PROTEIN ENRICHMENT OF CASSAVA (*MANIHOTESCULENTA*, CRANTZ) PEELSUSING CO-CULTURE OF *TRICHODERMA VIRIDE* AND *SACCHAROMYCES CEREVISIAE* BY SOLID STATE FERMENTATION

BY

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NOVEMBER, 2018

DECLARATION

I hereby declare that this dissertation entitled "**Protein enrichment of cassava (*ManihotesculentaCrantz*)peels using co-culture of *Trichoderma viride* and *Saccharomyces cerevisiae* by solid state fermentation**" was performed by me in the Department of Biochemistry, Ahmadu Bello University Zaria under the supervision of Prof. D.A. Ameh and Prof. S.E Atawodi. The information derived from the literature has been duly acknowledged in the text and a list of references provided. No part of this dissertation was previously presented for another degree or diploma at any university.

.

Stephen Paul EDOR …….. ……

(Name of Student) Signature Date

CERTIFICATION

This dissertation entitled **"PROTEIN ENRICHMENT OF CASSAVA (*MANIHOTESCULENTA*, CRANTZ) PEELSUSING *TRICHODERMA VIRIDE* AND *SACCHAROMYCES CEREVISIAE* CO-CULTURE BY SOLID STATE FERMENTATION"**by Stephen Paul EDOR meets the regulations governing the award of the degree of Master of Science in Biochemistry of the Ahmadu Bello University, and is approved for its contribution to knowledge and literary presentation.

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DEDICATION

This work is dedicated to God my Father and my Lord Jesus Christ in whom are hidden all the treasures of wisdom and knowledge

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ABSTRACT

Microbial processes have been used for the bioconversion of some agro-waste purposely to enhance their nutrient composition and for useful end products formation. Cassava peel is a major agricultural waste in Nigeria, considering the fact that Nigeria is the largest producer of cassava in the world. Solid state fermentation of cassava (*ManihotesculentaCrantz*) peel with emphasis on its protein enrichment using *Trichorderma viride* and *Saccharomyces cerevisiae* co-culture was evaluated in this study. Freshly peeled cassava peels were washed and oven dried to constant weight at 60^{o}C and milled into particle sizes of 1mm, 0.5mm and <0.5mm. The effects of substrate particle size, initial pH, incubation temperature and inoculums size on total protein yield were investigated. The optimum protein yield of 28.10mg/g of cassava peel (CP) was established at 1mm particle size, pH 5.0, inoculum size 3.0x10^{8}spores/ml, incubation temperature of 35^{o}C at day five of fermentation. Fermentation using co-cultures showed a significant ($p < 0.05$) increase in total protein yield (28.10mg/gCP) compared to their respective separate cultures (T. *viride* =12.85mg/gCP and *S. cerevisiae* =17.88mg/gCP$_{)}$crude protein content of unfermented cassava peel was significantly increased from (7.01% to 23.49%, 18.90% and 16.65%) when fermented with co-cultures of *S. cerevisiae* and *T. viride*, *S.cerevisiae* and *T. viride* respectively. Representing a 2.4 fold protein enrichment using the co-cultures of *S. cerevisiae* and *T. viride* as compared to 1.7 fold with *S. cerevisiae* and 1.4 fold with *T. viride* respectively. Fermentation also significantly reduced the antinutritional factors of cassava peel. Cyanidecontent was significantly reduced from 64.80mg/100g to 16.20mg/100g using co-culture, 51.80mg/100g using *S. cerevisiae* and 29.16mg/100gusing *T. viride*. Oxalate was also significantly reduced from 125.40mg/100g to 52.80mg/100g, 41.80mg/100g and 37.40mg/100g using *S. cerevisiae* and *T. viride,* co-culture, *S. cerevisiae* and *T. viride*, respectively. Essential amino acidsof the cassava peel also increased significantly in the fermented cassava peel as compared to the unfermented, with over 25% improvement for histidine, threonine, lysine, methionine and valine respectively. Fermentation with co-cultures of *S. cerevisiae* and *T. viride* significantly increased the nutritional value of cassava peel, making it a suitable means for protein enrichment.

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List of Abbreviations

A.O.A.C	=	Association of Official Analytical Chemists
ANOVA	=	Analysis of Variance
ATP	=	Adenosine Triphosphate
BSM	=	Basal Mineral Salt Medium
CP	=	Cassava Peel
FAO	=	Food and Agriculture Organization
FAOSTAT	=	Food and Agriculture Organization Statistics
HCN	=	Hydrogen Cyanide
IITA	=	International Institute of Tropical Agriculture
ILRI	=	International Livestock Research Institute
PDA	=	Potatoes Dextrose Agar
SCP	=	Single Cell Protein
SmF	=	Submerged Fermentation
SSF	=	Solid State Fermentation
TSM	=	Technicon Sequential Multi-Sample Amino Acid Analyzer
YPD	=	Yeast Peptone Dextrose

CHAPTER ONE

1.0 INTRODUCTION

1.1 Background of Study

Cassava (*ManihotesculentaCrantz*) is a perennial woody shrub, grown annually for its starchy roots. It is a cheap source of carbohydrates for human populations in the humid tropics (Onwueme, 2002). It is an important root crop in Africa, Asia, and South America, providing energy for about 500 million people. Cassava and its products are widely in use in the tropics to feed farm animals and for energy (Okpako*et al.,* 2008). Nigeria has consistently been ranked as the largest producer of cassava in the world since 2005 (FAO, 2012) followed by Brazil, Thailand and Indonesia with about 45 million metric tonnes grown annually. Cassava is grown throughout the tropics and could be regarded as the most important root crop, in terms of area of cultivation and total production (Ano, 2003). Cassava is strategically valued for its role in food security, poverty alleviation and as a source of raw materials for agro-allied industries in Nigeria with huge potential for exportation (Egesi*et al.,* 2007).Benue is the top region by production of cassava in Nigeria. As of 2005, productionof cassava in Benue was 3,548 thousand metric tones that accounted for 11.08% of Nigeria's production of cassava. The top five regions (others are Taraba, Kogi, Imo, and Cross River) account for 44.05% of it. Nigeria's total production estimated at 32,015.4 thousand metric tons on 2005 (Knoema, 2014).

In the processing of cassava, the roots are normally peeled to remove the two outer coverings, i.e. a thin brown outer covering and a thicker leathery parenchymatous inner covering. The peels constitute about 15-20% of the weight of the tuber (Obadina*et al.,* 2006). These peels are often regarded as waste and are usually discarded and allowed to rot. Vegetation and soil around the

heaps of the peels are rendered useless and devastated due to biological and chemical reactions that take place between the continuously fermenting peels, soil and the surrounding vegetation (Olanbiwoninu and Odunfa 2012). However, the peels have potentials for conversion into useful products of higher value or even as raw material for industries and they can be used as food or feed after biological treatment (Ajao*et al.,* 2009).In a bid to transform these agro wastes into value added products like single cell protein (SCP) mostly by solid state or submerged fermentation process; little attention has been given to co-culturing (Asmamaw and Fassil, 2014).

As the global human population increases, the world is facing tremendous pressure to meet its food, feed, chemicals, and energy needs; and also to balance the demand and supply in keeping with environmental safeguards (Rasul, 2016). Based on the population increase, conventional agriculture may well be unable to supply sufficient food (protein in particular) to satisfy such demands (Asmamaw and Fassil, 2014). The Food and Agriculture Organization (FAO) predicts a widening of the protein gap between developed and developing countries, this challenge becomes more daunting due to the changing climates, infertile soils and soil contamination through oil spillage and improper waste disposal (Smith, 2000). One possible solution to this problem is the productionof single cell proteins (SCP) which can be used in human food and animal feed (Adedayo*et al*., 2011). Single cell proteins, which are proteins obtained from microbial source is one of the alternatives that cannot be affected by climate change(Smith, 2000). Single cell protein has high content of protein containing all the essential amino acids (Asmamaw and Fassil, 2014).Microorganisms are an excellent source of SCP because of their rapid growth rate, their ability to use inexpensive raw materials as carbon source, and the

uniquely high efficiency, expressed as grams of protein produced per Kilogram of raw material, with which they transform these carbon sources to protein (Glazer and Nikaido, 2007).

