# ISOLATION OF *Aspergillus oryzae* AND SINGLE CELL PROTEIN PRODUCTION BY GROWN ON COFFEE CHERRY HUSK AND SAWDUST

**M.Sc. THESES**

**KUMA DIRIBSA**

**JANUARY 2019**

**HARAMAYA UNIVERSITY, HARAMAYA**

# ISOLATION OF *Aspergillus oryzae* AND SINGLE CELL PROTEIN PRODUCTION BY GROWN ON COFFEE CHERRY HUSK AND SAWDUST

**A Thesis Submitted to School Biological Sciences and Biotechnology**

**Postgraduate Program Directorate**

**HARAMAYA UNIVERSITY**

**In Partial Fulfillment of the Requirements for the Degree of MASTER OF SCIENCE IN BIOTECHNOLOGY**

**KUMA DIRIBSA**

**JANUARY 2019**

**Haramaya University, Haramaya**

**HARAMAYA UNIVERSITY**

**DIRECTORATE FOR POSTGRADUATE PROGRAM**

As research advisors, we hereby certify that we have read and evaluated the thesis prepared, Kuma Diribsa under our guidance, which is entitled **"Isolation of *Aspergillus oryzae* and Single Cell Protein Production by Grown on Coffee Cherry Husk and Sawdust"**. We recommend that the theses be accepted as fulfilling the theses requirement.

| | | |
|---|---|---|
| Ameha Kebede (PhD) | _____ | _____ |
| Major advisor | Signature | Date |
| Misrak Kebede (PhD) | _____ | _____ |
| Co-advisor | Signature | Date |

As members of the Board of Examiners of the M.Sc. thesis Open Defense Examination of KUMA DIRIBSA, we certify that we have read, evaluated the thesis and examined the candidate. we recommend that the thesis be accepted as fulfills the requirements for the Degree of Master of Science in Biotechnology.

| | | |
|---|---|---|
| _____ | _____ | _____ |
| Chairperson | Signature | Date |
| _____ | _____ | _____ |
| Internal Examiner | Signature | Date |
| _____ | _____ | _____ |
| External Examiner | Signature | Date |

Final approval and acceptance of the thesis is contingent up on the submission of the final copy to the council of Post Graduate Program (CPGP) through the candidates Graduate Committee (DGC or SGC).

# DEDICATION

This thesis work is dedicated to my beloved wife, Lalise Gudeta for her love and dedicated partnership in the success of my life.

# STATEMENT OF THE AUTHOR

By my signature below, I declare and affirm that this Thesis is my own work. I have followed all ethical and technical principles of scholarship in the preparation, data collection, data analysis and compilation of this thesis. Any scholarly matter that is included in the thesis has been given recognition through citation.

This Thesis is submitted in partial fulfillment of the requirements for Master of Science degree at Haramaya University. The Thesis is deposited in the Haramaya University Library and is made available to borrowers under the rules of the Library. I solemnly declare that this thesis has not been submitted to any other institution anywhere for award of academic degree, diploma or certificate.

Brief quotations from this thesis may be made without special permission provided that accurate acknowledgement of source is made. Requests for permission for extended quotation from it or reproduction of this thesis in whole or in part may be granted by the Head of the Department when in his or her judgment the proposed use of the material is in the interests of scholarship. In all other instances, however, permission must be obtained from the author of the thesis.

Name: Kuma Diribsa Signature: _____ Date_____

School: Biological Sciences and Biotechnology

# BIOGRAPHICAL SKETCH OF THE AUTHOR

The author was born on January 05, 1979 in Ambo Woreda, West Showa Zone, Oromia National Regional State, Ethiopia. He attended his elementary school education at Menkata Elementary School. He then continued his junior secondary school education (Grade 7-8) in the then Ambo Junior Secondary School (Chari), and his secondary school education (Grade 9-12) in the then Ambo Comprehensive Secondary School. Up on successful completion of his high school studies, he joined Jimma University Ambo College in 2004 and graduated on July 11, 2007 with diploma in Biology as a laboratory technician. Then, he was employed by Dilla University as technical assistant and served the institution for one year. While serving as a technical assistant, he got the opportunity to enroll for his BSc degree in 2012 at Dilla University and graduated on June7, 2015. After graduation, he had been serving Dilla University until he joined in 2016 the Postgraduate Program of the School of Biological Sciences and Biotechnology, Haramaya University, to follow his MSc. degree in Biotechnology.

# ACKNOWLEDGEMENTS

First and foremost, I would like to thank and praise the Almighty God who has saved our lovely soul by sending his only **SON** to this sinful world and helped me to accomplish my work despite the presence of several challenges standing on my way that could have potentially prevented me from achieving my goals.

Next, I thank my major advisor Dr. Ameha Kebede, for offering me constructive comments, guiding and encouraging me in all moments from topic selection up to thesis write up. I also equally thank my Co-advisor, Dr. Misrak Kebede for her comments, guidance and encouragements throughout the process of preparing this thesis. Indeed, this study would have not been possible without their support.

I also forward my gratitude to Wanago woreda, Bule Tokicha kebele administrative bureau staff workers for their valuable encouragements and the support provided while I was collecting both soil samples and substrates with my enumerators.

Finally, I would like to express my great gratitude to my family and all those people who have assisted me in various ways, including the academic and administrative staff of the School of Biological Sciences and Biotechnology, when I was doing my research work.

# LIST OF ACRONYMS AND ABBREVIATIONS

| | |
|---|---|
| ANOVA | Analysis of Variance |
| AOAC | Association of Official Analytical Chemists |
| BM | Biomass |
| BSA | Bovine Serum Agar |
| CCH | Coffee Cherry Husk |
| CYA | Czapeck Yeast Extract Agar |
| ECXA | Ethiopian Commodity Exchange Authority |
| EREDPC | Ethiopian Rural Energy Development and Promotion Center |
| FAO | Food and Agricultural Organization |
| GANA | Glucose Ammonium Nitrate Agar |
| GATT | General Agreement on Tariffs and Trade |
| ICT | International Trade Centre |
| LSD | Least Significant Difference |
| NA | Nucleic Acids |
| PDA | Potato Dextrose Agar |
| RPMI | Roswell Park Memorial Institute (culture medium) |
| RT | Room Temperature |
| SAS | Statistical Analysis Software |
| SCP | Single Cell Protein |
| SD | Sawdust |
| SDCCH | Sawdust and Coffee Cherry Husk |
| SNNPRS | Southern Nations Nationalities Peoples Regional State |
| SSF | Solid State Fermentation |
| USEPA | United States Environmental Protection Agency |
| WHO | World Health Organization. |

# TABLE OF CONTENTS

# LIST OF TABLES

# LIST OF FIGURES

# LIST OF TABLES IN THE APPENDIX

# LIST OF FIGURES IN THE APPENDIX



.

# Isolation of *Aspergillus oryzae* and Single Cell Protein Production by Grown on Coffee Cherry Husk and Sawdust

## ABSTRACT

*The rising global population pressure generates challenges to fulfill food requirements for the coming generations in adequate amounts. This suggests that the human population cannot continue to be entirely dependent on crops, animal husbandry and fisheries for food. Instead, efforts should be made to enhance the production of other potential alternative sources of food. To this end the current study has been undertaken to evaluate the production of single cell protein (SCP) from Aspergillus oryzae using coffee cherry husk and sawdust, to analyze the nutritional composition of the Aspergillus oryzae biomass produced and to determine the residual nucleic acid content of the biomass before and after nucleic acid reduction. Soil samples, coffee cherry husk and sawdust were collected from Southern Nations Nationalities Peoples Regional State, Gedio Zone, Dilla. Identification of Aspergillus oryzae was done using Aspergillus flavus and parasiticus agar (AFPA) to differentiate the isolates from Aspergillus flavus and Aspergillus parasiticus depending on the fungal colony reverse colors on plates. Two types of sterilized growth substrates, coffee cherry husk and sawdust, were transferred in to two sterile petri-dishes and inoculated with Aspergillus oryzae suspension containing $10^6$-$10^7$ spores/ml and incubated for seven days. To collect the biomass, 1gm fermented substrate from each petr-dish transferred to pre-weighed centrifuge tubes and 5ml of sodium sulphate (150 gm $1^{-1}$) were added to each tube. The tubes were centrifuged at 12000 rpm for 15 minutes. At the end of centrifugation, the fungal biomass with lower density than the substrate floated while the substrate settled to the bottom. The biomass were dried and grinded using a mortar and pestle. Three types of buffers were used for the extraction of total proteins from Aspergillus oryzae. The collected data were subjected to Analysis of Variance (ANOVA) with three replications using Statistical Analysis System (SAS) Version 9.0. The highest amount of protein (57.1mg), was recorded from Aspergillus oryzae grown on 60% CCH + 40% SD alone. Whereas the least amount of proteins were obtained from grown substrate 100% SD (24.9mg). 60% CCH + 40% SD is the appropriate combination that produce high amount single cell protein from Aspergillus oryzae.*

**Keywords:** Single Cell Protein, *Aspergillus oryzae,* Nucleic acid reduction, Cell viability test, Thermal shock, Coffee cherry husk, Sawdust

# 1.INTRODUCTION

The global human population has been increasing up to 250% in last six decades with a boost from 2.6 to 7 billion and it is expected that if the growth will continue with the same rate population may be 9 billion by 2042 according to Census Bureau of United States. The rising global population pressure generates challenges to fulfill the requirements of foodstuff. Population cannot entirely dependent over agriculture, animal husbandry or fisheries for food. However, agricultural sector has strengthened in most of the developed countries. However, some of them are still facing problems like hunger, malnutrition, food insecurity and food related diseases (Gabriel *et al.*, 2014).

The pioneering research conducted almost a century ago by Max Delbruck and his colleagues at the Institut for Garungsgewerbe in Berlin, first highlighted the value of surplus brewer's yeast as a feeding supplement for animals (Delbruck M., 1910). This experience proved more than useful in the ensuing First World War, when Germany managed to replace as much as half of its imported protein sources by yeast. Since brewers yeast from beer production was not produced in sufficient quantity to meet the demands as a protein feed, a very large proportion of yeast biomass was expressly produced by aerobic fermentations in a semi-defined medium containing ammonium salts as the nitrogen source (Hayduck F., 1913). This methodology was more efficient than brewing, but still resulted in some fermentation of the carbohydrate source, and suboptimal yield of biomass obtained per unit of substrate. In 1919, a process was invented by Sak in Denmark and Hayduck in Germany in which sugar solution was fed to an aerated suspension of yeast instead of adding yeast to a diluted sugar solution (Sak S., 1919). An incremental-feeding or fed-batch process was thus born which is still successfully used in todays fermentations.

After the end of World War I, German interest in fodder yeast declined, but was revived around 1936 by the 'Heresverwaltung', when both brewer's yeast, and a variety of yeast specially mass cultured, were used to supplement human and animal diets. By then the advantages of aerobic production of baker´s yeast in a rich wort had been fully recognised as a rapid means of producing food in large scale industrial installations. A radically different concept to that of agricultural production (Barnell H. R., 1974).

Around this time, the nutritive value of yeast was also the intense subject of study, with two important books published (Weitzel W. and Winchel M.,1932; Scholein J., 1937). By the begining of World War II, yeasts had been incorporated first into army diets, and later into civilian diets. Ambitious plans were laid for production of well over 100,000 tons per year. This figure never surpassed 15,000, probably because of the extensive disruption which typically accompanies wartime economies. The sustained interest in fodder yeast initiated in Germany in the inter-war years echoed elsewhere in the World. As part of a larger programme for utilizing natural sources, the Forest Products Laboratory of the United States Department of Agriculture undertook mass cultivation of yeast on sulfite waste liquor, the species used being Candida utilis. Production of fodder yeast in the mid-western states of the U. S. A. expanded steadily. The post war period was characterized by the recognised need to tackle the problems of humanity on a global scale. A number of international organisations emerged for this task under the leadership of the United Nations. One such organisation was The Food and Agriculture Organisation of the United Nations (FAO) which brought forward the hunger and malnutrition problem of the world population in 1960, introducing the concept of the protein gap (25% of the world population had a deficiency of protein intake in their diet). The population growth predictions, moreover showed that the number of inhabitants would double between 1960 and 2000, from 2.5 billion to 5 billion (the actual figure reaches 6 billion), and the greater part of this increase would take place in those countries suffering from malnutrition. The Malthusian prospect of a limiting food supply was reinforced by fears that agricultural production would fail to meet the increasing food requirements of humanity.

The resumption of peace had also procured a new atmosphere geared towards the academic study in civilian matters, and fermentation processes saw a very important period of progress. A greater involvement of private companies in the marketing of fermentation products had already begun. By the early 60´s a number of multi-national companies decided to investigate the production of microbial biomass as a source of feed protein. The basic kinetic mechanisms ruling the growth pattern of microbes had been elucidated (Monod J., 1942) and were being established for yeasts and filamentous fungi (Pirt S. J. and Kurowski W. M.,1970; Pirt, S. J.,1973). However, important technical challenges remained to be solved in industrial

fermentations, and the field was boosting with activity. The relatively low market selling price set for this non-conventional protein steered design towards low product cost and thus, large scale production. Abundant substrates with low prices were sought. By-products as wide ranging as cheese whey, molasses, starch, ethanol and methanol, hydrocarbon substrates and spent sulfite liquor were chosen to sustain commercial processes.

The novelty of unwanted waste product consumption added a new economic incentive to SCP production, as the idea of zero cost substrates, or even the obtainment of additional revenues through the concept of waste treatment were argued and incorporated favourably to reduce the production cost estimates. The benefits of SCP production were thus extended from the production of food to the preservation of the environment. However, this same reasoning also conditioned the production volumes to match substrate consumption, as we will discuss in section 5. By the mid 60´s, some quarter of a million tons of food yeast were being produced in different parts of the world and the Soviet Union alone planed an annual production of 900,000 tons by 1970 of food and fodder yeast, to compensate agricultural protein production deficits. By 1980, SCP production processes were operating on a large scale in developed countries, and plans to extend SCP production to underdeveloped countries were being made. But a number of technical and political developments that occurred in the 80´s conditioned the expansion of the promising SCP industry. Marked improvements in plant breeding and crop production on a global basis allowed for a continued increase in agricultural output, beyond the expected ceilings. Local effects such as agricultural reform implemented in China, also resulted in marked agricultural output. Finally, the prospect of the end of the cold war could first be foreseen at this time, with important liberation of agricultural reserve stocks for market trading (SFA., 2000). This trend was later materialised by the General Agreement on Tariffs and Trade (GATT) signed in Marrakesh, commiting 118 countries to a new open trade world market in 1994(GATT., 1994). This treaty effectively de-regulated the world distribution of goods, opening new market areas and connecting countries with surpluses, and countries with deficits. This treaty had an immense effect on agricultural product trade worldwide.

