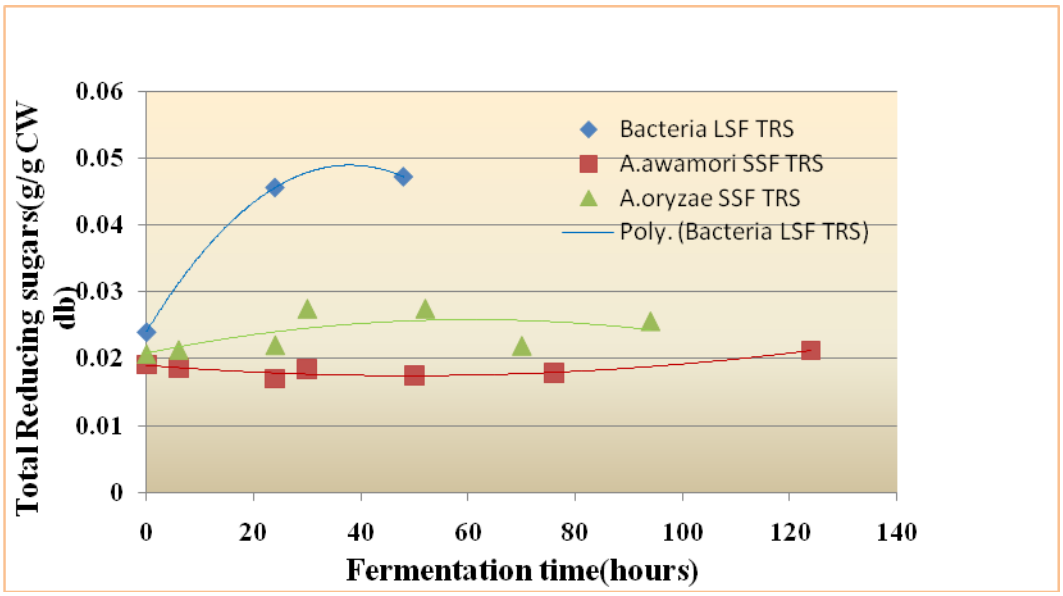



Figure 5.15: Scatter plot for comparison of change total reducing sugar concentration during solid state fermentation by *A. awamori* and *A. oryzae*; and liquid state fermentation by indigenous bacteria.

### 5.8.3. Free amino nitrogen

As observed for total reducing sugars concentration, emergence of hyphal tips influences the trend observed for free amino nitrogen concentration. Figure 5.16 below shows an increase in FAN by the 30$^{th}$ hour of *A. oryzae* fermentation, which is observed by the 70$^{th}$ hour of *A. awamori* fermentation. The trend observed for protease activity described in section 5.6.2.5 above confirms the release of proteolytic enzymes by *A. oryzae* at the 30$^{th}$ hour to release amino acids, thus the increase the FAN concentration obtained.

Liquid state fermentation by the isolated indigenous bacteria resulted in an increase of over 100% in FAN concentration, further indicating the significant synthesis of hydrolytic enzymes by the indigenous bacteria during fermentation. The results obtained may have been improved by the uniformity of the liquid state medium, which is hardly obtainable in solid state fermentation media.