Modernization has opened up an access to the efficient utilization of agro-industrial by-products, aiming at obtaining value added products like biofuels, biochemicals and biomaterials such as bio plastics. Bioprocessing of agro-wastes can help solve environmental problems associated with the disposal of these materials (Lin *et al.,* 2011).

One of the ways to appropriately utilize this waste is protein enrichment using Solid State Fermentation (SSF) technique to add value to it. Solid-state fermentation is defined as the fermentation involving solids in absence (or near absence) of free water; however, substrate must possess enough moisture to support growth and metabolism of microorganisms (Pandey *et al.,* 2001). Solid state fermentation offers numerous opportunities in processing of agro-wastes. This is partly because solid-state processes have lower energy requirements, produce lesser wastewater and are environmental-friendly as they resolve the problem of solid wastes disposal (Pandey, 2003).

Asmamaw and Fassil, (2014) reported that, fermentation using co-culture rather than monoculture of fungi increase biomass productivity by either efficient utilization of substrate or removal of inhibitory byproducts; it reduces the cost of enzymes and chemicals required for treatment of lignocellulosics agricultural wastes (Azmi*et al.,* 2009). The use of co-cultures could therefore be seen as a promising technology for improving fermentation with cellulolysic substrates for SCP production in combating the global challenge of food protein shortage; sinceagriculture alone cannot meet up with the high demands of protein required worldwide (Ukaegbu-Obi, 2016).

1.2 Statement of Research Problem

Cassava peel is a major waste in Nigeria, considering the fact that Nigeria is the largest producer of cassava in the world (FAO, 2012). The utilization of cassava peel is limited by its low digestibility and toxicity from extremely high levels of hydrocyanic acid (HCN) (Ofuya and Obilor, 1993).

The industrial and local processing of cassava as food has led to enormous generation of waste that are dumped in dumpsites and drainages instead of transforming them to value added products (Olabinduiwu and Odunfa, 2012). For example, about 2.96 million metric tons of cassava peels are generated and discarded in Nigeria from about 10 million metric tons of cassava processed for "Garri" alone (Aro*et al.*, 2010).Ninety eight percent (98%) of Nigeria's cassava peels annually are wasted due to constraints associated with drying and concerns about safety of use, particularly hydrogen cyanide and mycotoxins-related food poisoning.Consequently, peels are left to rot in heaps or set on fire-both polluting the nearby air, soil and groundwater and wasting a potential feed resource (ILRI, 2016).

1.3 Research Justification

The conversion of agro-waste into useful product by fermentation using monocultures of fungi and bacteria that synthesize hydrolytic enzymes that can degrade this biomass into useful products, such as ethanol, reducing sugars and other biochemicalshas been reported(Ezejiofor*et al.*, 2014).The production rates, and cost of these useful products could be further enhanced by using co-cultures withof two or more fungi.

Utilization of cassava peel waste into value added products like protein may not only solve the environmental challenge it currently poses, but it may also improve our nation's economy by providing food security. Due to the global shortage of food and the rate of population increase; there is a need to research into other alternative sources of protein production by cultivation of microbial biomass using renewable substrates which occur abundantly in nature such as cassava peels.

1.4 Aim and Objectives

1.4.1 Aim

The aim of this study was to carry out protein enrichment of cassava peel through Solid State fermentation using *Trichoderma viride* and *Saccharomyces cerevisiae* co-culture for inclusion in animal feed.

1.4.2. Specificobjectives

The specific objectives are to:

1. Produce protein enriched cassava peels by SSFusing co-culture of *Trichoderma viride* and *Saccharomyces cerevisiae* using different particle sizes
2. Study the effect oftemperature, pH, inoculum size, and fermentation periodon the protein enrichment of cassava peel.
3. Study the effect of fermentation on proximate composition and the antinutritional componentsof cassava peels
4. Study the effect of fermentation on amino acids profile of the protein enriched cassava peel.

1.5Null Hypothesis

The use of *Trichoderma viride* and *Saccharomyces cerevisiae* co-culture does not lead to protein enrichment of cassava peel using solid state fermentation.

CHAPTER TWO

2.0 LITERATURE REVIEW

2.1 Global Population Growth Challenge

According to the United Nations population report, the world population reached 7.3 billion by mid-2015 (Table 2.1), implying that the world has added approximately one billion people in the span of the last twelve years, between 2003 and 2015. Sixty percent of the global population lives in Asia (4.4 billion), 16 percent in Africa (1.2 billion), 10 percent in Europe (738 million), 9 percent in Latin America and the Caribbean (634 million), and the remaining 5 percent in Northern America (358 million) and Oceania (39 million). China (1.4 billion) and India (1.3 billion) remain the two largest countries of the world, both with more than 1 billion people, representing 19 and 18 per cent of the world's population, respectively (Nation, 2015).

More than half of global population growth between now and 2050 is expected to occur in Africa. Africa has the highest rate of population growth among major areas, growing at a pace of 2.55 per cent annually in 2010-2015. Consequently, of the additional 2.4 billion people projected to be added to the global population between 2015 and 2050, 1.3 billion will be added in Africa.

Among the ten largest population countries in the world, one is in Africa (Nigeria), five are in Asia (Bangladesh, China, India, Indonesia, and Pakistan), two are in Latin America (Brazil and Mexico), one is in Northern America (United States of America), and one is in Europe (Russian Federation). Amongst these, Nigeria's population, currently the seventh largest in the world, is growing at the most rapid rate. Consequently, the population of Nigeria is projected to surpass that of the United States by 2050, at which point it would become the third largest country in the world.

Table 2.1Population of the World and Major Areas, 2015, 2030, 2050 and 2100, According to the Medium-Variant Projection

Major areas	**Population in millions**			
	2015	**2030**	**2050**	**2100**
World	7 349	8 501	9 725	11 213
Africa	1 186	1 679	2 478	4 387
Asia	4 393	4 923	5 267	4 889
Europe	738	734	707	646
Latin America and the Caribbean	634	721	784	721
North America	358	396	433	500
Oceania	39	47	57	71

Source: Nation, 2015

2.2 Food Security

Food security exists when all people, at all times, have physical and economic access to sufficient, safe and nutritious food to meet their dietary needs and food preferences for an active and healthy life (FAO, 2013**).** For everybody to get enough healthy food, we need a food system that works well. The food system is made up of five parts (production, distribution, access, consumption and disposal), these parts cover everything from making food (production) to taking care of the waste (disposal). Each part of the food system is connected, and when there are problems in one part of the food system, it can affect the other parts of the system. This can cause changes in the cost of food, how fresh our food is, or how much food is in our stores (Lang and Heasman, 2015).

A food-secure world is one where all people have access to safe, nutritious and affordable food that provides the foundation for active and healthy lives. Food security affects more than human health and welfare – it also contributes to economic and political stability. Food security is a complex problem given interconnections and interdependencies in a global food system that is fundamentally dependent on soil, precipitation and water availability, climate and a host of services the earth provides and, at the same time, influenced significantly by trade, urbanization, changing demographics, and energy, water and land use policy (West *et al.*, 2014).The convergence of several factors magnifies the challenge of achieving food security, persistent poverty and undernourishment, expected population growth, income growth and changing consumption patterns, the required boost in agricultural production, climate change, and water scarcity.