However no research has been done on single cell protein production from *Aspergillus oryzae* using coffee cherry husk and sawdust as sole and mixed substrates. In Ethiopia, enormous amount of coffee husk and pulp are generated from coffee processing industries annually.

These materials have been poorly utilized and managed or are left to decompose or burned in open fields (Yisehak, 2009) or dumped in the environment including water bodies (Alemayeu Haddis and Rani, 2007). Yet, these activities cause and aggravate pollution of air, environment and water (Abebe Beyene *et al*., 2011) potentially undermining coffee certification since environmental considerations and sound coffee production systems are among the criteria and code of conduct required for the certification (Volkmann, 2008).

Thus, utilization of coffee husk and pulp would be an option to alleviate the environmental pollution problems (Yared Kassahun *et al.*, 2010). Although, in different regions of Ethiopia, this biomass is being consumed by households in place of firewood with inefficient open fire stoves, direct utilization of this type of biomass as a source of energy has been found not to be suitable because of its low density, high smoke, and low energy intensity (Abakr and Abasaeed, 2006). Moreover, smoke released from the biomass causes acute respiratory problems (Taylor and Nakai, 2012). A mass of 100 kg of red cherries picked at 65% moisture content will result in approximately 40 kg of sun-dried coffee cherries delivered to the processing plant. Of this mass, about 17 kg becomes the weight of sun-dried coffee beans while the remaining 23 kg will end up as residue at the processing plant (Yisehak Seboka *et al.*, 2009).

Furthermore, there are approximately more than 150 sawdust (*Cordia africana*) producing small furniture industries in Gedeo zone, the area where this study is planned to be was undertaken. This waste wood currently is not being used wisely and as a result its indiscriminate burning as a fire wood and charcoal. Hence, this study is aimed at producing SCP from these coffee cherry husk and sawdust and reducing the levels of waste disposal from both the coffee processing and furniture industries through useful consumption as substrates for cultivation of *Aspergillus oryzae*.

## General objective

The general objective of this study is to evaluate the production of single cell protein (SCP) from *Aspergillus oryzae* using cheap, cost-effective and easily available substrates, coffee cherry husk and sawdust.

## The specific objectives

1. To isolate *Aspergillus oryzae* from soil samples.
2. To determine the amount of single cell protein (SCP) that can be produced from *Aspergillus oryzae* grown on coffee cherry husk and sawdust used separately or in mixture as growth substrates.
3. To analyze the nutritional composition of the biomass produced by *Aspergillus oryzae*.
4. To determine the residual nucleic acid content after nucleic acid reduction.
5. To investigate the toxicity of *Aspergillus oryzae* using cell viability test.

# 2. LITERATURE REVIEW

## 2.1. Single Cell Protein Definition and Uses

Single cell protein (SCP) is the term used to describe microbial cells which are grown and harvested primarily for use as human or animal feed. SCP contains nucleic acids, carbohydrate cell wall material, lipids, minerals and vitamins. Microbial cells are produced as protein source for food or feed. Some edible eaten foods are in fact microorganisms. Many advantages have made microorganisms as protein source. Microbial cells are rich in protein and they can generate protein from inorganic nitrogen i.e. ammonia. Microorganisms can use alternative carbon sources i.e. $CO_2$ as by algae. Organic carbon is another carbon source that be used in wide range like waste products from industries or agriculture. Microorganisms grow much more rapidly than plants or animals which have given usage of microbial cells another advantages. But beside these advantages there are some disadvantages. The lack of sufficient quantities of essential amino acids like methionine in algal, little taste or smell, digestibility problem of some of microbial cells kinds, adverse effects in humans, limitation of microorganism's kinds because of pathogenic or toxicity and the high content of nucleic acid RNA which cause gout (Murray, 2004; Halasz Anna, 2000).

## 2.2. Application of Single Cell Protein

Single cell proteins (SCP) has applications in animal nutrition as fattening calves, poultry, pigs and fish breeding, feeds for laying hens, and feed of domestic animals. In the foodstuff area it is used as aroma carriers, vitamin carriers, emulsifying aids and to improve the nutritive value of baked products in soups, in readymade to serve meals, in recipes. In the technical field: in paper processing, leather processing and as foam stabilizers (Srividya *et al.*, 2013).

### 1.2.1. Nutritional values of single cell protein

Factors like nutrient composition, amino acid profile, vitamin and nucleic acid content as well as palatability, allergies and gastrointestinal impact should bring under consideration for the assessment of the nutritional value of single cell protein (Litchfield, 1968). In addition, long term feeding trials should also be tested for toxicological effects and carcinogenesis (Israelidis, 2008). In fact, nutritive values vary with the microorganisms used for single cell protein production. The way of harvesting, drying and processing also have impact on the nutritive value of the product. Single cell protein includes primarily proteins, fats, carbohydrates, ash ingredients, potassium. The composition depends upon the microorganism and the substrate on which it grows. Despite, the proteins as nutritional component in a food system they also perform several other functions (Mahajan, and Dua, 1995).

Single cell protein are good source of high quality protein with low fat content, vitamins predominantly B-complex, superior amino acid composition and furnished with thiamine, riboflavin, glutathione, folic acid and other amino acids but less in sulphur containing amino acids. Fungi and yeast possess up to 30–70% protein and rich in lysine, although deprived of cysteine and methionine amino acid content. However, bacteria can produced single cell protein more than 80% protein, although it is poor in sulphur containing amino acids with high nucleic acid content (Kurbanoglu, 2011; Adedayo *et al.*, 2011). Yeast single cell proteins are playing major role in the growth of aquaculture feeds. With admirable nutrient profile and capability to be large scale production economically, single cell proteins are partially replacing fishmeal as aquaculture feed (Coutteau and Lavens, 1989; Olvera-Novoa, *et al.*, 2002; Li and Gatlin, 2003). Some yeast strains such as *Saccharomyces cerevisiae* (Oliva and Goncalves, 2001).

### 2.2.2. Side effects of single cell proteins

Despite very striking features of single cell protein as a nutrient for humans and animals there are various problems associated that deters its adoption globally. Such problems include: the concentration of nucleic acid is higher than other conventional protein sources and it is the characteristics of all rapidly growing organisms. The problem which occurs with the consumption of high nucleic acid containing protein (18-25 g/100gm protein dry weight) is

the production of high concentration of uric acid level in the blood causing health disorders such as gout and kidney stone (Nasseri, *et al*., 2011). Single cell protein from bacteria has also been found to be associated with these pitfalls, which include: high ribonucleic acid content, high risk of contamination during the production process and recovering the cells is a bit problematic. About 70 to 80% of the total nitrogen is represented by amino acids while the rest occur in nucleic acids. It is also point to be noted that the microbial cell wall may be indigestible. There may be intolerable color and flavours (especially in algae and yeast there may also be possible skin reactions from consumption of foreign protein and gastrointestinal reactions may occur resulting in nausea and vomiting. Single cell protein from algae may not be suitable for human consumption because they are rich in chlorophyll (except *Spirullina*). It has low density *i.e*.1-2gm dry weight/litre of substrate and there is lot of risk of contamination during growth. The filamentous fungi show slow growth rate than yeasts and bacteria there is high Contamination risk and some strains produce mycotoxins and hence they should be well screened before consumption. All these detrimental factors affect the acceptability of single cell protein as global food.

## 2.3. Criteria of Choosing Microorganisms for SCP Production

The microorganism used for single cell protein production should have the following characteristics: High growth rate and biomass yield, high attraction for the substrate, low nutritional requirements, capacity to use complex substrates, capability to develop high cell mass, stability throughout reproduction, capacity for genetic modification, good tolerance to temperature and pH, balanced protein and lipid composition, low nucleic acid content, good digestibility and non-toxic (UIt, 2012).

## 2.4. Fungal Species Used for Single Cell Protein Production

The filamentous fungi that have been used include *Chaetomium celluloliticum, Fusarium graminearum, Aspergillus fumigatus, Aspergillus niger, Aspergillu oryzae, Cephalosporium*

*cichhorniae, Penicillium cyclopium, Rhizopus chinensis, Scytalidium acidophlium, Tricoderma viride, Tricoderma alba* and *Paecilomyces varioti* (Jaganmohan *et al*., 2013).

## 2.5. Growth Conditions Suitable for Fungal Species

There are some factors which (similar to bacteria) affect the growth of moulds: Carbon, nitrogen (C:N) ratio is required to be in the range of 5:1 to15:1. Ammonium salts are used as a nitrogenous source and phosphoric acid for phosphorus source. Their concentration may differ with respect to fungal species. pH required for growth medium ranges between 3.0 to 7.0 but the pH 5-6 or below is more desirable to most of the fungi due to bacterial contaminants. Generally, the temperature ranges between 25-30ºC and good quantity of oxygen is also required for the normal growth of fungi (Suman, 2016).

## 2.6. Description and Classification *Aspergillus oryzae*

*Aspergillus oryzae* is a filamentous fungus, or mold, that is used in East Asian (particularly Japanese and Chinese) food production, such as in soybean fermentation. *A. oryzae* is utilized in solid-state cultivation, which is a form of fermentation in a solid rather than a liquid state. This fungi is essential to the fermentation processes because of its ability to secrete large amounts of various degrading enzymes, which allows it to decompose the proteins of various starches into sugars and amino acids. A round vesicle with extending conidial chains, which appear as white and fluffy strands on the substrate that the fungi
inhabits (US EPA, 1997) characterizes this fungi.
Scientific classification Domain: Eukarya Kingdom: Fungi Division: Ascomycota Class: Eurotiomycetes Order: Eurotiales Family: Trichocomaceae Genus: Aspergillus Species: A. oryzae Binomial name Aspergillus oryzae (Ahlburg) E. Cohn)

## 2.7. Growth Substrates Used for Cultivation of Fungal Species

Many raw materials have been considered as substrate for SCP production (Nasseri *et al*., 2011). Conventional substrates such as starch, molasses, fruit and vegetable wastes have been used for SCP production, as well as unconventional ones such as petroleum by-products, natural gas, ethanol, methanol and lignocellulosic biomass (Martin, 1991; Bekatorou *et al*.,

2006). Carbohydrate substrates are the most widely used for SCP production due to the fact that carbohydrates are natural microbial substrates and also because carbohydrates constitute a renewable feed stock (Ugalde and Castrillo, 2002).

### 2.7.1. Coffee cherry husk and sawdust

Coffee pulp or husk is a fibrous mucilaginous material (sub-product) obtained during the processing of coffee cherries by wet or dry process, respectively. Coffee pulp/husk contains some amount of caffeine and tannins, which makes it toxic in nature, resulting the disposal problem. However, it is rich in organic nature, which makes it an ideal substrate for microbial processes for the production of value-added products. Several solutions and alternative uses of the coffee pulp and husk have been attempted. These include as fertilizers, livestock feed, compost, etc. However, these applications utilize only a fraction of available quantity and are not technically very efficient. Attempts have been made to detoxify it for improved application as feed, and to produce several products such as enzymes, organic acids, flavour and aroma compounds, and mushrooms, etc. from coffee pulp/husk. Solid state fermentation has been mostly employed for bioconversion processes. This paper reviews the developments on processes and products developed for the value-addition of coffee pulp/husk through the biotechnological means. Advances in industrial biotechnology offer potential opportunities for economic utilization of agro-industrial residues such as coffee pulp and coffee husk (Pandey *et al.,* 2000). Characteristics of the coffee husk is; moisture content 9.63%, bulk density 0.73mg/cm$^3$, Volatile matter 82.7%, fixed matter 7.65%, ash 7.65% total carbon 53.67%.

Sawdust or wood dust is a by-product or waste product of wood working operations such as sawing, milling, planing, routing, drilling and sanding. It is composed of fine particles of wood. These operations can be performed by wood working machinery, portable power tools or by use of hand tools. Wood dust is also the byproduct of certain animals, birds and insects which live in wood, such as the woodpecker and carpenter ant. In some manufacturing industries it can be a significant fire hazard and source of occupational dust

exposure. Sawdust is the main component of particleboard. Wood dust is a form of particulate matter, or particulates. Research on wood dust health hazards comes within the field of occupational health science, and study of wood dust control comes within the field of indoor air quality engineering.

The physicochemical properties, such as particle size distribution, bulk density, moisture concentration, water absorption rate, moisture evaporation rate and in vitro ammonia emission (Ahn *et al.*, 2015).

### 2.7.2. Availability of coffee cherry husk and sawdust in Ethiopia

Coffee is one of the most important agricultural export commodities in the world economy, next to oils and it is the most important and strategic commodity on which Ethiopia's economy depends on. It has always been the most important cash crop and largest export commodity, which account 90% of exports and 80% of total employment in Ethiopia (Addis Ababa, 2008). Ethiopia had been the origin of coffee since coffee plant was initially found and cultivated in the Kaffa province (Bonga, Makira) of Ethiopia (UNCTAD/WTO, 2002). Coffee in Ethiopia contributes 41% of the country's total foreign exchange earnings and about 10% of the gross domestic product. Over 25% of the populations of Ethiopia are dependent on coffee for their livelihoods. There are four types of coffee production system in Ethiopia: forest coffee (10%), semi-forest coffee (35%), garden coffee (35%), and plantation coffee (20%) (5% government, 15% private) ( ECXA, 2008).

The agro-industrial and the food sectors produce large quantities of waste, both liquid and solid. Due to the great demand of coffee, coffee industries are responsible for the generation of large amount of residues, which are toxic and represent serious environmental problems (Solange *et al*., 2011).

The wastewater generated from coffee processing plant contains organic matter like pectin, proteins, and sugars (Bello-Mendoza, and Castillo-Rivera, 1998). Coffee pulp, is one of the principal by-products of wet processed coffee constitutes almost 40% of the wet weight of the coffee berry, is rich in carbohydrates, proteins, amino acids, poly-phenols, minerals, and appreciable quantities of tannins, caffeine and potassium. The poly-phenols and caffeine are

reported to be the anti-physiological factors on animal feed. Hence, coffee pulp has to follow a preliminary treatment before it is used (Sebastianos *et al*., 1998). Coffee pulp is generated to the extent of 40% in the fermentation of coffee berries poses many problems in the coffee producing countries. Its disposal in nature, without any treatment, causes severe environmental pollution due to putrefaction of organic matter.