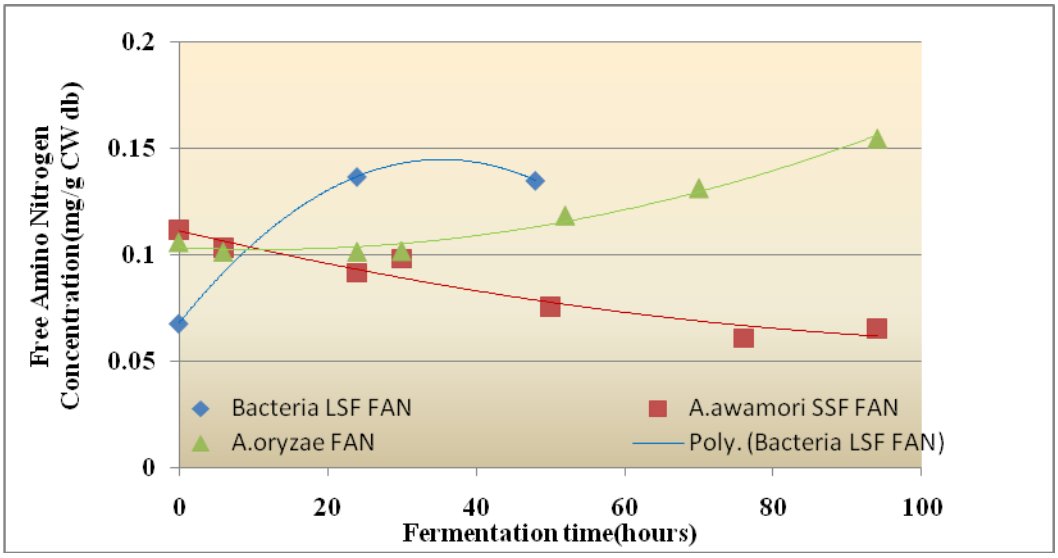



Figure 5.16: Scatter plot for comparison of change in free amino nitrogen concentration during solid state fermentation by *A. awamori* and *A. oryzae*; and liquid state fermentation by indigenous bacteria.

## *5.9. Summary*

Quite interesting results were obtained from the experiments carried out during this study. The preliminary growth experiment produced positive results, which influenced the continuation of this study. Change in the initial chemical characteristics of coffee residues was observed to occur during solid and liquid state fermentation of coffee residues by *Aspergillus awamori*, *Aspergillus oryzae* and indigenous bacteria, in different proportions, depending on the metabolism of the micro-organism used. Autolysis of solid state fermentation products was observed to increase the free amino nitrogen content, indicating an increase in total protein content with fermentation. The conclusions drawn from the results obtained from the experiments are presented in the next chapter.

# Chapter 6: Conclusions and Recommendations

Coffee residues, which are by-products of the secondary processing of coffee are generally considered as waste and to a large extent, are disposed of through non-sustainable waste management practices. These by-products contain chemical compounds which qualify them as potential raw materials for a number of processes; bio-processing being an area of interest which presents a less expensive and more sustainable means of managing these residues.

The experiments carried out in this study showed that Bacteria and fungal strains can be employed in biological valorisation of coffee residues, such as solid state fermentation and liquid state fermentation by these micro-organisms, where applicable. The proliferation of these micro-organisms on coffee residues is indicative of the presence of substances which can be exploited to produce value added products.

Increase in the concentration of Total reducing sugars and free amino nitrogen concentration were obtained during solid state fermentation with *Aspergillus awamori* and *Aspergillus oryzae*, which indicate the generation of these compounds from the metabolic activities of these micro-organisms. Although the increase observed may not be considered as significant, the ability of these micro-organisms to utilize the nutrients available in coffee residues presents the potential of obtaining various value-added products through bio-processing of coffee residues.

A value-added product which has been identified from this study is Protease, obtained from solid state fermentation of coffee residues by *Aspergillus oryzae*. This is an important enzyme which makes up about 60% of the enzymes' market, with wide application in various industries. Generation of this enzyme from this study confirms the feasibility of the concept of 'transforming waste to product', in application to coffee residues.

Optimization of the fermentation conditions may result in a more significant yield of metabolic by-products. Conditions, such as moisture content is an important factor in solid state fermentation, which has varying optimum requirements for different organisms. The basic moisture content employed in this study may have influenced the growth pattern obtained for the two strains of fungi used. Further work on factorial optimization of fermentation conditions for the different organisms may result in more significant metabolite yield. Pre-treatment of the coffee residues e.g. acid treatment, may improve the accessibility

of the polysaccharides by the micro-organisms, as evident in the increased concentration of total reducing sugars measured in acid digested coffee residues from the initial chemical characterization experiment performed.

Considering that *Aspergillus awamori* and *Aspergillus oryzae* are just two out of a large variety of fungal species available, fungal solid state fermentation of coffee residues presents a large opportunity for further work, to obtain various value-products using other fungal strains. The ability of different strains of fungi to metabolize coffee residues is evident from the different colonies of indigenous fungi identified. An interesting example is the generation of mannase from the mannan component of coffee residues. Mannase is required in the manufacture soluble instant coffee for the hydrolysis of coffee mannan, in order to reduce the viscosity of coffee extract and simplify the production process (Arya and Rao, 2007a). The application of solid state fermentation by mannase producing fungi such as *Sclerotium rolfsii* (Arya and Rao, 2007a) to coffee residues, in instant coffee manufacturing sites would provide a less expensive way of obtaining this enzyme, and the impact of collecting and transporting coffee residues to other sites for valorisation, which is an important consideration in waste valorisation, may be reduced.