The United Nations estimates that about 842 million people – approximately one in eight – are undernourished today, majority live in developing countries, where more than 14 percent of people are unable to meet their daily dietary energy requirements(Kakwani and Son, 2016). The highest prevalence of undernourishment is in sub-Saharan Africa; undernourishment also remains a significant challenge in western Asia. Globally, the total number of undernourished people has fallen 17 percent since 1990. Progress has been made in southern Asia, northern Africa and most countries of eastern and southeastern Asia, as well as in Latin America (Nations, 2015).

Globally, there has been a challenge of food and nutrition adequacy mostly caused by climate change, sharp rise in food demand to falling supply, neglect of investment in agriculture, high cost of production due to the need to buy agrochemicals. These factors have serious implications on rising insecurity and conflict induced instability in many countries, Nigeria inclusive (Etim*et al.*, 2017). Prior to the advent of colonial rule, in 1900 and the discovery of crude oil in the 70s, Nigeria's economy relied majorly on agriculture and was doing well based on exports across the borders of the country (Duke *et al.*, 2017).Nigeria's agricultural productivity experienced a diminishing return since the discovery of crude oil as major attention were directed towards oil exploration and its revenue generation. Today Nigeria is listed among the major importer of finished agricultural products, we currently remain a net importing nation, spending about two (2) billion dollar on importing food items annually (Amaechi, 2016). The central bank of Nigeria posted that Nigeria is yet to reach self-reliance and sufficiency in productivity (CBN, 2015). Food demands have outgrowntotal supply, which have attracted global attention (Abu and Soom, 2016).

Population growth makes the challenge of feeding everyone much more difficult. The Food and Agriculture Organization (FAO) of the United Nations estimates the world's population will reach 9.6 billion people by 2050 and 10.9 billion by 2100 (FAO, 2013). Much of this increase is projected to come from developing countries, where populations will rise 39 percent by 2050 and an additional 17 percent by 2100. The most dramatic growth is likely to occur in the least developed countries of the world. Feeding this growing global population in the years to come will require producing more food and distributing it in a manner that reaches more people (Godfray*et al.*, 2010).Therefore the need to boost global food production will be necessary to meet the growing demand. Hence, the need to seek alternative means of utilizing the little we have produced is imperative. We have a lot of food materials that are discarded as wastes and some as by-products of food processing; cassava peel is a typical example of this. Adding value to these foods and agricultural by-products to further utilize them as animal feeds could contribute significantly in reducing the food security challenge (Parfitt*et al.*, 2010).

2.3 CassavaPlant

Cassava (*ManihotesculentaCrantz*) is a dicotyledonous plant, belonging to the family Euphorbiaceous (Alves, 2002). It is a perennial shrub, 2 to 4 m in height and is mainly propagated from stem cuttings. Cassava is the world's seventh most important crop in terms of production and provides the staple food for more than 500 million people in the humid tropics (Beeching, 2013). It is widely grown in most countries in the tropical regions of Africa, Latin America and Asia (Nassar and Ortiz, 2007).

The crop's ability to thrive in adverse environmental conditions and its high efficiency at incorporating carbon dioxide into carbohydrates compared with other crops, makes cassava to

play a vital role in a planet faced with the interrelated challenges of population growth, food scarcity, and climate change (Beeching, 2013).Cassava is an important source of carbohydrate for humans and animals, having higher energy (610 kJ/100 g fresh weight) than other root crops. Dried cassava root has energy similar to the cereals (FAO, 1990). In Africa, the continent with the largest cassava production, about 93% of the produce is used as food (Nweke*et al.,* 2002).



Figure 2. 1: CassavaRoot Tuber

2.3.1 The origin and taxonomy of cassava

Cassava originated fromBrazil and Paraguay. Earlysites and farms of cassava existed in Northern Brazil and Central America but its greatest staple status is in tropical Africa (Leon, 1977; Wheatley *et al.,* 1993). It is from these sites that cassava was introduced into other parts of the World including Africa, during the early trade movements and explorations by the Portuguese. Cassava was introduced into Nigeria by the Portuguese traders and explorers from Fernando-Po

to Warri in the then Mid-Western Nigeria in the late 18th Century (Njoku and Muoneke, 2008). It later spread to Lagos, Badagry, Abeokuta and Ijebu in the early 19th Century by slaves returning from West Indies and Sierre Leone who settled in these towns (Agboola, 1979). These returnees processed cassava into 'gari', 'lafun' and 'iwa-panya' (roast and eat) for food. Cassava and cassava products were later introduced into Eastern Nigeria along the coastal towns of Calabar and Yenagoa by traders from Western Nigeria. Thus, cassava may have been introduced in Nigeria to different regions about three hundred and thirty years ago (Agboola, 1979).

The importance of cassava cannot be overemphasized, althoughthe site of its origin is far away from Africa and Nigeria in particular, its highest staple utility rests in Africa. It is a basic staple food to more than 70% of Nigerian population and it is consumed at least once every day (Njoku and Muoneke, 2008). Over 90% of cassava produced in Nigeria is used locally for food, animal feed, industrial and pharmaceutical uses and unspecified quantities for export. As human food, cassava is processed into about fifty food forms - gari, lafun, bread, flakes, flour etc(Nassarand Ortiz, 2007).

An estimate of 50million tonnes of cassava peel waste are generated annuallyin Africa (ILRI, 2015). While the processing of cassava in Nigeria generates about 14million tonnesof by products, comprising of peels, stumps, woody and undersized tubers currently disposed offas wastes (Okike*et al.,* 2015). Africa's 50 million tonnes of cassava peel waste after value addition could generate at least 15 million tonnes of High Quality Cassava Peel (HQCP), which could substantially address the shortfalls in the supply of animal feed, eliminate environmental pollution and has the potential to generate $2billion a year industry in the continent (ILRI, 2015).

2.3.2 Importance of cassava as a staple food

Cassava, also called “manioc”, “yucca”, or “tapioca”, is a major staple root crop in many tropical and subtropical developing countries, especially West Africa.



Figure 2. 2Cassava Peels Accumulated in an Unofficial Dump.

Source: ILRI, 2015.

It is grown in more than ninetycountries; it is ranked as the 6th most important source of energy in human diets globally and the 4th supplier of energy after rice, sugar and corn/maize (Heuberger, 2005).As a crop whose by-products have a wide array of uses, cassava is the most important food crop in Nigeria by production quantity next to yam, which is the most important

food crop by value (FAOSTAT, 2012). Though Cassava is an important staple food, it contains toxic antinutritional substances that interfere with its digestion and nutrients uptake (Wobeto*et al.*, 2007).

Staple food products such as cassava products, rice and yam account for larger shares of the total food budget in low income countries. In Nigeria, cassava can be processed into different forms usable by man. IITA (2002) highlighted the common forms of cassava products available in Nigeria. These include gari, fufu, cassava chips, cassava flour, starch, farina, tapioca, macaroni, cassava bread and pudding (Echebiri and Edaba, 2008).

2.3.3 Toxicity of cassava

Cassava like other foods contains antinutritional components, which are of particular concern, particularly the Cyanogenic glycosides (linamarin and lotaustralin). When hydrolysed, they release hydrocyanic acid (HCN). The presence of cyanide in cassava is of concern for both human and animal consumption (Zealand, 2005). Linamarin is the major Cyanogenic glucoside found in cassava, during processing, linamerase comes in contact with linamarin and catalysesits hydrolysis to glucose and acetone cyanohydrin, which are subsequently degraded to acetone and cyanide (Ikpesu*et al.*, 2016).

Although cassava possesses toxic materials, different processing methods exist to remove cyanogens and their effectiveness depends on the processing steps and the sequence utilized. Crushing, draining and sun drying cassava roots made into flour removes 96% to 99% of total cyanogen, whereas soaking and sun drying or soaking and fermentation removes about 98% of cyanogens (Montagnac*et al.*, 2009).