Gedeo Zone is one of the leading coffee producer and exporter since Yirga chaffe coffee is found in these Zone. Coffee harvesting is processed by the wet method in which the coffee berry is subjected to mechanical and biological operation in order to separate the bean or seed from the exocarp (skin), mesocarp (mucilaginous pulp) and the endocarp (parchment). There are 142 wet coffee and 64 dry coffee processing industries are present in Gedeo Zone. From these industries, 12,018 tonnes of wastes are removed through the year to the environment and causes healthy problem to the surrounding society. (Source: Gedeo Zone coffee, tea and spices office). Residue pulp is mostly dumped in streams, although a small amount of it is sold as fuel or for 'tea' making in rural areas (Yisehak Seboka, 2009).

## 2.8. Production of Single Cell Protein

Three types of fermentation processes, namely submerged fermentation, semisolid state fermentation (Varavinit *et al*., 1996; Ageitos *et al*., 2011) and Solid state fermentation, can produce single cell protein. In the submerged fermentation, the substrate to be fermented is always in a liquid phase with the nutrient required for growth of microorganism. The substrate is held in the fermenter which is operated continually whereas the product biomass is also harvested continuously. The product is filtered or centrifuged and dried for the production of single cell protein. However, in semisolid fermentation process, the preparation of the substrate much elaborated; it is more favourable to a solid substrate, for e.g. cassava waste. Submerged culture fermentations are more investment concentrated and with higher operating cost as compared to semisolid fermentations which have a lower protein yield. The microbial cultivation occupies various basic process engineering operations, such as stirring and mixing of a multiphase system (gas-liquid-solid), transport of oxygen from the gas bubbles through

the liquid phase to the organisms, and heat transfer from the liquid phase to the environment. Solid state fermentation (SSF) has been extensively studied with thousands of publications describing various types of bioreactor designs, process conditions and microorganisms for the production of various value added products like SCP, feeds, enzymes, ethanol, organic acids, B-complex vitamins, pigments, flavours, (Singhania *et al.*, 2009). This process consists of depositing a solid culture substrate, such as rice or wheat bran, on flatbeds after seeding it with microorganisms; the substrate is then left in a temperature-controlled room for several days. Liquid state fermentation is performed in tanks, which can reach 1,001 to 2,500 square metres (10,770 to 26,910 sq ft) at an industrial scale. Liquid culture is ideal for the growing of unicellular organisms such as bacteria or yeasts. To achieve liquid aerobic fermentation, it is necessary to constantly supply the microorganism with oxygen, which is generally done via stirring the fermentation media. Accurately managing the synthesis of the desired metabolites requires regulating temperature, soluble oxygen, ionic strength and pH and control nutrients (Capalbo *et al.*,2001).

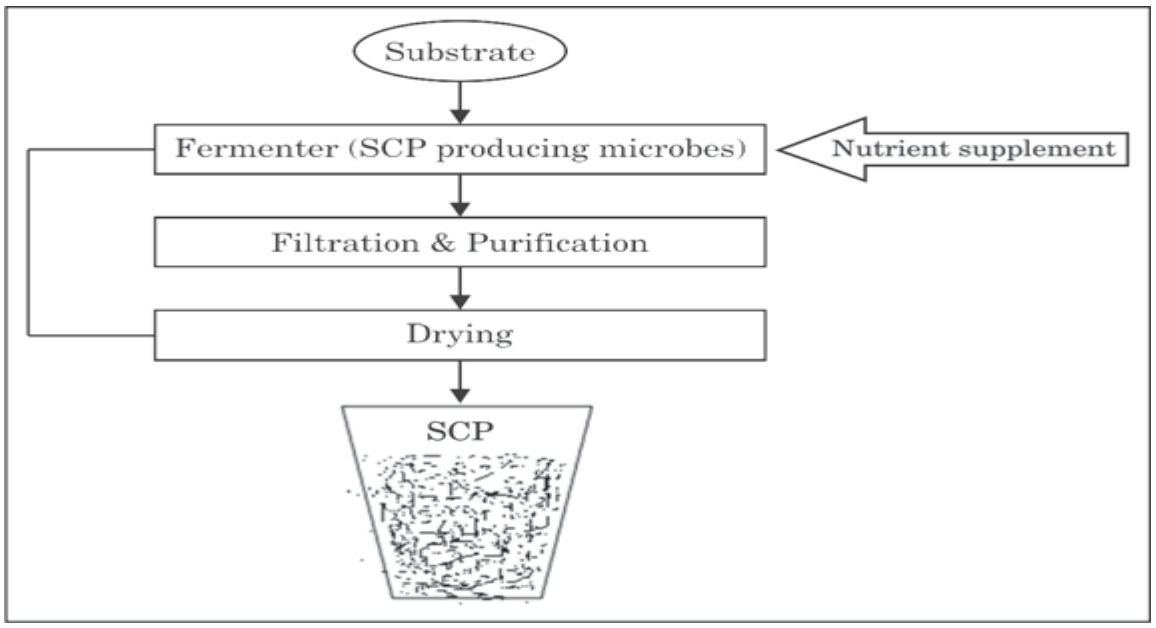



Figure 1. Flow chart of Single cell protein production (Ageitos *et al*., 2011).

### 2.8.1. Factors that affect single cell protein production

A factor that affecting single cell protein productions are includes; carbon source, nitrogen source, inoculums size and age, aeration, temperature and pH (Fatemeh *et al.*, 2019)

### 2.8.2. Growth rate and biomass

High growth rate is needed for high output (weight of biomass produced per unit of time). However, this will give also disadvantage of high RNA content in cell because nucleic acid content is proportional to the growth rate. The biomass yield coefficient is weight of cells produced per unit of substrate consumed. High yield coefficient will be a target to give high output in order to utilize as much as possible of substrate (especially if the substrate is expensive) Leuenberger H. (1971).

# 3. MATERIALS AND METHODS

## 3.1. Description of the Study Area

The Gedeo zone is located at 369 km from Addis Ababa, the capital city of Ethiopia, and 90 km from Hawassa, the capital of Southern Nations Nationalities and Peoples' Regional State (SNNPRS). Administratively, it lies within SNNPRS, one of the nine self-administering regions of Ethiopia. Geographically, the zone is located north of the equator from 5°53'N to 6° 27'N latitude and from 38° 8' to 38° 30'E longitude. The altitude ranges from 1500 to 3000 m above sea level. The Gedeo highland receives both equatorial and monsoon trade winds in the region (Tadese, 2002). It has a sub humid tropical climate and receives a mean annual rainfall of 1500mm. The mean annual temperature ranges from 12.6–22.4°C (Bishaw *et al.*, 2013). Gedeo is one of the major coffee (*Coffee arabica*) and *enset* (*Ensete ventricosum)*- producing zones of the region. Coffee and enset are the dominant perennials in Gedeo agroforestry. This agroforest is also the home for the internationally recognized organic coffee, i.e. Yirgachefe Coffee (Sileshi Degefa, 2016).

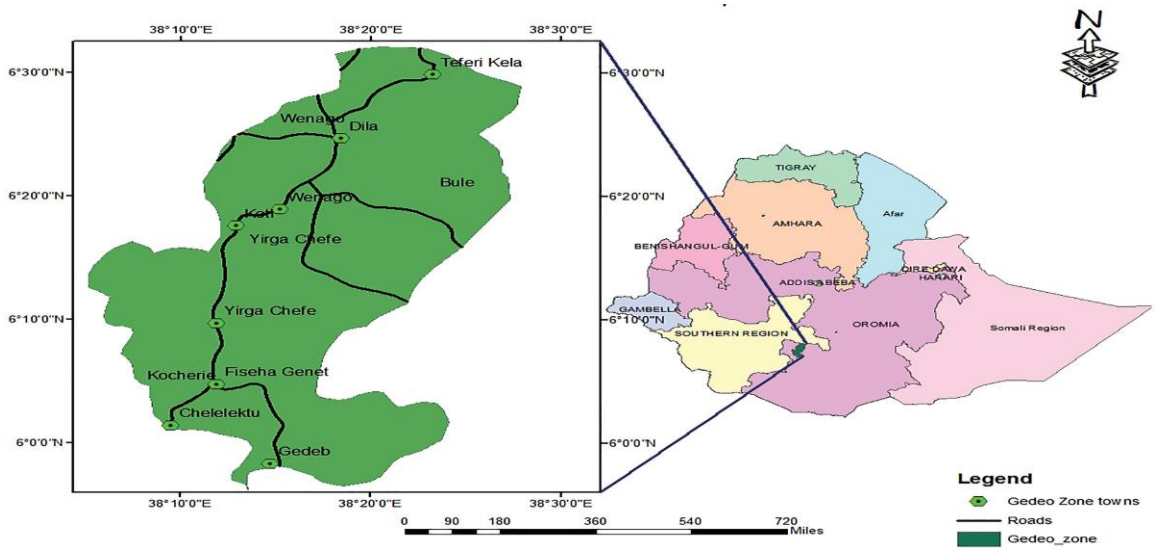



Figure 2. Map of the Study Area

## 3.2. Research Design

The experiments were carried out in a completely randomized design (CRD) using laboratory-based bioassay. The treatments included two substrates (Coffee cherry husk and sawdust) and extracted proteins from *Aspergillus oryzae* is in triplicates.

## 3.3. Data Collection

Soil samples, Coffee cherry husk and sawdust were collected from wheat cultivated lands, coffee processing industries and sawdust producing small furniture houses, respectively, from Wanago Woreda, Bule tokicha Kebele in Gedio Zone, Ethiopia. From each sampling site, 100gm of samples were collected and kept into sterilized polyethylene bags and brought into Haramaya University research laboratory for fungal isolation and SCP production.

## 3.4. Isolation and Identification of *Aspergillus oryzae*

Isolation of *Aspergillus oryzae* was done using the following procedures. 100gm of soil samples collected from each wheat cultivated farm land were dried at Haramaya University Microbiology Laboratory and ground using mortar and pestle and sieved using 2 mm mesh size sieve. From each sieved soil sample, 1gm of soil was suspended into 9 ml of sterile distilled water using a test tube and subsequently serially diluted up to $10^{-4.}$ From each dilution 1ml of soil suspension was withdrawn and pour plated into a sterile Petri-dish containing autoclaved glucose ammonium nitrate agar (GANA: glucose 20gm, $NH_4NO_3$ 1gm, difcobacto yeast extract 1gm, $K_2HPO_4$ 0.5gm, rose Bengal 0.06gm, MgSO4 $7H_2O$ 0.05gm, Streptomycin 0.03gm, Agar 20gm in 1L distilled water) and then incubated for 3-5 or longer days at room temperature (27-30°C).

Identification of *Aspergillus oryzae* was done using Aspergillus flavus and parasiticus Agar (AFPA) to differentiate the isolates (*Aspergillus oryzae*) from *Aspergillus flavus* and *Aspergillus parasiticus* depending on the reverse color of colonies. In addition, Czapeck Yeast Extract Agar (CYA) was also used for morphological identification. After incubation, the plates were observed for macroscopic characters such as: colony diameter, colony color, conidial color, mycelial color, colony reverse color, colony texture and nature of spores according to Sooriyamoorthy *et al*. (2004). The microscopic characteristics were observed by preparing slide cultures as described by Leck (1999).

## 3.5. Preparation of Inoculum from PDA Slant Cultures

Zero point seventy eight (0.78) grams of powdered commercial PDA was suspended into a Erlenmeyer flask containing 200 ml of distilled water. The suspension was mixed and boiled until the medium completely dissolved. The resulting boiled medium was apportioned into 5 ml by placing into clean test tubes and sterilized at 121$^{o}$C for 15 minutes using an autoclave. After autoclaving, the test tubes were placed on a board tilted up to produce slants upon cooling the molten PDA. The slants were then inoculated with mycelia of the isolated *A. oryzae* and incubated at room temperature (28$^{o}$C) until sporulation (up to 7 days). The spores of *A. oryzae* were released from the mycelia by adding sterile distilled water on the slant and shaking the tube to produce a spore suspension. The resulting suspension was used as the source of inoculum for subsequent experiments, and was kept in a refrigerator at 4$^{0}$C until use (Cicik Supyani, 2009).

## 3.6. Pre-treatment of Coffee Cherry Husk and Sawdust

The substrates were subjected to steaming at 100$^{o}$C for one hour and later the moisture content and the pH was adjusted to 60% and 4.5, respectively. pH adjustment was done using acetate buffer. After adjustment, the substrates were also sterilized and subjected to alkali treatment (1% NaOH, 10 min at room temperature). All experiments were carried out in triplicates (Pushpa *et al*., 2010).

## 3.7. Substrate Preparation

Sawdust and coffee cherry husks were ground into small pieces (approximately 1 cm$^{3}$) mechanically with mortar and pestle. The resulting substrates were then used separately and in mixture for the growth of *Aspergillus oryzae*. Thus, a total of five preparations, i.e. 50% Sawdust and 50% coffee cherry husk, 60% sawdust and 40% coffee cherry husk, 40% sawdust and 60% coffee cherry husk, 100 % coffee cherry husks (CCH), and 100% sawdust, were arranged and washed before use as growth substrate. From each substrate preparation, 10 gm were weighed and transferred into petri-dishes and autoclaved at 121$^{o}$C for 15 minutes (Atikpo *et al*., 2008) for use in the cultivation of *Aspergillus oryzae*.

## 3.8. Production of Fungal Biomass Using Solid State Fermentation (SSF) and Harvesting

Solid state fermentation (SSF) was carried out in Petri-dishes containing the above-mentioned substrate preparations. All substrate preparations were inoculated with spore suspensions ($10^6$-$10^7$ spores/ml) of *Aspergillus oryzae* grown on PDA and mixed thoroughly until the inoculum is distributed homogenously throughout the substrate.

Duplicate samples of l gm of fermented substrate from each petri-dish were transferred to pre-weighed centrifuge tubes and 5 ml of sodium sulphate (150 gm $1^{-1}$) was added to each tube. The tubes containing the mixture were centrifuged at 12000 rpm for 15 minutes. Centrifugation was repeated thrice under similar conditions to achieve complete separation of fungal biomass from the substrate. At the end of centrifugation, the fungal biomass which has a density lower than the growth substrate floated while the substrate settled to the bottom of the centrifuge tube. The biomass alone was transferred to a pre-weighed filter paper and dried in hot air oven for 72 h at 85 ± 1°C to obtain a constant weight (Asha-Augustine *et al*., 2006).