Autolysis of solid state fermentation products resulted in an increase in the free amino nitrogen concentration, indicating an increase in protein content of the fungi/coffee residues products. This product may serve as enhanced feed for animals. Fungal metabolism may result in the degradation of anti-nutritional compounds in coffee residues, whilst increasing the protein content, thus improving its quality as animal feed. However, the generation of toxic metabolites from fungal metabolic activities is also obtainable, thus further work needs to be done to determine the degradation of anti-nutritional compounds, the production of toxic metabolites, as well as the effect of fermentation time on the degradation and generation of these materials.

Liquid state fermentation of coffee residues by bacteria produced interesting results in terms of the increase in concentration of free amino nitrogen and total reducing sugars obtained. These results suggest the ability of bacteria to rapidly hydrolyse the macro molecular compounds present in coffee residues. Although the issue of water use and disposal, as well as downstream processing arise in LSF, it should be further investigated for the potential of increasing the yield of reducing sugars, and the possibility of generating bio-ethanol from the sugar produced using ethanol producing yeast e.g. *saccharomyces cerevisiea* . Also, a high

significant enzyme activity may be obtained from LSF, thus presents the possibility of extracting useful enzymes as value- added products.

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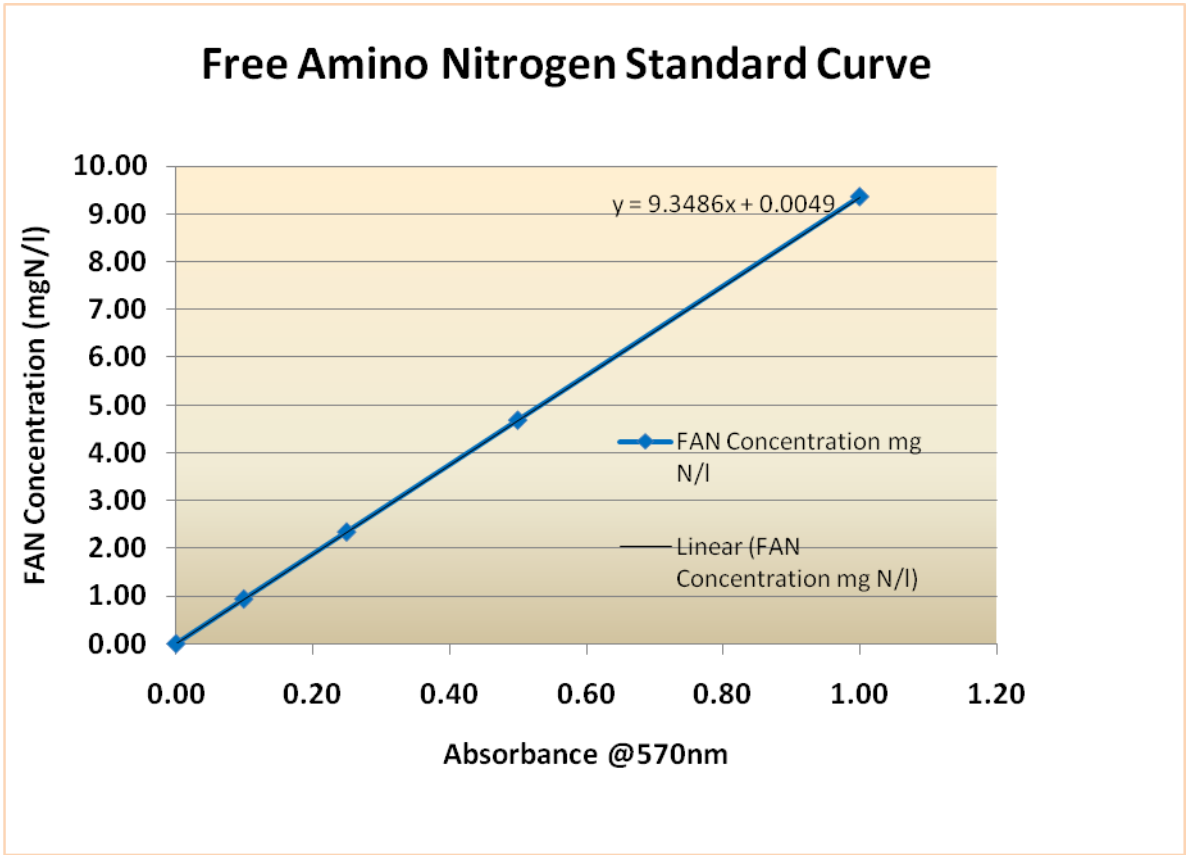
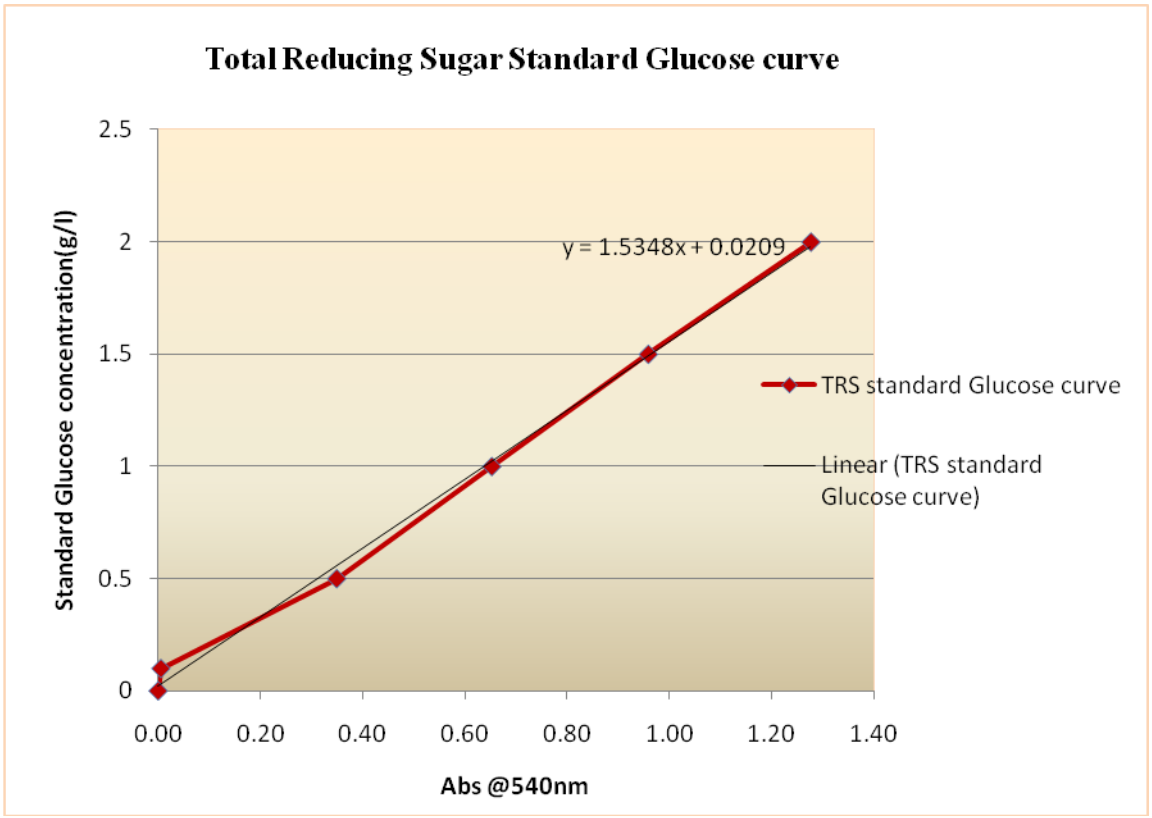
# Appendices


Free Amino Nitrogen Standard Curve
FAN Concentration (mgN/l)
10.00
9.00
8.00
7.00
6.00
5.00
4.00
3.00
2.00
1.00
0.00
0.00
0.20
0.40
0.60
0.80
1.00
1.20
Absorbance @570nm
y = 9.3486x + 0.0049
FAN Concentration mg N/l
Linear (FAN Concentration mg N/l)


Total Reducing Sugar Standard Glucose curve
Standard Glucose concentration(g/l)
2.5
2
1.5
1
0.5
0
y = 1.5348x + 0.0209
TRS standard Glucose curve
Linear (TRS standard Glucose curve)
0.00
0.20
0.40
0.60
0.80
1.00
1.20
1.40
Abs @540nm