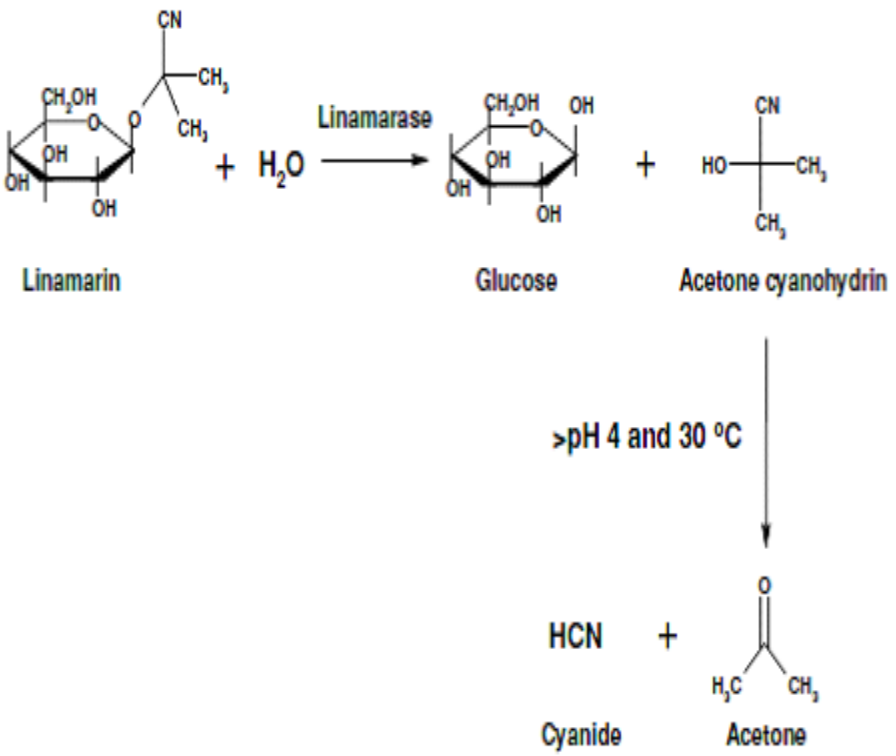


Figure 2. 3: CyanogenSynthesis in Cassava.

Fermentation and oven-drying are efficient processing methods to remove cyanide and other anti-nutrients; since cassava is a nutritionally strategic famine crop in developing countries, reducing its toxicity and improving its nutritional value is crucial and fermentation is the most suitable method to achieve this goal (McMahon *et al.*, 1995). Fermentation of food products is an effective method of improving the starch and protein digestibility and reduces the level of anti-nutrients present (Ikpesu*et al.*, 2015).

2.4 Fermentation

Fermentation is a process that is important in anaerobic conditions where there is no oxidative phosphorylation to maintain the production of ATP (Adenosine triphosphate) by glycolysis. During fermentation pyruvate is metabolised to various different compounds. Homolactic fermentation is the production of lactic acid from pyruvate; alcoholic fermentation is the conversion of pyruvate into ethanol and carbon dioxide; and heterolactic fermentation is the production of lactic acid as well as other acids and alcohols. Fermentation has become an essential technology that is necessary for converting lignocellulosic materials such as cassava peel into value added products with potential for providing solution to the challenge of animal nutrition such as inadequate protein intake (Nasseri*et al.*, 2011).

Fermentation is an important process in the production and processing of most cassava based products in Africa, though limited number of techniques are being used. Fermentation has been reported to be responsible for product stability, flavor development and cyanide elimination (Motarjemi, 2002). It improves the nutritional content of food through biosynthesis of vitamins, essential amino acids and protein by enhancing protein quality and fibre digestibility. It also improves micronutrients bioavailability and helps in degrading antinutritional factors in food of plant source (Achinewhu*et al.*, 1998).

Fermentation is broadly classified into two major types, namely; solid state fermentation (SSF) and submerged fermentation (SmF). Both types differ considerably in substrate type, microorganisms and technology employed (Subramaniyam and Vimala, 2012). Solid state fermentation utilizes solid substrate, lacking in free flowing liquid but sufficiently moistened to support the growth of microorganisms. Usually, cellulosic wastes such as bagasse, bran and

paper pulp are used for the production of target compounds (Pandey, 2003). Submerged fermentation however utilizes free flowing liquid substrate for the cultivation of microorganisms, particularly those requiring high water activities. Usually, industrial liquid wastes such as molasses, waste water and broths are used for the growth and production of target product (Subramaniyam and Vimala, 2012).

2.4.1 Submerged andsolid state fermentation

Technologies of both submerged and solid state fermentation had advanced at different pace. Until lately, submerged fermentation was favoured over solid state fermentation largely owing to technical advancement in the area of submerged fermentation relative to solid state fermentation. Availability of cheaper cellulose rich agro-waste and industrial bye products has however attracted attention to the method of solid state fermentation. Hence, increasing advancement in the field has been reported (Hölkerand Lenz, 2005). More so, solid state fermentation holds a few advantages over submerge fermentation and as well addresses some challenges of the submerged fermentation system like large effluent volume (Pandey *et al.*, 1999). In addition, solid state fermentation simulates the condition of natural habitat for fungal growth which has promoted the use of the method for the production of large quantity of industrially important enzymes like cellulase (Pandey *et al.*, 1999). Solid state fermentation processing poses the greatest socio-economic potential by raising the standards through the production of protein enriched foods and feeds for both humans and animals (Raimbault, 1998).

2.4.2 Microbial co-culture fermentation in industrial biotechnology

Microbial co-culture occurs when two or more known microbial strains combine their metabolic activity for the degradation and metabolism of substrates under aseptic conditions. Mixed

cultivations are often found in nature under septic conditions with unspecified microbial strains (Bader *et al.*, 2010). In a habitat, different microorganisms may compete for substrates as well as act symbiotically. Microorganisms have evolved mechanisms to protect their substrates and to defend their habitat against competitors.

Though the natural cooperation of different microorganisms are utilized in only a few applications in industrial biotechnology, in most cases, pure cultures are utilized for formation of the desired product(s), and the undesired microorganisms are eliminated by sterile working conditions; co-culture fermentations may result in increased yield, improved control of product qualities and the possibility of utilizing cheaper substrates. Co-cultivation of different micro-organisms may also help to identify and develop new biotechnological substances (Bader *et al.*, 2 010).Other advantages of co-culture cultivation are the possibilities of utilizing secondary products (e.g. whey, molasses) cheaper than glucose as substrates for biotechnological production of chemicals. Using substrates other than glucose offers potential to develop biological production processes at competitive costs.

Furthermore, co-cultivation processes can help find new substances of industrial interest, because a number of secondary metabolites are produced during co-cultivation (Oh *et al.*, 2007).Besides having the industrial importance, co-culture systems have medical implications as well. Penttinen*et al.*, (2005) reported that there was a significant increased induction of apoptosis in mammalian cells by spores produced in a co-culture of *Streptomyces californicus* with *Stachybotryschartarum* in comparison with the spores of *Streptomyces californicus* grown in pure culture.Co-cultivation may result in increased yields, a reduction of process costs because of cheaper (sometimes even unsterile) substrates and control of product quality (Kleerebezem

and Van Loosdrecht 2007). In some cases, production of substances normally not formed by pure cultures can be observed through the induction of appropriate genes in co-cultivation processes. Co-culture and mixed culture fermentation may have a great impact on the development of biofuels, bioenergy and bio-based products. Hence, they can be utilized in the production of foods, food additives, pharmaceuticals, enzymes, bulk and fine chemicals, bioremediation and degradation of lignocelluloses. They avail the opportunity to use cheap substrates, increase yields and product quality (Goers *et al.*, 2014).