## 3.9. Dry Weight of the *Aspergillus oryzae*

The fungal mycelium was harvested at the $8^{th}$ day of growth and separated from the growth substrate for direct biomass estimation. The mycelia pellets were repeatedly washed with distilled water and dried in an oven at $70^0$C overnight. The dry weight of the fungus was then calculated using the following formula.

Dry weight = (weight of the filter paper + mycelium) − (weight of the filter paper).

## 3.10. Analysis of the Nutritional Composition of *A. oryzae* Biomass

Biomass samples of *Aspergillus oryzae* were analyzed for chemical composition (moisture, protein, fat, carbohydrates, and ash) using the AOAC (1995) procedures.

### 3.10.1. Determination of crude protein content

The crude protein in samples was determined using Kjeltec distillation unit. A finely grounded 0.8 gm of biomass of *Aspergillus oryzae* sample powder was transferred to a digestion tube and then, adds 0.5gm of digestion mixture and 12 ml of concentrated $H_2SO_4$. The sample was digested in a digestion unit till it became colorless. Then the digestion tubes were cooled and transferred to the distillation unit. 30 ml of 40% NaOH solution was allowed into the tube and liberated ammonium gas was absorbed in 4% boric acid solution containing mixed indicator. The pink color of the boric acid solution was turned into green and this was titrated against 0.01N HCl until the pink color was obtained. Finally, the crude protein in percent was obtained by using the following formula (Rashida *et al.2014*).

$$Protein\% = \frac{TV \times 0.014 \times 100(ml) 1 \times 100 \times 6.25}{Weight\ of\ the\ sample\ (gm)\ x\ Aliquot\ used\ for\ distillation\ (ml)} \text{x}100$$

where, TV = Titre Value

### 3.10.2. Determination of crude fat content

The crude fat content of the samples were estimated by Soxhlet extraction method using SOCS – PLUS apparatus.

Sample of 2gm was weighed using electronic weighing balance and transferred to a thimble. The weight of the empty beaker was recorded and all the beakers were loaded into the system. Eighty ml of petroleum ether was poured into the beaker from the top and boiled for about 80-90 min at 80$^{o}$C. After the completion of process time, the temperature was doubled to 160$^{o}$C for 15–20 min to collect the petroleum ether. All the beakers were removed and placed in a desiccator for about 5 min. The final weight of the beaker was noted down (Rashida *et al.2014*).

$$Crude\ fat\% = \frac{W_4 - W_3}{W_2 - W_1} \times 100$$

where,

$W_1$ = Weight of empty thimble, (gm) $W_2$ = Weight of thimble + sample, (gm)

$W_3$ = Weight of empty flask, (gm) $W_4$ = Weight of flask + fat, (gm)

### 3.10.3. Determination of total ash content

The total ash content of the sample was determined using muffle furnace. Five (5) gm of the sample was accurately weighed into a crucible (which was previously heated to about 600°C and then cooled). The crucible was placed on a clay pipe triangle and heated first over a low flame till all the material was completely charred, followed by heating in a muffle furnace for about 3 - 5 h at 600°C. It was then cooled in desiccator and weighed. The percentage of ash was calculated by using the following expression (Rashida *et al. 2014*).

$$\text{Total ash (\%)} = \frac{\text{weight of ash (gm)}}{\text{Weight of sample(gm)}} \times 100$$

### 3.10.4. Determination of total carbohydrate content

The procedure for carbohydrates estimation was carried out as follows. The total carbohydrate was estimated by phenol sulphuric acid method of (Dubois *et al.,* 1956). 5gm of dried samples of cells were taken for carbohydrates analysis. The dried fungal cells were taken in a test tube and 1ml of phenol (5%) and 5ml concentrated $H_2SO_4$ were added in quick succession. The tubes were kept for 30 min. at $30^0$C and the optical density (OD) of the colour developed was measured at 490 nm against the blank. D-Glucose was used as a standard and carbohydrate content was calculated by using the following formula (Rashida *et al.2014*).

$$\text{Carbohydrate \%} = \frac{X}{0.1} X100$$

where, X= Concentration of glucose from standard graph.

### 3.10.5. Determination of moisture content

The moisture content in the dry biomass of *Aspergillus oryzae* was determined after drying at 105°C for 24h. The percentage moisture content was then calculated using the following formula (Rashida *et al. 2014*).

$$\text{MC}\ \% = \frac{\text{Wet Weight} - \text{Dry Mass}}{\text{Weight of the samples}} \times 100$$

## 3.11. Toxicity Testing Methods

Biomass of *Aspergillus oryzae* at concentration levels of 20, 40 and 80 µl/ml were tested in vitro to evaluate the effects on the viability of human lymphocytes after 24, 48 and 72 h of exposure. A one-way analysis of variance (ANOVA) was performed on the cytotoxicity data using a pairwise multiple comparison procedures. Cytotoxicity testing was done using human blood. Human Blood was collected by using a sterile syringe and immediately transferred into a 10 ml heparin tube. The blood was diluted with an equal volume of RPMI-1640. Ten millilitre of the mixture was layered onto 5 ml of histopaque in a 15 ml polypropylene conical tube and then centrifuged at 1,500 rpm for 30 mins at ambient temperature. The interface layer consisting of mononuclear cells was carefully removed using a sterile pipette and washed twice with Salt Solution by centrifugation at 4°C. About 20 µl of cell suspension, 80 µl of 0.2% trypan blue solution were mixed in an Eppendorf tube and incubated for 2 mins at room temperature. Using a sterile pipette, 10 µl of the trypan blue cell suspension mixture was then transferred to a haemocytometer chamber and covered with a cover glass slip. Viable and non-viable cells were counted: coloured (blue) cells were considered dead while uncoloured cells excluding the dye were considered viable. Viability was determined as (Iheanacho, 2015).

$$\%\ \text{Cell viability} = \frac{\text{viable cell counted}}{\text{total number of cells}} \times 100$$

## 3.12. Protein Extraction Using Buffers

Three different types of buffers, i.e. citrate buffer (pH 5.0), phosphate buffer (pH 7.0) and carbonate-bicarbonate buffer (pH 10.0), were used for the extraction of total proteins from *Aspergillus oryzae*. The fungal mycelia were harvested by centrifugation at 2500 rpm. Then the resulting mycelia were homogenized at 6000 rpm using citrate buffer and centrifuged subsequently at 6000 rpm for 45 min. The temperature during the course of extraction was maintained at $4^{o}$C. The supernatant obtained was used for estimating the protein content.

## 3.13. Protein Determination

The protein content of the extract was determined using the Bradford assay method. In this method, changes in the absorbance of a Coomassie Brilliant Blue Dye-G250 at 595nm were measured as the dye binds with the protein and results in a change in the protein from cationic to anionic state. The changes in absorbance are proportional to the protein concentration. Coomassie Brilliant Blue Dye-G250 binds primarily to basic amino acids such as arginine, lysine and histidine. Therefore, there is a difference in absorption level of different concentrations of proteins. For this reason, a standard curve (see Appendix Table 1**)** was prepared using different concentrations of Bovine Serum Albumin (BSA) to indirectly determine the concentration of protein from the absorption reading obtained using a spectrophotometer (Bradford, 1976).

## 3.14. Methods of Nucleic Acid Reduction

About 70-80% of the total cell nitrogen is found in amino acids while the rest occurs in nucleic acids. This concentration of nucleic acids in single cell proteins is higher than other conventional proteins and is characteristic of all fast growing organisms. The problem which occurs from the consumption of proteins with high concentration of nucleic acids (18-25 gm/100 gm protein dry weight) is the high level of uric acid production in the blood, sometimes resulting in a disease called gout (Calloway, 1974).

Man, birds and some reptiles lack the enzyme uricase and the end product of purine degradation is uric acid. The removal or reduction of nucleic acid content of various SCP's is achieved with one of the following treatments (Zee *et al.,* 1974).

A. Chemical treatment with NaOH (0.20N NaOH at 37°C for 1 hr)

B. Treatment of cells with 10% NaCl

C. Thermal shock

In case of heat shock method, samples were taken and heated in water bath at 64°C for 20 min (Maul *et al*., 1970) then, nucleic acid content was measured after 30 min.

## 3.15. Determination of NA Content before and after NA Reduction

Nucleic acid content was measured before and after nucleic acid reduction using a spectrophotometer, which is programmed for nucleic acid measurements according to the method of Prakash, (2017).

## 3.16. Statistical Analysis

All the collected data were subjected to Analysis of Variance (two way anova) following the methods described in Gomez (1984) with three replications and the analysis was done using the Statistical Analysis Software (SAS Institute and Cary NC) Version 9.0. Means were compared for significant difference using Fisher`s LSD (FLSD) at $P<0.05$.

# 4. RESULTS AND DISCUSSION

## 4.1. Isolation of *Aspergillus oryzae*

Samples of hyphae, assumed to be *Aspergillus oryzae* was isolated based on colony color. All plates assumed to be *Aspergillus oryzae* pure cultures on PDA, gradually becoming yellow green colony color. This indicated that the fungi were *Aspergillus oryzae*. In addition to this, the isolate produced creamy reverse color on AFPA medium within 48h of incubation at $30^0$C. The reverse color of the isolates did not change after the additional incubation period for one week and indicated that the organisms were strains of *Aspergillus oryzae*.

### 4.1.1. Morphological characteristics

#### 4.1.1.1. Macroscopic characteristics

Macroscopic Characteristics such as, colony diameter 64 mm, colony color yellow, conidia color yellow green, mycelia color whitish, Colony reverse yellowish white and colony texture was wet. All the macroscopic characteristics of the isolates were indicating that the isolates were strains of *Aspergillus oryzae* see table below.

Table 1 Macroscopic characteristics of *Aspergillus oryzae* observed after 7 days of incubation at 25°C on CYA medium.

| Macroscopic Characteristics | CYA |
|---|---|
| | Isolate strain |
| Colony Diameter (mm) | 64 |
| Colony color | Yellow |
| Conidia color | Yellow green |
| Mycelia color | Whitish |
| Colony reverse | Yellowish white |
| Colony texture | Wet |
| Nature of spore masse | Powdery |

#### 4.1.1.2. Microscopic characteristics

The microscopic characteristics of the isolates were also evaluated and the results are shown below. As can be seen from the table, the conidia heads are radiate to loosely columnar, vesicles pyriform and 23.14µm wide; stipes 527.5 x 11.7µm, metulae 11.25 x 4.37µm, phialides 11.78 x 4.16µm in length and width, respectively. Conidia smooth to rough and ovoidal in shape, which measures 4.25µm in length. In addition, cream reverse color was produced within 48h when the isolate was inoculated on AFPA medium. This was in agreement with the characteristics reported by Maren, (2002) for *A. oryzae*. All these comparable microscopic characteristics also indicated that the isolate was species of *Aspergillus oryzae*.

Table 2. Microscopic characteristics of *Aspergillus oryzae* observed after 7days of incubation at 25$^{o}$C on czapeck yeast extract agar (CYA) medium.

| Microscopic characteristics | Length(µm) | Diameter(µm) |
|---|---|---|
| Vesicles | __ | 23.14 |
| Stipes | 527.5 | 11.7 |
| Metulae (1$^{st}$ sterigmata) | 11.25 | 4.37 |
| Phialides(2$^{nd}$ sterigmata) | 11.78 | 4.16 |
| Conidia | 4.25 | ___ |

## 4.2. Measurement of the Biomass of *Aspergillus oryzae*.

Aspergillus oryzae showed a significant increase ($P<0.05$) in biomass in the SSF of CCH mixed with SD. As shown in the table, the highest biomass (1.2gm/100gm of substrate) were produced when *A. oryzae* was grown 60% CCH + 40% SD, whereas the lowest biomass (0.3gm/100gm of substrate) was obtained in 100% SD.

Table 3. The mean value of biomass grown on coffee cherry husk, saw dust and their combinations.

| Substrates Combinations | Gram of Biomass/100gm of substrate |
|---|---|
| 60% CCH + 40% SD | 1.2 |
| 100% CCH | 0.8 |
| 50% CCH + 50% SD | 0.6 |
| 60% SD + 40% CCH | 0.4 |
| 100% SD | 0.3 |
| Mean | 0.66 |

CCH = Coffee Cherry Husk SD = Sawdust

## 4.3. Determination of the Extracted Protein from *Aspergillus oryzae.*

The results of the amounts of single cell proteins produced by *Aspergillus oryzae* are presented in table 4 below. *Aspergillus oryzae* grown on 60% CCH + 40% SD, 100% CCH, 50% CCH + 50% SD, 60% SD + 40% CCH and 100% SD were significantly ($p<0.05$) different from one another. The highest amounts of protein (51.3mg) were recorded from *Aspergillus oryzae* grown on 60% CCH + 40% SD alone, whereas the least amount of proteins was obtained from growing substrate 100% SD (24.9mg). *Aspergillus oryzae* grown on 100% CCH (42.3mg), 50% CCH + 50% SD (37.1mg) and 60% SD + 40% CCH (29.8mg) were recorded on pH 5, respectively.

*Aspergillus oryzae* grown on 60% CCH + 40% SD, 100% CCH, 50% CCH + 50% SD, 60% SD + 40% CCH, and 100% SD were also significantly ($p<0.05$) different. The highest amounts of protein (53.3mg) were recorded from *Aspergillus oryzae* grown on 60% CCH + 40% SD alone followed by 100% CCH (47.0mg), 50% CCH + 50% SD (39.9mg) and 60% SD + 40% CCH (33.4mg) recorded on pH 7. Whereas the least amounts of protein were obtained from growing substrate 100% SD (26.9mg).

In the case of pH 10, *Aspergillus oryzae* grown on 60% CCH + 40% SD, 100% CCH, 50% CCH + 50% SD, 60% SD + 40% CCH and 100% SD were significant ($p<0.05$) . The highest amounts of protein (57.1mg) were recorded from *Aspergillus oryzae* grown on 60% CCH +

40% SD alone. While the least amounts of proteins were obtained from growing substrate 100% SD (29.6). *Aspergillus oryzae* grown on 100% CCH (50.7mg), 50% CCH + 50% SD (46.6mg) and 60% SD + 40% CCH (37.9mg) were recorded, respectively.
*Aspergillus oryzae* grown on 60% CCH + 40% SD, extracted by pH5, pH7 and pH10 were significant ($p<0.05$). The highest amounts of protein (57.1mg) were recorded from pH 10, whereas the least amount of proteins was obtained on pH 5 (51.3mg).