2.5: Fermenting Organisms used for Agro-waste Bioconversion

Microorganisms are excellent tools used to convert most lignocellulosic agro-wastes such as such as cassava peel, rice husk, sugarcane bagasse, orange peel, etc. into valuable products. Although these lignocellulosic materials are harsh to ferment, due to the lignin degradation products as well as the presence of acids. The mycelial organisms are capable of producing a range of extracellular enzymes required for the hydrolysis of complex, polymeric solid substrates listed above. These mycelia organism include a large number of filamentous fungi and a few bacteria of actinomycetes (Dashtban*et al.*, 2009).

2.5.1*Trichoderma viride*

Trichoderma species are efficient producers of many extracellular enzymes; hence, they are used for the production of cellulase and other enzymes in commercial quantity. These enzymes degrade complex polysaccharides, which are used in food and textile industries for these purposes (Harman, 2006).*Trichoderma viride* is sometimes regarded as a microorganism which is capable of breaking down cellulose and has the ability to synthesize some essential factors to dissolve the cellulose that is tied up by hydrogen bonds. *Trichoderma viride* is a fungus, with the

following taxonomy: kingdom: fungi, phylum: Ascomycota, class: Sordariomycetes, order: Hypocreales, family: Hypocreaceae, genus: Trichoderma and species: Trichoderma viride. They produces cellulase in large amounts which have capacity to degrade cellulose (Mandel and Higa, 1970). The benefit of the fungus is its ability to produce cellulosic enzymes which was required to hydrolyze crystalline cellulose and can produce fairly high protein biomass (Volk*et al.,* 2004).

2.5.2 *Saccharomyces cerevisiae*

Saccharomyces is a genusof fungithat includes many species of yeasts. The taxonomy are: kingdom: fungi, phylum: Asomycota, class: Saccharomycotina, order: Saccharomycetales, family: Saccharomycetaceae, genus: Saccharomyces, species: *Saccharomyces cerevisae*. Many members of this genus are considered very important in food production. It is known as the brewer's yeast or baker's yeast. They are unicellular and saprophytic fungi. Cerevisiae is used in making wine, bread, beer, and for human and animal health (Spellman *et al..,* 1998). *Saccharomyces cerevisiaeis* a mold which is often used to feed bioconversion *process.* This Utilization of *Saccharomyces cerevisiae* is based on the ability of the microbes to produce vitamins, enzymes, and nutrients (Kamarudin*et al..,* 2013). They also produce glutamic acid that can improve feed palatability, assimilate and secrete proteins and essential amino acids and contains vitamin B complex (Fardiaz, 1992).

2.6 Protein

Proteins are biological macromolecules made up of amino acids as their building blocks, and are found in cells and parts of cells. All proteins whether from the most ancient lines of bacteria or from the most complex forms of life, are constructed from the 20 amino acids (Nelson *et al.,* 2005).

The diversity of the thousands of proteins found in nature arises from the commonly occurring 20 amino acids. Proteins are polymers of amino acids, with each amino acid residue joined to its neighbour by a specific type of covalent bond. There are over 300 naturally occurring amino acids in nature, 20 constitute the monomer units of proteins,from these building blocks different organisms can make such a widely diverse products such as enzymes, hormones, antibodies, transporters, muscle fibers, the lens of the eye, feathers, spider webs, rhinoceros horn, milk proteins, antibiotics and mushroom poison and other substances having distinct biological activities (Nelson *et al.*, 2005).

Proteins are a diverse and abundant class of biomolecules, constituting more than 50% of the dry weight of cells. This diversity and abundance reflect the central role of proteins in virtually all aspects of cell structure and function. Biologically occurring polypeptides range in size from small to very large, consisting of two or three to thousands of linked amino acid residues. All 20 amino acids (Table 2.2) are biologically important. Humans can synthesize 12 (nutritionally nonessential) of the 20 common amino acids from the amphibolic intermediates of glycolysis and of the citric acid cycle. Of the 12 nutritionally nonessential amino acids, nine are formed from amphibolic intermediates and three (cysteine, tyrosine and hydroxylsine) from nutritionally essential amino acids (Nelson *et al.*, 2005).

Essential amino acids are "essential" not because they are more important to life than the others, but because the body does not synthesize them. They must be present in the diet or they will not be present in the body.

Table 2. 2List of Essential and Nonessential Amino acids

	Essential	**Non-essential**
1	Histidine	Alanine
2	Isoleucine	Arginine
3	Leucine	Aspartic acid
4	Lysine	Cysteine
5	Methionine	Glutamic acid
6	Phenylalanine	Glutamine
7	Threonine	Glycine
8	Tryptophan	Proline
9	Valine	Serine
10		Tyrosine
11		Asparagine
12		Slenocysteine
13		Pyrrolysine

Source: Adapted from Nelson *et al.*, (2005).

2.5.1 Single cell proteins

Single cell proteins (SCP) are proteins derived from a culture of single-celled organisms or unicellular microorganisms, used especially as a food supplement. The biomass or protein extract from single or mixed cultures of algae, yeasts, fungi or bacteria are being used as an ingredient or a substitute for protein-rich foods, and is suitable for human consumption or as well as animal feeds (Goldberg, 2013). They are the dried cells of microorganisms (such as yeast) that could be grown in large-scale culture systems for use as protein for human or animal consumption. Microorganisms have been used for the synthesis of high protein products like cheese and fermented soybean products. They have natural ability to convert low protein organic mass into high protein organic products by various processes. This ability of microorganisms is being used to fight the problems of malnutrition and poor protein diet in third world countries, and to manufacture protein-rich food for animals (Friedman, 1996).

2.5.2 Trends of single cell protein production

Single cell protein is a term coined in the 1960´s to embrace microbial biomassproducts which were produced by fermentation. Its production technologies arose as apromising way to solve the problem of worldwide protein shortage (Suman*et al.*, 2015). They evolved as bioconversion processes which turned low value by-products, often wastes, into products with added nutritional and market value. Intensive research into fermentation science and technology for biomass production, as well as feeding, has resulted in a profound body ofknowledge, the benefits of which now span far beyond the field of SCP production.

The widespread application of plant breeding programmes and agricultural crop productiontechniques resulted in a high availability of plant food sources, such as soya, maize, wheat and rice in the second half of the 20th century. However, the combination of sophisticated production with food processing technology yielded a new generation of SCP products which may be used as meat substitutes, texture providing agents and flavour enhancers (Suman*et al.,* 2015).Future application of heterologous protein expression may further develop the potential of this food line, resulting in precisely tailored products which will meet specific dietary requirements, or simulate high added value specialty products.Single cell protein productions from agricultural wastes possess attractive features as a nutrient supplement for both human and animals feed. Single cell protein has various benefits over animal and plant proteins since its requirement for growth are neither seasonal nor climate dependent hence, it can be produced all-round the year(Ukaegbu-Obi, 2016).

Several investigations were carried out using cellulose and hemicellulose waste as a suitable substrate for increasing single cell protein production. Many raw materials have been considered as substrate (carbon and energy sources) for SCP production (Nasseri*et al.,* 2011). Further in many cases, these raw materials have been hydrolyzed by physical, chemical and enzymatic methods before use. Various hydrocarbon, nitrogenous compounds, polysaccharides and agricultural wastes such as hemicelluloses and cellulose waste (Azzam, 1992; Zubi, 2005) from plants and fibrous proteins such as horn, feather, nail, and hair from animals have thus been used for the production of SCP (Pandey *et al.*, 2000). These waste products have been converted to biomass, protein concentrate or amino acids using proteases derived from many microorganisms and are rich in some growth factors required by microorganisms.