*Aspergillus oryzae* grown on 100% CCH, were significantly ($p<0.05$) different. The highest amounts of protein (50.7mg) were recorded from pH10 alone. Whereas the least amount of protein was obtained from pH5 (42.3mg). *Aspergillus oryzae* grown on 50% CCH + 50% SD, extracted by pH5, pH7 and pH10 also significant ($p<0.05$). The highest amounts of protein (46.6mg) were recorded from pH10, while the least amount of protein was obtained from pH5 (37.1mg). Similarly, single cell protein produced from *Aspergillus oryzae* grown on 60% SD + 40% CCH and extracted by pH5, pH7 and pH10 were significant. The highest amounts of protein (37.9mg) were recorded from pH 10 alone, whereas the least amount of protein was obtained from pH5 (29.8mg). Likewise, *Aspergillus oryzae* grown on 100% SD and extracted by pH 5, pH 7 and pH 10 were also significantly ($p<0.05$) different from one another. The highest amounts of protein (29.6mg) were reported from pH 10 alone. Whereas the least amount of protein was obtained from pH5 (24.9mg) along the last row.

All the above results were in agreement with those of Mahmood, (2012) who reported the values of protein content which grown on different fruit wastes ranging from 24.2-57.3mg with the same species of the organisms. Among substrates, 60% CCH + 40% SD alone provided higher amount protein content than others, whereas 100% SD provided the least amount of protein content.

Many researchers in their investigation have used inorganic supplements for the mycelium growth on waste materials. Dimmling, and Seipenbusch, (1978) studied the raw material used for the production of SCP. Ojokoh, and Uzeh (2005) utilized glucose (2%w/v) and
(NH4)2 HPO4 (0.25%w/v) as a nitrogen source supplement for the production of *Sachharomyces cerevisiae* biomass in papaya extract medium. Adoki (2008) studied various factors influencing cell biomass production with *Candida* species. Several different fruit

wastes have uitilized as substrate by a number of researchers such Kamel, (1979) used dates, Ghanem, (1992) used beet pulp, Azin, and Moazami (1989) used sugarcane bagasse, Enwefa, (1991) used banana skins and Moharib (2003) used guava peel for the production of single cell protein. Yousufi *et al*. (2003) studied effect of moisture content on the production of single cell protein using *Rhizopus oligosporus* and *Aspergillus oryzae* grown on wheat grit combinations. Bellamy (1995) studied the conversion of insoluble agricultural wastes to SCP by thermophilic microorganisms. Mahat and MacRae, (1992) investigated on production of SCP on natural rubber waste serum using *Rhizopus oligosporus.* Yabaya and Ado (2008) studied the mycelia protein production by *Aspergillus niger* using banana peels. Steinkraus (1986) used edible substrates for the production of microbial biomass protein. The degree of mycelia biomass growth depends on the type of substrate used. Thus, in the present investigation coffee cherry husk, saw dust, and their combinations were explored for biomass production (SCP) instead of dumping them. In this study, coffee cherry husk and sawdust were used to produce single cell protein as feed and food.

Among the extraction buffers used Carbonate-bicarbonate at (pH10) was found to be most efficient in extracting out total proteins of *Aspergillus oryzae.* The protein yield with various buffers was as follows (mg/g sample)*:*citrate buffers,37.0gm; Phosphate (pH7) 40.1% ,carbonate- bicarbonate,44.3%.

Table 4.The mean value of extracted protein, extracted from *Aspergillus oryzae*

| Substrate Combinations | Protein content (SCP) (in mg/100gm of substrate) | | |
|---|---|---|---|
| | Citrate(pH5) | Phosphate(pH7) | Carbonate-bicarbonate (pH10) |
| 60% CCH + 40% SD | $51.3 \pm 4.2^{Aa}$ | $53.3 \pm 9.7^{Fb}$ | $57.1 \pm 4.5^{Kc}$ |
| 100% CCH | $42.3 \pm 6.0^{Bd}$ | $47.0 \pm 5.2^{Ge}$ | $50.7 \pm 6.2^{Lf}$ |
| 50% CCH + 50% SD | $37.1 \pm 7.0^{Cg}$ | $39.9 \pm 11.5^{Hh}$ | $46.6 \pm 6.5^{Mi}$ |
| 60% SD + 40% CCH | $29.8 \pm 8.3^{Dj}$ | $33.4 \pm 6.3^{Ik}$ | $37.9 \pm 4.9^{Nl}$ |
| 100% SD | $24.9 \pm 5.2^{Em}$ | $26. 9 \pm 6.2^{Jn}$ | $29.6 \pm 15.8^{Oo}$ |
| Means | 37 | 40.1 | 44.3 |

CCH = Coffee Cherry Husk SD = Sawdust

The values in the table show mean ± SEM (n=3). Superscripts in capital letters compare between means within the column, and means with different capital letters represent have significant difference, and means with different small letters are also significantly different at $p< 0.05$.

## 4.4. The Proximate Composition of *A. oryzae* Biomass

### 4.4.1. Crude Protein

*Aspergillus oryzae* grown on 50% CCH + 50% SD, 60% CCH + 40% SD, 100% CCH, 100% SD and 60% SD + 40% CCH showed significant difference ($p>0.05$) in crude protein content. The highest crude protein (45.0%) was recorded from *Aspergillus oryzae* grown on 60% CCH + 40% SD alone, whereas the least crude proteins were obtained from grown on 100% SD (30.0%). *Aspergillus oryzae* grown on 100% CCH (40.4%), 50% CCH + 50% SD (38.0%), and 60% SD + 40% CCH (34.0%) recorded respectively. The results were in agreement with those of Sikander *et al*. (2017) who reported values of crude protein content ranging from 30-45%. From these crude protein content values, it was possible to rank growth substrates or substrate combinations were used in the current study.

### 4.4.2. Crude fat

*Aspergillus oryzae* grown on 60% CCH + 40% SD and 100% CCH were not significantly different from each other. The highest (8.0%) crude fat was recorded from *Aspergillus oryzae* grown on 60% CCH + 40% SD. While the least fat contents were obtained from growing substrate on 100% SD (2.0%) and followed by 5.0%, 4.6% and 2.0% for *Aspergillus oryzae* grown on 50% CCH + 50% SD, 60% SD + 40% CCH and 100% SD were also not significant. However, single cell protein obtained from 100% CCH compared with 50% SD + 50% CCH, 60% SD + 40% CCH and 100% SD were statistically different. The biomass of *Aspergillus oryzae* obtained from growing subsrate 60% CCH + 40% SD compared with 50% CCH + 50% SD, 60% SD + 40% CCH and 100% SD were also significant. These results were in agreement with Sikander *et al.* (2017) who reported that the crude fat content of fungi containing 2-8%.

### 4.4.3. Total ash content

*Aspergillus oryzae* grown on 60% CCH + 40% SD, 100% CCH, and 50% CCH + 50% SD were not significantly different. However, *Aspergillus oryzae* grown on 50% CCH + 50% SD, 60% CCH + 40% SD and 100% CCH were significant when compared with 60% SD + 40% CCH and 100% SD. The highest total ash contents were 14.0% for *Aspergillus oryzae* grown on 60% CCH + 40% SD alone and followed by 11.3%, 11.0% and 9.4% for *Aspergillus oryzae*, grown on 100% CCH, 50% CCH + 50% SD and 60% SD + 40% SD respectively. While the least total ash contents were obtained from *Aspergillus oryzae* grown on 100% SD (9.1mg). These results were in agreement with Sikander *et al.* (2017) who reported that the crude fat content of fungi containing 9-14%.

### 4.4.4. Total carbohydrate content

The total carbohydrate was estimated by a phenol sulphuric acid method of Dubois *et al.* (1956) procedures. The value of total carbohydrate in *Aspergillus oryzae* grown on 60% CCH + 40% SD, 100% CCH, 50% CCH + 50% SD, 60% SD + 40% CCH and 100% SD were significantly ($p<0.05$) different. Among substrates, 60% CCH + 40% SD (48.8%) alone provided higher carbohydrate content than others, whereas 100% SD (25.2%) alone provided the least carbohydrate content. Among the growth substrate 100% CCH, 50% CCH + 50% SD and 60% SD + 40% CCH, obtained the value of total carbohydrate 37.8%, 31.0% and 29.5%, respectively.

The nutrient composition of the substrate is one of the factors that can affect quantitative and qualitative yield of cultivated fungi, supplements containing sugars and starch (easily available carbohydrates). The difference in carbohydrate content of species grown on different substrates could be due to the difference in carbon content of the substrate, temperature, pH level, moisture content, and substrate combinations and variation in selected fungi species.

Fungal chemical composition depends upon the species as well as upon the substrate, age and fructification(Irina *et al*., 2015)

### 4.4.5. Moisture content

*Aspergillus oryzae* grown on 60% CCH + 40% SD, 100% CCH, and 50% CCH + 50% SD, 60% SD + 40% CCH and 100% SD were not significantly different from each other. The highest values of moisture contents (6.0%) for *Aspergillus oryzae* grown on 60% CCH + 40% SD alone, while the least moisture contents were (4.5%) obtained from *Aspergillus oryzae* grown on 100% SD. The next moisture contents obtained from the growing substrate 100% CCH, 50% CCH + 40% SD and 60% SD + 40% CCH were 5.0%, 4.8% and 4.6 respectively. These results were closely related to the finding of Anupama and Ravindra (2000) in which fungi moisture contents of 4.5-6% reported.

Some researchers reported the proximate composition of fungi in their study. Israelidis (2013) report the amount of chemical composition of fungi; i.e. 30-45% protein, 9-14% ash and 2-8% fat content in his study. Nasser (2011) also report similar results with the above researcher in his research work.

Table 5. The Proximate Composition of *Aspergillus oryzae* biomass

| Substrate Combinations | Proximate Compositions (%) | | | | |
|---|---|---|---|---|---|
| | Crude Protein | Crude Fat | Crude Ash | Carbohydrate | Moisture |
| 60%CCH+40%SD | $45.0 \pm 3.3^{A}$ | $8.0 \pm 1.0^{A}$ | $14.0 \pm 2.7^{A}$ | $48.8 \pm 1.1^{A}$ | $6.0 \pm 0.0^{A}$ |
| 100% CCH | $40.4 \pm 0.3^{B}$ | $5.7 \pm 2.0^{A}$ | $11.3 \pm 3.1^{BA}$ | $37.8 \pm 1.6^{B}$ | $5.0 \pm 1.0^{A}$ |
| 50%CCH+50%SD | $38.0 \pm 7.3^{C}$ | $5.0 \pm 2.0^{B}$ | $11.0 \pm 1.1^{BA}$ | $31.0 \pm 1.0^{C}$ | $4.8 \pm 0.6^{A}$ |
| 60%SD+40%CCH | $34.0 \pm 2.7^{D}$ | $4.6 \pm 2.8^{B}$ | $9.4 \pm 0.9^{B}$ | $29.5 \pm 1.8^{D}$ | $4.6 \pm 0.9^{A}$ |
| 100% SD | $30.0 \pm 1.5^{E}$ | $2.0 \pm 1.0^{B}$ | $9.1 \pm 1.0^{B}$ | $25.2 \pm 2.8^{E}$ | $4.5 \pm 0.5^{A}$ |

CCH = Coffee Cherry Husk SD = Sawdust

The values in the table show mean ± SEM (n=3). Superscripts in capital letters compare between means within the column, and means with similar capital letters represent no significant difference, whereas means with different capital letters are significantly different at $p < 0.05$.

## 4. 5. Reduction of Nucleic Acids Levels in *A. oryzae* (% of dry cells).

*Aspergillus oryzae* grown on 60% CCH + 40% SD, 50% CCH + 50% SD, 60% SD + 40% CCH, 100% SD and 100% CCH were significantly ($p<0.05$) different across column. The highest amounts of nucleic acids reduction (83.6%) were reduced from *Aspergillus oryzae* grown on 60% CCH + 40% SD, whereas the least amounts of nucleic acid were reduced from growing substrate 100% SD (59.9%) by the method of 0.20N NaOH. The highest amounts of nucleic acids (87.1%) were reduced from *Aspergillus oryzae* grown on 60% CCH + 40% SD alone, while the least amount of nucleic acid was reduced from growing substrate 100% SD (65.2%) by the methods of 10% NaCl. The nucleic acid content of the biomass removed by thermal shock, was significant ($p<0.05$) different. The highest amounts of nucleic acid reduction (94.0%) were reduced by thermal shock, whereas the least amount of nucleic acid (68.8%) was reduced by the same method through the final column.

The amounts of nucleic acids analysis were presented in table 6 below. The nucleic acid content of the biomass obtained by thermal shock, 10% NaCl and 0.20N NaOH were significantly ($p<0.05$) different. The highest amounts of nucleic acid were reduced by thermal shock (94.0%) alone; whereas 0.20N NaOH reduced the least amount of nucleic acid (83.6%). The highest amounts of nucleic acids (90.6%) were also reduced by thermal shock alone, whereas the least amounts of nucleic acids (75.9%) were reduced by the method of 0.20N NaOH. The highest amounts of nucleic acids (87.4%) were also reduced by thermal shock alone; whereas 0.20N NaOH (67.7%) reduced the least amount of nucleic acid. Similarly, the highest amounts of nucleic acid were reduced by thermal shock (84.2%) alone, while 0.20N NaOH reduced the least amount of nucleic acid (64.9%). Likewise, the highest amounts of nucleic acid were reduced by thermal shock (82.8%) alone, while 0.20N NaOH reduced the least amount of nucleic acid (59.6%).

The percentage of nucleic acid reduction was lower than the work of other's. This may be due to the difference among PH, temperature and procedures used during the reduction

process. The outcomes of the current study have been compared with previous studies conducted using similar standard methods for quantitative determination of nucleic acids in yeast cells. Antonio, (2011) found the significance of the process to the pH and temperature effects on RNA extraction yield. In addition to this, he reported that in 24hrs of autolysis with 10% NaCl, the RNA extraction yield was 89.7%.

In general, comparison of the current study with previously conducted studies showed that nucleic acid composition and content vary within Species Khalil *et al*. (2008). The variation may be due to the difference in species of the organisms (Khalil *et al*. (2008) and due to the contaminant such as protein, carbohydrate, lipids or other nucleic acids like DNA free of RNA or RNA free of DNA (Siun Chee Tan and Beow Chin Yiap, 2009). These explanations are supported by the findings of previous researchers. Eighty-six percent of nucleic acids were extracted from cells by 0.20N NaOH at 37°C for 1hr as reported by Makoto and Seiji, (1975). Khalil *et al*. (2008) reported that the heat shock treatment at 64°C for 20 min followed by 30 min incubation caused a decrease in nucleic acids ranged from 65.2 to 88.8 % .The reduction in nucleic acid content was 65.2, 71.6, 74.0, 83.0 and 88.8 % in case of *Pichia anomala, Candida rugosa, Candida blankii, Kluyveromyces lactis and Rhodotorula glutinis*, respectively. It is evident that the growth product of *Rhodotorula glutinis* was the most affected in nucleic acid reduction followed by *Kluyveromyces lactis* then the other strains. It was reported (Kurbanoglu and Algur, 1996) that one-step heat shock was carried out at $90^0$C for 2hrs and the highest nucleic acid reductions achieved for *Saccharomyces cerevisiae, Candida utilis, Fusarium moniliforme* and *Bacillus subtilis* were 95.2, 89.6, 95.5 and 75.3 %, respectively. On the other hand, three step heat shock was carried out at $68^0$C for 3s, $45^0$C for 2hrs and $55^0$C for 1hr and the maximum nucleic acid reductions obtained were 95.8, 86.4, 91.0 and 78.5 % for these species, respectively. It was also reported that a heat shock treatment at $65^0$C for 5-10 min followed by incubation at $55^0$C for 2 hrs resulted in 81.0- 85.0 % reduction in nucleic acid content (Abu-Ruwaida *et al.,* 1988).

Table6. Nucleic acid reduction level by 0.20N NaOH, 10% NaCl and thermal shock treatments.

| Substrate Combinations | Concentration before treatment | Amount after treatment | | |
|---|---|---|---|---|
| | | 0.20N NaOH % Reduction in NA content | 10% NaCl % Reduction in NA content | Thermal shock % Reduction in NA content |
| 60%CCH+ 40% SD | 16.4mg | $83.6 \pm 3.6^{Aa}$ | $87.1 \pm 6.5^{Fb}$ | $94.0 \pm 7.9^{Kc}$ |
| 100% CCH | 14.4 mg | $75.7 \pm 3.2^{Bd}$ | $79.3 \pm 0.4^{Ge}$ | $90.6 \pm 4.8^{Lf}$ |
| 50% CCH+50% SD | 12.7 mg | $67.0 \pm 8.8^{Cg}$ | $70.0 \pm 4.3^{Hh}$ | $87.4 \pm 10.6^{Mi}$ |
| 60%SD+ 40% CCH | 12.2 mg | $64.8 \pm 11.4^{Dj}$ | $68.1 \pm 1.7^{Ik}$ | $84.2 \pm 5.3^{Nl}$ |
| 100% SD | 11.2 mg | $59.9 \pm 0.9^{Em}$ | $65.2 \pm 12.6^{Jn}$ | $82.8 \pm 13.5^{Oo}$ |
| Average | 13.3 | 70.2 (4.0mg) | 73.9 (2.5mg) | 87.8 (1.6mg) |

The values in the table show mean ± SEM (n=3). Superscripts in capital letters compare between means within the column, and means with similar capital letters represent have significant difference, and means with different small letters are also significantly different at $p < 0.05$.

**4.6. Toxicity Test.**

It was observed, as data obtained showed that the viability of cells without any treatment with toxin was 97.2%, 97.3% and 97.2% after 24, 48 and 72 h, respectively, of incubation. The viability of cells after 24,48 and 72 hrs exposure were not strongly influenced by the concentration of toxin. This showed almost similar results in cell viability when compared to the control with no toxin contents.

Table 7. Percentage of toxicity test by cell viability

| Substrate Combinations | Exposure time | % Cell Viability ± SD | | |
|---|---|---|---|---|
| | | 20 µl/ml | 40 µl/ml | 80 µl/ml |
| 60% CCH and 40% SD | 24hrs | 97.2 | 97.5 | 97.0 |
| | 48hrs | 98.0 | 96.8 | 96.4 |
| | 72hrs | 97.0 | 98.2 | 97.0 |
| Control | 24hr | 97.1 | 97.4 | 97.1 |
| | 48hr | 97.1 | 97.2 | 97.4 |
| | 72hr | 97.4 | 97.3 | 97.1 |
| | Means | 97.2 | 97.3 | 97.2 |

CCH = Coffee Cherry Husk, SD = Sawdust, SD = Standard Deviation

The values in the table show mean ± SEM (n=3). Superscripts in capital letters compare between means within the column, and means with similar capital letters represent no significant difference, whereas means with different capital letters are significantly different at $p< 0.05$.

The safety of any food-grade product is carefully evaluated before its commercialization and is assured throughout the manufacturing, processing, transportation, storage, and use of the product. Safety assurance measures range from the selection of manufacturing raw materials to a series of toxicological tests. To ensure the safety of food-grade enzymes, the Joint FAO/WHO Expert Committee on Food Additives(Joint FAO/WHO., 1989) required that food enzyme preparations derived from fungal sources should not contain detectable amounts of aflatoxin B1, ochratoxin A, sterigmatocystin, T-2 toxin (a major trichothecene toxin), or zearalenone. The safety of *Aspergillus oryzae* as production organisms for food-grade products has long been recognized. To ensure the safety of the products, the industry carefully selects, maintains, and sometimes modifies the production strains. Fermented foods produced by *Aspergillus oryzae* have been shown to be aflatoxin-free (Liu, and Chen, 1966). No soy sauce production strains of *Aspergillus oryzae* tested produced detectable amounts of cyclopiazonic acid (Matsushima *et al.,* 2001). Today, it is agreed by the majority of the scientific community that *Aspergillus oryzae* does not produce aflatoxins (Kozakiewicz, 1982).

In the field of enzyme production, the safety and toxigenicity of *Aspergillus oryzae* as a production organism have been assessed (Barbesgaard *et al.,* 1992). Toxicity tests of several enzyme preparations derived from *Aspergillus oryzae* indicate that *Aspergillus oryzae* is safe for use in its intended applications (Lane, 1997). In the USA, a number of enzyme preparations derived from *Aspergillus oryzae* have been granted ‘‘generally recognized as safe’’ status in various food-processing applications on the basis of publicly available. When a new production strain of *Aspergillus oryzae* is developed, it is of primary importance that the strain to be tested for the production of toxicologically significant amounts of relevant secondary metabolites.

Toxicity has not been detected in any *Aspergillus oryzae* cultures (Kusumoto *et al.*, 1990; Manabe *et al*., 1968). Therefore, it is thought that the toxin gene homolog cluster in *Aspergillus oryzae* is not functional. It is important to prove at the molecular level that *Aspergillus oryzae* is incapable of producing toxin in order to continue to use strains of this species with confidence in the food-processing industry.

# 5. SUMMARY, CONCLUSIONS AND RECOMMENDATIONS.

## 5.1. Summary

Coffee cherry husk and sawdust are naturally found by-product of agriculture in Ethiopia. They are believed to have organic compound that used for single cell protein production. Therefore, this study was aimed to produce single cell protein from coffee cherry husk and sawdust as a sole and in various combinations; analyzing the chemical composition of the biomass produced from *Aspergillus oryzae.*

Coffee cherry husk and sawdust samples were collected, washed, dried and ground. Then, the obtained dry powder of the two plants were weighed, sterilized and added into sterilized petri-dishes and inoculated with *Aspergillus oryzae.* The separation of mycelia and substrate was performed following standard procedure and determine the amount of single cell by spectrophotometric method. Crude protein, crude fat, total ash content, total carbohydrate and moisture content were determined by following the standard procedure of kjeldahl method. Besides, the nucleic acid content of the biomass were determined using techniques of the different treatments.

The protein content of the SCP isolates was highest for those obtained from the substrate combination 60% CCH + 40% SD which contain 57.1mg/100gm of substrate and for those obtained via the Bradford method of extraction. Thermal shock methods of treatment were highest Reduction methods of nucleic acid content of single cell protein. The general objective of this study is to evaluate the production of single cell protein (SCP) from *Aspergillus oryzae* using coffee cherry husk and sawdust. The specific objective is to determine the amount of single cell protein (SCP) that can be produced from *Aspergillus oryzae* using coffee cherry husk and sawdust as growth substrates. Isolation and identification of *Aspergillus oryzae* for single cell protein production is the crucial methods. Production of Fungal Biomass Using Solid State Fermentation (SSF) and harvesting is the main methods in this process. This would help to analyses raw material coffee cherry husk and sawdust for *Aspergillus oryzae* growth by their independently or in mixture. *Aspergillus oryzae* was able to convert both substrates into biomass and protein

concentration. Samples of *Aspergillus oryzae* were analyzed for chemical composition (moisture, protein, fat, carbohydrates, and ash) using the AOAC procedures. Buffers such as citrate (pH 5.0), phosphate (pH 7.0) and carbonate-bicarbonate (pH 10.0) were used for the extraction of total proteins from *Aspergillus oryzae* and Protein determination was done by Bradford Assay. The reduction of nucleic acid content of various SCP's is achieved with all of the following treatments. Chemical treatment with 0.05N NaOH, treatment of cells with 10% NaCl and thermal shock. The result obtained shows that *Aspergillus oryzae* strain can produce maximum protein yield on 60% CCH + 40% SD. The raw material coffee cherry husk is a potential substrate for single cell protein production than sawdust. 60% CCH + 40% SD is the best combination to produce high amount single cell protein from *Aspergillus oryzae*. The nucleic acid reduction by heat shock is the most suitable reduction methods than others. Cell viability assay is an easy and effective method used for toxicity testing of single cell protein.

## 5.2. Conclusion

The bioconversion effect of coffee cherry husk and sawdust into SCP was evaluated using *Aspergillus oryzae*. The biomass level was increased with the increase in concentration coffee cherry husk and sawdust at 60% CCH + 40% SD ratios. Hence the availability of nutrients in coffee cherry husk and sawdust has rapidly promoted growth of fungal cells. The highest biomass (dry biomass 57.1mg, 53.3mg and 51.3mg/100gm) substrate was extracted by citrate (pH 5), phosphate (pH7) and Carbonate-bicarbonate (Ph10) recorded, respectively on the $7^{th}$ day of fermentation at 60% CCH + 40% SD ratios, where *Aspergillus oryzae* was used as inoculum. The present findings reveal that coffee cherry husk and sawdust can be used as effective alternate carbon source for SCP production.

## 5.3. Recommendations.

Based on these findings the following recommendation were forwarded.

1. Coffee cherry husk and sawdust at 60% CCH + 40% SD level performed better than sawdust alone in inducing single cell protein from *Aspergillus oryzae* species. It is possible that this substrate could even be made to induce high level of single cell protein. It is, therefore, recommended that the fermentation of CCH and SD can be studied using *Aspergillus oryzae.*
2. Coffee cherry husk and sawdust have been shown to be as effective as 1% NaOH forpre-treating CCH and SD biomass production. Generally, it was found to improve fungal biomass production. Therefore, suggested that further investigation be done in this area before substrate can be used in the single cell protein production process.
3. Pre-treated CCH and SD have been shown to be valuable for fungal biomass production. It is possible to increase the food and feed values of this substrate by growing the mycelia on them using solid state fermentation processes.
4. It also recommended that the economic assessment of the possibility of using CCH and SD as growth substrates for single cell protein production can be made. It is also interesting to know what actually prevents the release of single cell protein from *Aspergillus oryzae* species when it was grown on CCH and SD. Therefore, studies should also be conducted in this direction to make this substrate industrially valuable.

# 6. REFERENCES.

Abakr,Y. A. and Abasaeed, A. E. (2006). Experimental evaluation of a conical-screw briquetting machine for the briquetting of carbonized cotton stalks in Sudan. *Journal of Engineering Science and Technology*, 4(1): 212 – 220.

Abebe B., Yared K., Taffere A., Fassil A., Aklilu A., Worku L., Helmut K. and Ludwig T. (2011).The impact of traditional coffee processing on river water quality in Ethiopia and the urgency of adopting sound environmental practices. *Environ Monit Assess.*184 (5): 7053 – 7063

Abu-Ruwaida, A.S., Banat, M.I. and Hamdan, Y.I. 1988. Reduction of nucleic acid content in cell biomass of methanol utilizing mixed bacterial culture by heat treatment. *Biotechnology Letters*, 10(3):597-602.

Adedayo, M.R., Ajiboye, E.A., Akintunde J.K. and Odaibo, A. 2011. Single Cell Proteins: As Nutritional Enhancer. *Adv. App. Sci. Res.*, 2 (5): 396–409.

Adoki, A. (2008). Factors affecting yeast growth and protein yield production from orange plantain and banana wastes processing residues using *Candida* species. *African Journal of Biotechnology*, 7(3): 290-295.

Ageitos, J.M., Vallejo J.A., Veiga-Crespo P. and Villa T.G. 2011. Oily yeasts as oleaginous cell factories. *J. Am. Sci*., 90(7): 1219–1227.

Alemayeu Haddis and Rani Devi. 2007. Effect of effluent generated from coffee processing plant on the water bodies and human health in its vicinity. *Journal of Hazardous Materials*, 152(5): 259 - 262.

Andersen and Jorgensen SB (2005) U-loop reactor modelling for optimization, part 1:estimation of heat loss. *J. Environ. Iss.*, 9(3): 88–90.

Anupama and Ravindra P. 2000. Value-added food: Single cell protein. *Biotech. Adv*., 18 (6): 459-479.

Antonio Martins Oliveira and Pedro de Oliva Neto. (2011). Improvement in RNA extraction from *S. cerevisie* by optimization in the autolysis and $NH^3$ hydrolysis. *Braz. arch. biol. Technol*., 54 (5) 1- 7.

AOAC (1995). Official methods of the analysis, (16th ed.). Arlington, V.A: Association of Official Analytical Chemists. Washington, DC.360.

Asha-Augustine, I. Imelda-Joseph and R. Paul Raj. 2006. Biomass estimation of *Aspergillus niger* S,4 a mangrove fungal isolate and *Aspergillus oryzae NCIM* 1212 in solid-state fermentation. *J. Mar. Biol. Ass. India,* 48 (2): 139 – 146.

Atikpo M., Onokpise O., Abazinge M., Louime C., Dzomeku M., Boateng L., Awumbilla. 2008. Sustainable mushroom production in Africa: A case study in Ghana. *Afr. J. Biotechnol*., 7(1):249 -253.

Azin, M., and N. Moazami. 1989. Production of SCP from sugarcane bagasse. Second Congress of Forest and Technology. Faculty of Engineering. Tehran University, 7(4): 19-21.

Barbesgaard P., Heldt-Hansen H., Diderichsen B. 1992. On the safety of *Aspergillus oryzae*: a review. *Appl Microbiol Biotechnol*, 36(1): 569–572.