Various forms of organic waste such as celluloses, hemicelluloses, hydrocarbon and different types of agricultural wastes were used in the production of SCP (Adedayo*et al.*, 2011**)**.The degree of SCP production depends on the type of substrate used and also on media composition (Mondal, 2006). The highest amount of protein (690 mg) was obtained from *Saccharomyces cerevisiae* when grown on beetroot extract supplemented with basal media, and the highest biomass yield per gram of substrate (16%) was obtained from *Aspergillusniger*when grown on banana extract supplemented with basal media. For *Saccharomyces cerevisiae*,cucumber waste has been proved better substrate followed by orange peels. *Aspergillusterreus*possesses a high protein value and has been used as a better choice for SCP production using cheap energy sources like eichorniaand banana peel (Jaganmohan*et al.*, 2013). The cladodes of *Opuntiaficus-indica*(cactus pear) were one such lignocellulosic raw material that has potential for production of SCP in arid and semi-arid regions (Gabriel *et al.*, 2014).

CHAPTER THREE

3.0 MATERIALS AND METHODS

3.1 Materials

3.1.1 Chemicals and reagents

All chemicals were of analytical grade purchased from either British drug House (BDH Chemicals Ltd, Poole, England) or Hopkins and Williams (Essex, England).

3.1.2 Sample collection

Cassava sample was obtained from Kazzabed Farms, Idrisu-Ajonailo, Omala LGA of Kogi State and authenticated in the Department of Biological Sciences, Ahmadu Bello University, Zaria, Nigeria, the sample was assigned with a voucher specimen number 2347.

3.1.3 Microorganisms

Trichoderma viride and *Saccharomyces cerevisiae*(both wild type) wereobtained from the Department of Microbiology, Ahmadu Bello University, Zaria.These strains were cultured and maintained on potato dextrose agar (PDA) and YPG slants, supplemented with chloramphenicol (250mg) to prevent bacterial growth and stored at 4^{o}C for further use.

3.2 Methods

3.2.1Cassava peels preparation

The Cassava tubers were washed and peeled. They peels were dried immediately in an oven at 60^{o}C to constant weight. The sampleswere milled to particle sizesof 1mm, 0.5mm and below 0.5mm, then stored in an air tight container.

3.2.2 Inoculapreparations**:**

3.2.2.1 Preparation of spore inoculum of Trichodermaviride

Inoculum suspensions were prepared from fully sporulated fungi (5 -7 day old) cultures grown onPotatoe Dextrose Agar plates (Ezekiel and Aworh, 2013). The colonies were covered with 10ml of sterile distilled water containing 2.5% Tween 80 (polyoxyethlenesorbitanmonooleate), to facilitate the uniform distribution of *Trichoderma viride*spores (Petrikkou*et al.*, 2001). The spores were dislodged by aseptic gentle shaking for 15 minutes and transferred to a sterile test-tube (Sabo *et al.*, 2005).

3.2.2.2: Preparation of Yeast cells of Saccharomyces cerevisiae

Saccharomyces cerevisiae were grown in Yeast Peptone Dextrose (YPD) broth (1% yeast extract, 2% peptone and 2% dextrose) containing 0.05mg/ml of chloramphenicol. Ten milliliter of the YPD medium was inoculated with a single selected colony of the desired yeast strains using a wire loop and then incubated overnight at 30^0C with an agitation rate of 300rpm. After incubation, then 1 ml of the yeast cell culture was transferred into 200ml YPD medium. The yeast cells were allowed to grow at 30^0 C for 4 hours on a shaker. The cells were harvested by centrifugation at 3000rpm for 5 minutes. The pellets formed in the centrifuge was re-suspended in 30ml of sterile distilled water (Byaruagaba-Bazirake*et al.*, 2013).

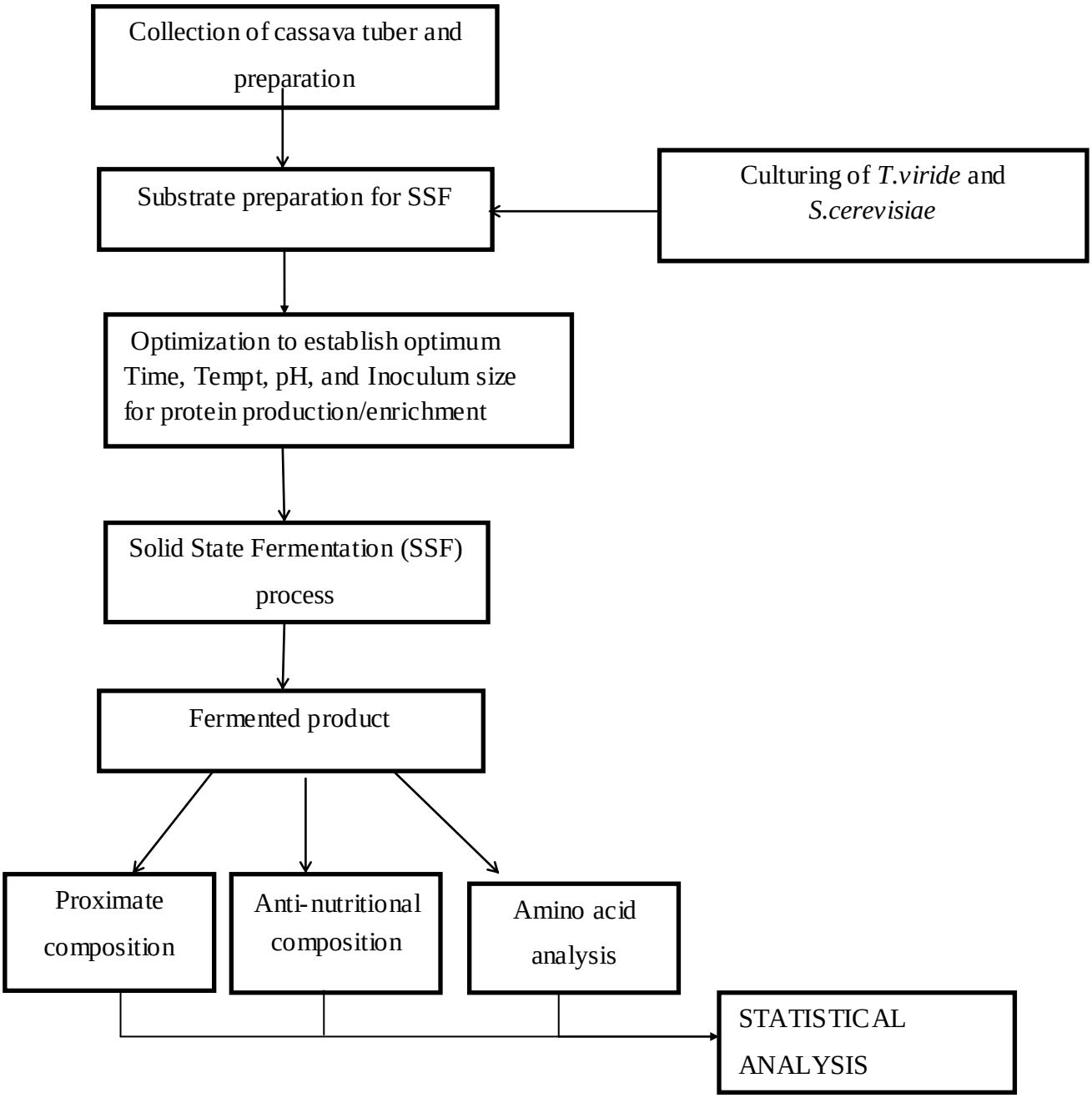


Figure 3. 1Experimental Design

3.2.3Measurement of spores of microorganism:

The conidial were counted on a Haemocytometer slide bridge as described by Sharma, (1989).The inoculums concentration was microscopically enumerated using the cell counting heamocytometer (Neubauer Chamber, Merck, S.A, Madrid, Spain) as described by Petrikkou*et al.*, (2001). The spore and yeasts suspension was prepared by proper mixing and approximately nine micro liters of the cell suspension was pipetted into one of the two counting chambers, all of the spores in each of the four (4) 0.1 mm^3 corner squares were counted.

Average cell count was calculated as:

Spore/ml = (n) × 10^6.

Where n = the average cell count per square of the four corner squares counted.