Barnell, H. R. (1974) Biology and the Food Industry. Studies in Biology No. 45,

Bekatorou, A., Psarianos, C., Koutinas, A. A. 2006. Production of food grade yeasts. *Food Technol. Biotechnol*., 44(4): 407- 415.

Blochl S. E., Burggraf G., Fiala G., Lauerer G., Huber R., Huber R., Rachel

A., Segerer K. O., Stetter P. and Völkl .1995 Isolation, taxonomy and phylogeny of hyperthermophilic microorganisms. *World Journal of Microbiology and*

*Biotechnology*, 11 (1): 9–16.

Bello-Mendoza, R. and Castillo-Rivera, M. F. 1998. Start-up of an anaerobic hybrid UASB filter reactor treating waste water from a coffee processing plant. *Anaerobe*, 4 (4): 219-225.

Bishaw, B., Neufeldt, H., Mowo, J., Abdelkadir, A., Muriuki, J., Dalle, G. & Luedeling, E. 2013. 'Farmers' strategies for adapting to and mitigating climate variability and change through agroforestry in Ethiopia and Kenya', Forestry Communications Group. Edited by Davis C.M., Bernart B., Dmitriev A., Corvallis, Oregon: Oregon State University.

Bradford, M. M. 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal Biochem*, 72(1): 248-254.

Bunker, H. J. (1966) New Foods. 2nd Int. Congr. Food Sci. and Technol., Warsaw. In Biology and the Food Industry. Studies in Biology No. 45 (Barnell, H. R. Ed., 1974) p. 48. Edward Arnold Ltd. London

Calloway.1974. Single cell protein, Academic Press, New York, 129–146.

Capalbo, F.H., Moraes, I.O., Pelizer, M.H. 2001. Solid-state fermentation of *Bacillus thuringiensistolworthi* to control fall armyworm in maize. *Electr. J. Biotechnol.,* 4 (2): 1-5.

Cicik S., supyani. 2009. Linoleic and linolenic acids analysis of soybean tofu with *Rhizopus oryzae* and *Rhizopus oligosporus* as coagulant. *International Journal of Microbiological Research*, 3 (5): 303-405.

Coutteau P. and Lavens P. 1989. The use of yeast as a single-cell protein in aquacultural diets. *Gentics,* 54(5):1583–1592.

Dimmling, W. and Seipenbusch, R. 1978. Raw material for the production of SCP. *Precess Biochem*., 13 (3): 9-15.

Dubois, M., K. A., Gilles, J.K., Hamilton, P.A., Rebers and F. Smith. 1956. Colorimetric method for determination of sugars and related substances. *Anal. Chem*., 28(6): 305-356.

Enwefa, Chijioke. 1991. Biomass production from banana skins. *Applied Microbiology and Biotechnology*, 36(2):283-284.

Ethiopian Commodity Exchange Authority, Addis Ababa. 2008. Analysis of Coffee Supply, Production, Utilization and Marketing Issues and Challenges in Ethiopia.

Fatemeh S.S.Reihani, and Kianoush Khosravi-Darani. 2019. Influencing factors on single-cell protein production by submerged fermentation: A review. *Electronic Journal of Biotechnology*, 37 (2):34-40.

Franze Vorreiter, Silke Richter, Michel Peter, Sven Baumann, Martin von Bergen, Janina M. and Tomm. 2016. Comparison and optimization of methods for the simultaneous extraction of DNA, RNA, proteins, and metabolites. *Analytical Biochemistry,* 508 (8): 25-33.

Gabriel A., Victor N. and James C. du P. 2014. Cactus pear biomass, a potential lignocelluloses raw material for single cell protein production (SCP): A Review. *Int. J. Curr. Microbiol. App. Sci*., 3(7): 171–197.

Ghanem, K.M. 1992. Single cell protein production from beet pulp by mixed culture. *Microbiology*, 8(1): 39 - 43.

Gomez and Gomez.1984. Statistical procedures for agricultural research. John Wiley and Sons, New York. 265.

Halasz, Anna. 2000. Use of yeast biomass in food production. CRC press. Inc.

Florida. 312.

Harris, E. E. (1949) Food Yeast Production from Wood Processing By-Products. United States Department of Agriculture Forest Service.

Iheanacho H. 2015. Cytotoxic Effects of Aflatoxin B1 Standard in Relation to Aflatoxin Extracts from South African Compound Feeds on Human Lymphocytes. *Int. J. Biomed Data Min.*, 108 (3): 2090-4924.

Irina A. Kadnikova, Rui Costa, Tatina K. Kalenik, Olga N. Gurulena, Shi Yanguo. (2015). Chemical Composition and Nutritional Value of Auricularia auricular-Judae. *Journal of Food and Nutrition Research,* 3(8):478-482.

Israelidis C.J. 2008. Nutrition-Single cell protein, twenty years later. http*:// www. Biopolitics gr/biopolitics/html/pubs/vol1/isreali.htm.* Jan 5, 2017.

ITC (International Trade Centre) UNCTAD/WTO. 2002. International trade statistics, Geneva, Switzerland. http://www. intracen.org/tradestat/site3-3d/ index.html. Jun 21, 2018.

Jaganmohan, P., Purushottam, B., Prasad, S.V. 2013. Production of SCP with Aspergillus terrus using Solid State fermentation. *Eur. J. Biol. Sci*., 5(2): 38-45.

James, C.S. 1995. Analytical Chemistry of Foods 1st Edition. Chapman and Hall, New York Cultivation of Edible and Medicinal Fungi, Penn State University, Pennsylvania, 431.

Joint FAO/WHO Committee on Food Additives (JECFA), 1989. Specifications for identity and purity of certain food additives. JECFA 35th session. Rome, Italy.

Kamel, Basil S. 1979. Dates as a potential substrate for single cell protein production.

*Enzyme and Microbial Technology*, 1(3): 180-182.

Khalil, M. S. Hassouna and R. H. Zaanon. 2008. Utilization of Food Industries Wastes for the Production of Single Cell Protein by Yeasts. Alexandria, Egypt.

Kozakiewicz, Z. 1982. The identity and typification of *Aspergillus parasiticus. Mycotaxon* 15 (4):293–305.

Kurbanoglu, E.B. and Algur, O. F. 1996. The effect of methods of one and three step heat shock on the reduction of nucleic acid content of single cell protein. *Turkish Journal of Biology*, 20 (1):183-190.

Kusumoto, K., T. Goto, and M.Manabe**.** 1990. Evaluation for conversion of sterigmatocystin to aflatoxin in koji-molds. *Rep. Natl. Food Res. Inst*., 54(6)**:** 14–17.

Lane, R.W., Yamakoshi, J., Kikuchi M., Mizusawa K., Henderson L., Smith M. 1997. Safety evaluation of tannase enzyme preparation derived from *Aspergillus oryzae*. *Food Chem Toxicol.*, 35(4): 207–212.

Leck, A. 1999. Preparation of lactophenol cotton blue slide mounts. *Journal Community Eye Health*, 12(30): 24.

Li, P. and Gatlin D.M. 2003. Evaluation of brewer's yeast (*Saccharomyces cerevisiae*) as a feed supplement for hybrid striped bass (*Moron chrysops -M. saxatilis*). *Aquaculture* 219(1–4): 681–692.

Liu P. and Chen, S. 1966. Studies on toxin production by fungi used in fermentation industry. *Chung-Kuo Nung Yeh Hua Hsueh Hui Chih.*, 4(1): 65–73.

Lichfield, J.H. 1968. In. Single cell protein (Eds: Mateles RI and Tannenbaum SR), M.I.T. Press, Cambridge Massachusetts, 122.

Machida, M., Yamada O., and Gomi K.2008. "Genomics of Aspergillus oryzae: Learning from the History of Koji Mold and Exploration of Its Future." *Oxford Journals*: DNA Research, 15(4):173-183.

Mahajan, A., Dua, S. 1995. A perspective on biotechnological potential. *J. Food Sci. Technol.*, 32(2): 162-165.

Mahat, M.S. and I.C. MacRae. 1992. *Rhizopus oligosporus* grown on natural rubber waste serum for the production of single cell protein: a preliminary study. *World J. of micro and Biotech.*, 8(1):63-64.

Mahmood khan yousufi. 2012. To determine protein content of single cell protein produced by using various combinations of fruit wastes and two standard food fungi. *International journal of advanced biotechnology and research,* 3(1): 533-536.

Mahmood khan yousufi. 2012. Utilization of various fruit wastes as substrates for the production of single cell protein using *Aspergillus oryzae* and *Rhizopus oligosporus*. *International journal of advanced biotechnology and research*, 5(2): 92-95.

Makoto Tajima and Seiji Yoshikawa. 1975. Changes of Nucleic Acids in the Preparation Process of Cell Protein Isolates from Yeast (*Saccharomyces cerevisiae*). *Agricultural and Biological Chemistry*, 39(3): 611-616.

Manabe, M., S. Matsuura and M. Nakano. 1968. Studies on the fluorescent compounds in fermented foods. Part I. Chloroform soluble fluorescent compounds produced by Koji-molds. *Nippon Shokuhin Kogyogakkaishi*, 15(2)**:** 341– 346.

Martin, M.A. 1991. Bioconversion of waste materials to industrial products, *Elsevier*

*Applied science*, 98(6): 187-223.

Matsushima K., Chang P-K., Yu J., Abe K., Bhatnagar D., Cleveland T.E. 2001. Pre-termination in aflR of *Aspergillus sojae* inhibits aflatoxin biosynthesis. *Appl Microbiol Biotechnol*, 55(3): 585–589.

Maren A., Klich. 2002. Identification of common A*spergillus* species, 1st Edition. Central bureau Voor Schimmelcultures, Utrecht, The Netherlands.124.

Maul, S.B., Sinskey, J.A. and Tannenbaum, S. R. 1970. A new process for reducing the nucleic acid content of yeast. *Nature*, 22(2): 181-182.

Moharib Sorial A. 2003. Utilization of guava peel waste as substrate for protein and protease production by *Saccharomyces cerevisiae*. *Advances in food sciences*, 25(2):49-55.

Monod, J. (1942) Recherches sur la croissance des cultures bacteriennes. Hermann, Paris

Murray Moo-Young. 2004. Single cell protein. Concise encyclopedia of bio resource technology. 293-305.

Najah Mahdi Mohammed Al-Mhanna. 2012. Single Cell Protein (SCP) Production from Date Juice. MSc. THesis, warzaw University of life Sciences- SSGW Forestry and wood technology, Poznan, 1-177.

Nasseri A.T., Rasoul-Amini S., Moromvat M. H. and Ghasemi Y. 2011. Production of single cell protein from fruits waste. *Am. J. Food Technol.*, 6(2): 103–116.

Ojokoh, A.O. and R.E., Uzeh. 2005. Production of *Saccharomyces cerevisiae* biomass in papaya extract medium. *African Journal of Biotechnology*, 4(11):1281-1284.

Oliva-Teles, A., Gonçalves, P. 2001. Aquaculture enhancement. *J. Chem. Eng.*,

202(4): 269- 278.

Olvera-Novoa M.A., Domiminguez-cen L.J. and Olvera-castillo L. 2002. Effect of the use of the microalgae *Spirullina maxima* as fishmeal replacement in diets for tipalia, *Oerochromis mossambicus* (Peters), fry. *Aquacul. Res*, 29(10): 707-715.

Pandey A., Soccol C.R., Nigam P., Brand D., Mohan R., Roussos S. 2000. Biotechnological potential of coffee pulp and coffee husk for bioprocesses. *Biochem Eng J.* 6(2):153-162**.**

Peterson, W. H., Snell, E. E. and Frazier, W. C. (1945) Fodder yeasts from wood sugar. Ind. Eng. Chem. 37.

Pirt, S. J. (1973) Estimation of substrate affinities (Ks values) of filamentous fungi from colony growth rates. J. Gen. Microbiol.75.

Pirt, S. J. and Kurowski, W. M. (1970) Extension of the theory of the chemostat with feedback of organisms. Its experimental realization with a yeast culture. J. Gen. Microbiol. 63.

Pitt,J.I. and Hocking,A.D.1985. Fungi and Food spoilage. Academic press, Autralia. 259-288.

Prakash Babu M. and Mohamed Ilyas M.H. 2017. SCP production from *Saccharomyces cerevisiae* isolated from mangrove sediment. *Int. J. Adv. Multidiscip. Res.*, 4(1):1-10.

Pushpa S. Murthy and Madhava Naidu. 2010. Protease production by *Aspergillus oryzae* in solid state fermentation utilizing coffee by products. *World applied science Journal*, 8(2): 199-205.

Rashida Rajuva TA and Joy pp. 2014. Pineapple Research station(Kerala agricultural University), vzhakulam.

Sebastianos, R., Isabelle, P.G. and Sylvain, D. 1998. Biotechnological management of coffee pulp. *Applied Mycology and Biotechnology, 7(3)*:34-52.

Sikander ali, javeria mushtaq, farzana nazir and hira sarfraz. 2017. Production and processing of single cell protein (scp). *European journal of pharmaceutical and medical research ejpmr*, 4(7): 86-94.

Sileshi Degefa. 2016. Home garden agroforestry practices in the Gedeo zone, Ethiopia: a sustainable land management system for socio-ecological benefit Tokyo, Japan 28-36.

Singhania, A.K., Soccol, C.R., Pandey, A. 2009. Recent advances in solid state fermentation. *Biochem. Eng. J*., 8(9): 667-789.

Siun Chee Tan and Beow Chin Yiap. 2009. DNA, RNA and Protein Extraction: The past and The present. *J.Biomed and Biotechnol.*2009; 2009: 574398.

Scholein, J. (1937) The Brewer's Yeast as a Medicine and Feeding Stuff. Verlag Steinkopf, Dresden

Solange, I.M., Ercília, M. S. M., Silvia, M. and José, A.T. 2011. Production, Composition, and Application of Coffee and Its Industrial Residues. *Food Bioprocess Technol*ogy, 4(1):661–672.

Sooriyamoorthy, S., K. Silva, M. Gunawardhane and C. Liieperuma. 2004. Isolation and identification of indigenous *Aspergillus oryzae* for saccharification of rice starch. *Trop. Agric. Res*., 21(16): 121-127.

Suman Upadhyaya, Shashank Tiwari, N.K. arora, D.P. Singh .2016. Microbial protein: a valuable component for future food security. *Research gate*, 4(2):259-279.

Srividya, A. R., V. J., Vishnuvarthan, M. Murugappan, Prajakt Gopal Dahake. 2013. Single Cell Protein- A Review: *International Journal for Pharmaceutical Research Scholars (IJPRS),* 4(2): 472-485.