3.2.4 Substrate preparation for solid state fermentation:

The method ofEzekiel and Aworh, (2013) was adapted. Five grams (5g) ofdried cassava peels powder were weighed into fourteen (14) 250 ml conical flasks per parameter. Samples weremoistened with 12.5ml of Basal Mineral Salt Medium (BSM) g/100ml;theinitialpH of the samples were adjusted using 1M HCl and 1M NaOHuntil the desired pH was achieved. Each flasks were clogged with cotton wool and then covered with aluminum foil. The flasks containing the substrates were autoclaved at 121^{o}C for 15 minutes. The autoclaved samples were allowed to cool to ambient temperature before inoculation with *T. viride* and *S. cerevisiae* simultaneously. All experiments were performed in triplicates.

3.2.5 Setting up of fermentation process:

The basis for fermentation was chosen for all the fermentation conditions: Time profile for protein enrichment, incubation temperature, initial pH, and inoculums size on the protein

enrichment of cassava peel. Each conical flask contained the prepared dried cassava peel powder and were inoculated with the prepared inoculums of *T.viride*, *S.cerevisiae* and co-culture of T.*viride and S.cerevisiae* respectively. Subsequently, the fermentation parameters were varied as indicated in the substrate preparation procedure and samples withdrawn on the noted day corresponding to the day with the highest total protein for subsequent analysis to evaluate the effect of the variables on protein enrichment. This procedure is expected to help facilitate the establishment of suitable fermentation conditions for cassava peels using *Trichoderma viride and Saccharomyces cerevisiae* under solid state fermentation process. (Methodology of Ezekiel and Aworh, 2013 was adopted).

3.2.5.1 Determination of the effect of time profile for protein enrichment

To establish a time profile for the fermentation process, co-culture of *T. viride and S. cerevisiae* were grown at constant temperature, pH and inoculum size. Samples were withdrawn daily for ten (10) days respectively and analyzed for total protein using modified Lowry method (Lowry *et al.,* 1951); the optimum fermentation period corresponding to the day with the highest protein content were noted.

3.2.5.2 Determination ofthe effect of temperature on protein enrichment

Co-culture of *T. viride and S. cerevisiae* were grown at different temperatures in order to study the effect of temperature on protein enrichment of cassava peel, but other factors were kept constant. The cultures were incubated at 25, 30, 35 and 40°C for five days in incubators. The protein content were quantified using modified Lowry method to identify the best temperature level at which highest protein content were obtained was also noted .

3.2.5.3 Determination ofthe effect of pH on protein enrichment

The effect of initial pH on protein enrichment of cassava peel by *Trichoderma viride* and *Saccharomyces cerevisiae* were investigated within the range of 3-9. The pH were adjusted with 0.1M NaOH or 0.1M HCl to pH values of 3, 4, 5, 6, 7, and 9. The protein content were quantified using modified Lowry Method to identify the best pH value at which highest protein content were obtained.

3.2.5.4 Determination of the effect of inoculum sizes on protein enrichment

The effect of different inoculum sizes on protein enrichment was studied by adding spore's suspension of 1.5 x 10^8, and 3 x 10^8spores/ml respectively for each organism. Other parameters were kept constant.

3.2.6Preparation of sample for protein estimation

Crude soluble protein was extracted from the fermented cassava peel through simple contact method, using 0.05M citrate-phosphate buffer (pH 6.8) according to the method of Kashyap*et al.,* (2002). The fermented cassava peel in each conical flask were thoroughly mixed with 20 ml of the buffer solution using a rotary shaker set at 150 rpm for 1 hour. The entire contents of the flask was squeezed through a 50 mesh size sieve, the pooled extract was then centrifuged for 10 min at 10,000 xg (4^{o}C) using a refrigerated centrifuge (model)and the collected supernatant was used to determine the total protein content.

3.2.7 Determination of total protein content

Protein concentration in the fermented cassava peel was estimated with Folin-Ciocalteu reagent using Lowry modified protocol as describe by Hartree (1972) with bovine serum albumin (BSA), as standard protein. Protein concentration was expressed as mg/g of fermented cassava peel.

Principle: Under alkaline conditions the divalent copper ion forms a complex with peptide bonds in which it is reduced to a monovalent ion. Monovalent copper ion and the radical groups of tyrosine, tryptophan, and cysteine react with folinreagent to produce an unstable product that becomes reduced to molybdenum/tungsten blue.

Procedure - Hartree-Lowry assay: Reagents

- Reagent A consists of 2 g sodium potassium tartrate x 4 H20, 100 g sodium carbonate, 500 ml 1N NaOH, H_20 to one liter (that is, 7mM Na-K tartrate, 0.81M sodium carbonate, 0.5N NaOH final concentration).
- Reagent B consists of 2 gm sodium potassium tartrate x 4 H_20, 1 g copper sulfate (CuSO4 x $5H_20$), 90 ml H_20, 10 ml 1N NaOH (final concentrations 70 mM Na-K tartrate, 40 mM copper sulfate).
- Reagent C consists of 1 volume Folin-Ciocalteau reagent diluted with 15 volume water.

Assay

Serial dilutions of 0.3 mg/ml bovine serum albumin in the same buffer containing the unknowns were prepared, to give concentrations of 30 to 150 micrograms/ml (0.03 to 0.15 mg/ml).

One milliliter (1.0 ml) of each dilution of standard protein-containing unknown, or buffer (for the reference) were added to 0.90 ml reagent A in separate test tubes and mixed.

The mixture were Incubated inside the tubes for 10 minutes in a 50 ^{o}C water bath, then cooled to room temperature (28 ^{o}C).

The measure of reagent B (0.1 ml) were added to each tube, mixed, then incubated for 10 minutes at room temperature(28 ^{o}C).

Reagent C (3 ml) were rapidly add to each tube, mixed, then incubated 10 minutes in the 50 ^{o}C water bath, and cooled to room temperature; with final assay volume of 5 ml each.

Absorbance was measured at 650 nm in 1 cm cuvettes using Spectrophotometer (model)

Standard curves was plotted, shown in appendix 1, the regression equation was used to determine the concentration of the unknown samples.

3.2.8Proximate analysis of cassava peel and fermented product

Cassava peel were analysed for crude protein, ash, fat, crude fibre and total carbohydrate before and after protein enrichment by Fermentation based on AOAC standard procedures.

3.2.8.1 Determination of Moisture (AOAC, 2000)

The method described by A.O.A.C (2000) was used. An empty dish and lid were dried in an oven at 105^{o}C for 3h and transferred to a desiccator to cool. The empty dish and lid were weighed. Three grams of sample were weighed and spread uniformly on the dish. The dish containing the samples was placed in the oven and dried at 105^{o}C for 3h. After drying, the dish with partially covered lid was transferred to the desiccator to cool. The dish containing the dried sample was reweighed.

The moisture content was calculated as follows:

$$\% \, Moisture \; Content = \frac{W1 - W2 \times 100}{W1}$$

W1 = weight (g) of sample before drying

W2 = weight (g) of sample after drying

3.2.8.2 Determination of crude protein content (AOAC, 2000)

Reagents**:** Kjeldahl catalyst: a mix of 9 part of potassium sulfate (K_2SO_4) with 1 part of copper Sulfate ($CuSO_4$), Sulfuric acid (H_2SO_4), 40% NaOH solution, 0.2N HCl solution, 4%H_2BO_3, indicator solution: a mix of 100ml of 0.1% methyl red (in 95% ethanol) with 200ml of 0.2% bromocresol green (in 95% ethanol)

Method: Sample (1g) was placed in a digestion flask and 5g of Kjeldahl catalyst and 200ml of concentrated H_2SO_4 added. A tube containing the above chemicals except sample as blank was prepared; the flask was placed in inclined position and heated using heater gently until frothing ceases; then boiled briskly until solution clears. The above was cooled and 60ml of distilled water was added cautiously. Immediately the flask was connected to digestion bulb on condenser with the tip of the condenser immersed in standard acid and 5-7 drops of mix indicator in receiver. Flask was rotated to mix content thoroughly, and then heated until all NH_3 is distilled. The receiver was removed, and the tip of condenser was washed and the excess standard acid distilled was titrated with standard NaOH solution.