Steinkraus, K.H. 1986. Microbial biomass protein grown on edible substrates: the indigenous fermented foods. In microbial biomass protein (M. Moo-Young and K.F. Gregory Eds.) Selection I. *Elsevier Applied Science*. London: 33.

Tadese, K.K. 2002. 'Five thousand years of sustainability? A case study on Gedeo land use, Southern Ethiopia, Treemail publishers, Heelsum, the Netherlands.169.

Taylor, E. T. and Nakai, S. 2012. Prevalence of Acute Respiratory Infections in Women and Children in Western Sierra Leone due to Smoke from Wood and Charcoal Stoves. *Int. J. Environ.Res. Public Health,* 9(6): 2252 – 2265.

The General Agreement on Tariffs and Trade (GATT 1994). This document may be obtained from the World Trade Organisation at http://www.wto.org.

The State of Food and Agriculture 2000. This document may be obtained from FAO at http://www.fao.org

US EPA, Attachment--I Final Risk Assessment.1997. Attachment I-final risk assessment of *Aspergillus oryzae. Food Technol. Biotechnol*., 5(4):105-321

Varavinit A., Srithongkum P., De-Eknamkul C., Assavanig A. and Charoensiri K. (1996) Production of single cell protein from cassava starch in air lift fermentor by *Cephalosparium eichhorniae*. *Starch-Starkem*, 48(10): 379–382.

Volkmann, J. 2008. Assessment of Certification Options for Wild Forest Coffee in Ethiopia CoCE Project Report Sub-project. *J.Africa*, 23(4):21-43.

Weitzel, W. and Winchel, M. (1932) The Yeast, its Nutritive and Therapeutic Value. Verlag Rothgiese und Diesing, Berlin

Yabaya, A. and S.A. Ado. 2008. Mycelial protein production by production by *Aspergillus niger* using banana peels. *Science world Journal*, 3(4): 9-12.

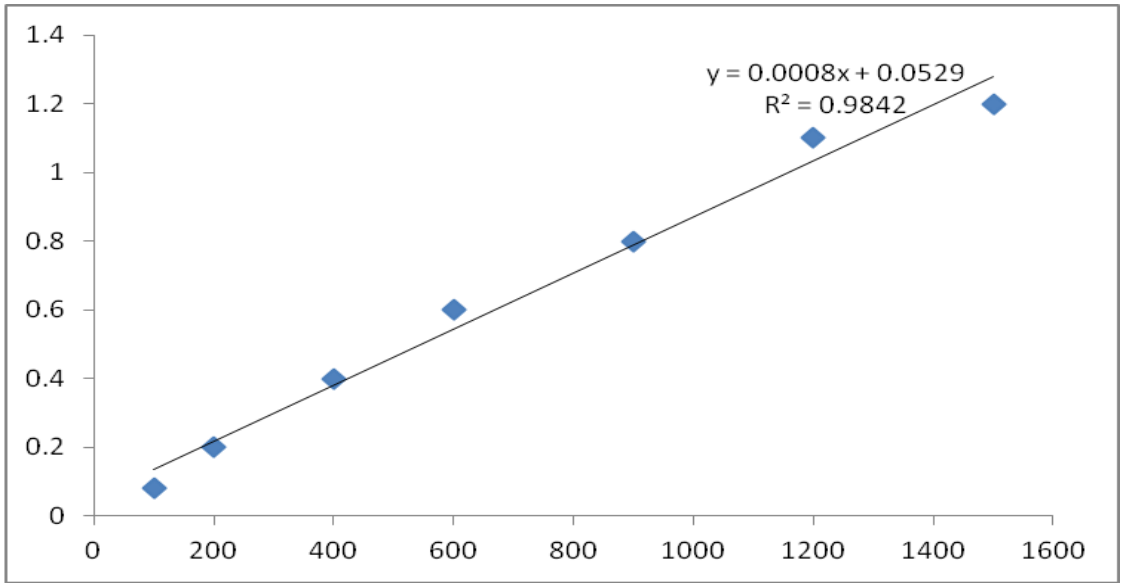
Yared Kassahun, Kebede, Tesfu Kebede, Fassil Assefa and Aklilu Amsalu. 2010. Environmental impact of coffee processing effluent on the ecological integrity of rivers found in Gomma Woreda of Jimma zone, Ethiopia. *Journal of Ecology and Hydrology*, 10(3) 259 - 270.

Yisehak Seboka. 2009. Bio-carbon Opportunities in Eastern and Southern Africa, Charcoal production: Opportunities and barriers for improving efficiency and sustainability. United Nations Development Programme 1 UN Plaza, USA, New York.431.

Yousufi, Mahmood Khan, Khan, Shaukat Saeed and Jha, Krishna. 2003. The Effect of moisture content on the production of single cell protein using *Rhizopus oligosporus* and *Aspergillus oryzae* grown on okara – wheat grit. Combinations. *Indian J. Applied and Pure Bio.,* 18(1): 81-84.

Zee, J.A. and R.E., Simard. 1974."Simple process for the reduction in the nucleic acid content in yeast". *Applied Microbiology*, 29 (1):59-62.

# 7. APPENDIX




Appendex figure1. Protein standard carve by using BSA

Appendix table 1. Two-way ANOVA for protein over substrate and PH.

| Anova | pH 5 | | | | |
|---|---|---|---|---|---|
| Source | df | Mean square | Mean | F. value | p.value |
| Model | 4 | 323 | 37.1 | 8.0 | 0.00 |
| Error | 10 | 40.1 | | | |
| Corrected total | 14 | | | | |
| Anova | pH7 | | | | |
| Source | Df | | | | |
| model | 4 | 330 | 40.1 | 4.9 | 0.01 |
| Error | 10 | 66.9 | | | |
| Corrected total | 14 | | | | |

Appendix table 1 .Two-way ANOVA for protein over substrate and PH

| Anova | pH10 | | | | |
|---|---|---|---|---|---|
| Source | Df | | | | |
| Model | 4 | 349*** | 44.4 | 4.6 | 0.02 |
| Error | 10 | 75.7** | | | |
| Corrected total | 14 | | | | |

*** =Highly significant
* *= Moderately significan

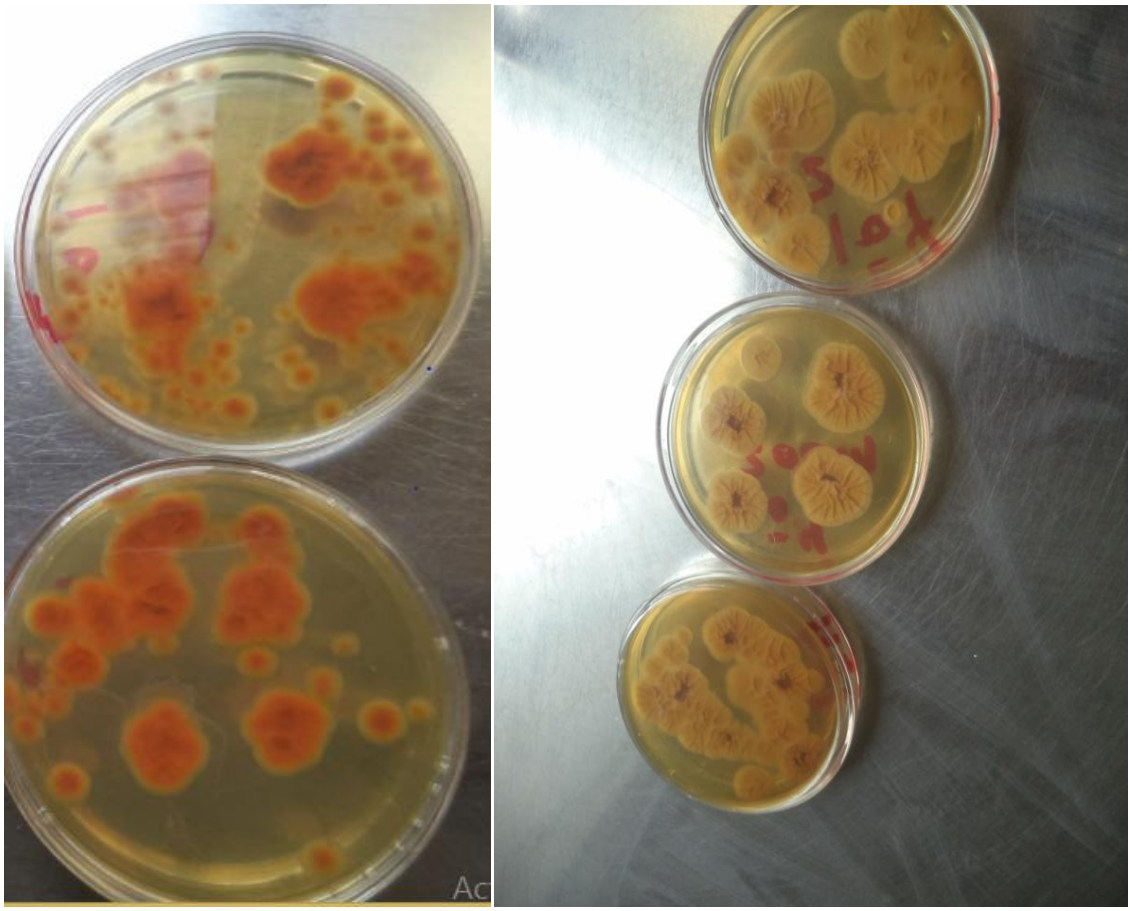
Appendix table 2. Proximate Composition of Aspergillus oryzae biomass.

| Crude protein | | | | | |
|---|---|---|---|---|---|
| source | Df | Mean square | mean | F.value | P.Value |
| model | 4 | 85.1 | 39.3 | 5.7 | >0.01 |
| Error | 10 | 5.6 | | | |
| Corrected total | 17 | | | | |
| Moisture Content | | | | | |
| source | df | Mean square | mean | F.value | P.Value |
| model | 4 | 1.6 | 4.9 | 1.3 | >0.04 |
| Error | 10 | 0.2 | | | |
| Corrected total | 14 | | | | |
| Fat content | | | | | |
| source | df | Mean square | mean | F.value | p.value |
| model | 4 | 13.9 | 5.0 | 3.7 | >0.04 |
| Error | 10 | 3.7 | | | |
| Corrected total | 14 | | | | |
| Total carbohydrate | | | | | |
| source | df | Mean square | mean | F.value | p.value |

| | | | | | |
|---|---|---|---|---|---|
| Model | 4 | 210.9 | 35.3 | 6.5 | >0.00 |
| Error | 10 | 5.6 | | | |
| Corrected total | 14 | | | | |

Ash content

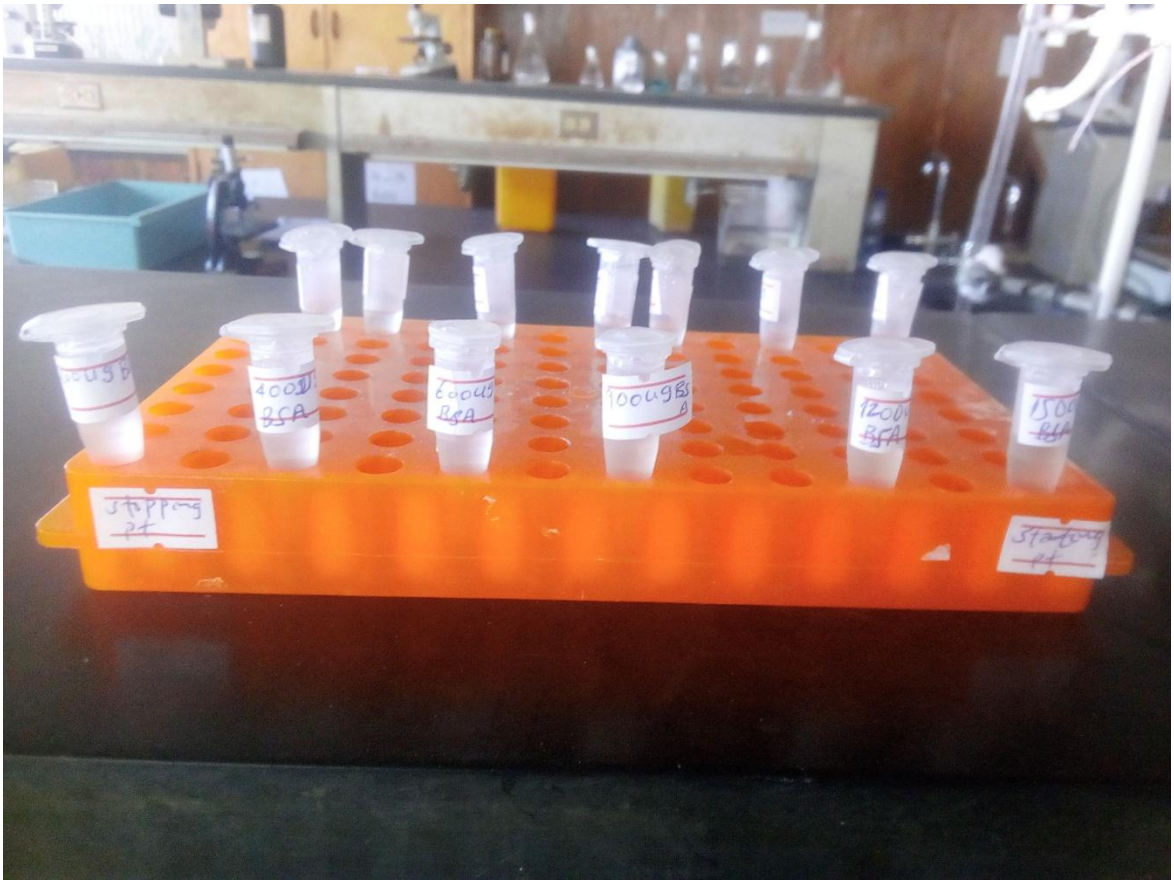
| Source | df | Mean square | mean | F.value | p.value |
|---|---|---|---|---|---|
| Model | 4 | 6.8 | 10.7 | 1.8 | >0.02 |
| Error | 10 | 3.7 | | | |
| Corrected total | 14 | | | | |



Appendix figure 1. Differentiation of *Aspergillus oryzae* by reverse color from *A. flavus* and *A. parasiticus.*

Figure 4. The yellow green color of *Aspergillus oryzae spores.*

Figure 5. The yellow whitish reverse color of *Aspergillus oryzae*.



Appendix figure 6. While I was prepared standard curve by BSA.