Calculation:

$$Crude\ Protein(\%) = \frac{(A - B) \times N \times 1.4007 \times 6.25}{W}$$

Where: A= Volume (ml) of 0.2N HCl used sample titration

B= Volume (ml) of 0.2N HCl used in blank titration

N= Normality of HCl

W= Weight (g) of sample

14.007 = atomic weight of nitrogen

6.25 = the protein nitrogen conversion factor

3.2.8.3 Determination of ash content (AOAC, 2000)

A crucible and its lid were placed in the furnace at 550^{o}C overnight to ensure all impurities on the surfaces was burnt off. The crucible was cooled in a desiccator for 30min; and then weighed to 3 decimal places. About 5g of sample was weighed and placed into the crucible, heated over low bunsenflame with lid half covered. When fumes were no longer produced, the crucible and lid was placed in the furnace. Heated at 550^{o}C overnight, the lid was not covered during heating, but placed after complete heating to prevent loss of fluffy ash; then cooled in the desiccator. The ash was weighted with the crucible and lid when the sample turned to gray. If not sample were returned to the furnace for further ashing.

Calculations

$$Ash\ (\%) = \frac{Weight\ of\ ash\ (g)\ \times 100}{Weight\ of\ sample\ (g)}$$

3.2.8.4 Determination of fat content (AOAC, 2000)

Reagent: Petroleum ether

Method:About 3-5g of sample were weighed to filter paper and wrapped. Sample was placed in the extraction thimble and transferred into soxhlet apparatus. About 250ml of petroleum ether were filled into the bottle and taken by the heating mantle. The soxhlet apparatus was connected and water turned on to cool them and then the heating mantle was switched on. Sample was heated for about 14hours (heat rate of 150drop/min) the solvent was evaporated using vacuum

condenser. Bottle was incubated at 80-90^{o}C until the solvent was completely evaporated and bottle was completely dried. After drying, the bottle was transferred with partially covered lid to the desiccator to cool. Bottle was reweight and its dried content.

Calculation

$$Fat(\%) = \frac{Weight\ of\ fat\ (g) \times 100}{Weight\ of\ Sample\ (g)}$$

3.2.8.5 Determination of crude fibre

This was based on continuous extraction of fat content from the sample using petroleum ether in a soxhlet extractor. In this principle, non-polar components of the sample are easily extracted into the organic solvent. The fat content of each sample was determined by the procedure described by AOAC (2000) a clean dry round bottom flask containing antibunping granules was used. Exactly 210 ml of petroleum ether (40 – 60 ^{o}C) was put into a flask fitted with soxhlet extraction unit. Cold water was put into circulation, the heating mantle was then switched on and the heating rate adjusted until the solvent is refluxed at a steady rate. Extraction was carried for two and a half hours. The sample was removed and dried to a constant weight in an oven. It was thencooled in a desiccator, reweighed and the percentage crude fat content was determined.

$$Crude\ fat\ (\%) = \frac{Weight\ of\ lipid\ extracted}{Weight\ of\ dry\ sample}\ x\ 100$$

Where the weight of lipid extracted was the loss in weight of the sample after extraction, drying in an oven and cooled ina desiccator.

3.2.8.6 Determination of carbohydrate content

The percentage carbohydrate was determined by difference as the sum of ash, protein, lipid, crude fibers subtracted from the value of organic matter.

3.2.9 Determination of antinutrient components

3.2.9.1 Determination of cyanogenic glycoside

The Cyanogenic glycoside was determined using alkaline picrate according to the method described by Onwuka and Olapade (2005). Five grams of dried samplepowder of both fermented and unfermented cassava peel in each case were weighed and dissolved in 50ml distilled water. The cyanide extraction was allowed to stay overnight and then filtered and the filtrate was used for cyanide determination (Inuwa*et al.,* 2011).Different concentrations of KCN solution containing 0.2 to 1.0µg/ml cyanide were prepared from a stock solution of KCN prepared by dissolving 0.2g of KCN salt in 1000ml distilled water.To 1ml of the aqueous extract and standard cyanide solution in test tubes, 4ml of alkaline picrate (obtained by dissolving 1g of picrate and 5g of Na_2CO_3 in 200ml of distilled water) was added and the whole setup was incubated in a water bath at a temperature of 50^{o}C for 30 min. The formation of a dark red colour was read spectrophotometrically at 490nm against a reagent blank whose content was 1ml of distilled water and 4ml of alkaline picrate solution. The cyanide content of the flours was subsequently extrapolated from the cyanide standard curve.

3.2.10.2 Determination of phytate:

The method of Reddy and Love, (1999) was adopted. Each ground sample (2g) was dissolved in 100ml of 2% hydrochloric acid for 5hours and filtered. The filtrate (25ml) was placed in a conical flask and 5ml of 0.3% Ammonium thiocyanate solution was added. The mixture was

then titrated with standard iron (III) chloride solution which contained 1.95mg of iron per milliliter, until a brownish-yellow colour persisted for at least five minutes. The percentage phytate was calculated using the formula:

Phytate (%) = (Y × 1.19× 100)/ (Sample weight)

Y = Titrevalue x 1.95mg.

3.2.10.3 Determination of tannin

The tannin extract was prepared by weighing 0.2g of the sample into 100ml conical flask, 50ml of distilled water was added and boiled for 1hr, filtered and the solution made to the volume on cooling. Aliquot of both tannic acid and the sample were prepared respectively. 2.5ml of folin-Denis reagent was added to each followed by 10ml of the 17% sodium carbonate solution and was diluted appropriately and then placed in a water bath at 25°C for 20minutes. This was determined calorimetrically at 760nm using spectrophotometer, as described by Price and Butler, 1977.

3.2.9.4 Determination of oxalate

Oxalate contents of the sample was determined by titrimetric method as described by Dye (1956). Two grams (2g) of ground sample was used for extraction purposes. To the aliquot of the extract was added 20ml of 6M hydrochloric acid, evaporated to half its volume and then filtered. To the filtrate was added 3 dropsof methyl red indicator was added and concentrated ammonia solution turned faint yellow. The resultant solution was then heated at 90°C with 10ml of 5% (w/v) calcium chloride to precipitate the oxalate and subsequently filtered off. The residue

was then washed into a beaker using hot 25% (v/v) sulphuric acid solution and diluted. This was warmed to 90°C and titrated while hot with 0.01M potassium permanganate solution.

3.2.9.4 Determination of saponins

Saponin content was determined using the AOAC (1990) method. Saponin extraction was done using acetone and methanol. Crude lipid content of the sample was extracted with acetone while methanol was used to extract saponin. Cassava peels powder sample (2g in triplicates) were folded in a filter paper and put in a thimble and extracted by refluxing in a soxhlet extractor. Extraction was done with acetone in 250ml capacity round bottom flask for three hours, after which the apparatus was dismantled and another 150 ml capacity flask containing 100mlmethanol was fitted to the extractor and extraction was sustained for another 3 hours. Weight of flask before and after second extraction was taken to note the change in weight. Methanol was recovered by distillation after the second extraction and the flask was oven-dried, allowed to cool at room temperature and weighed. Saponin content was calculated from the formula.

$$Saponin\ \left(\frac{mg}{100g}\right) = A - \frac{B}{Sm} \times 100$$

Where A = Mass of the flask and extract

B =Mass of empty flask

Sm = Mass of the sample

3.2.10 Determination of amino acid profile of samples:

The amino acid profile in the sample were determined before and after fermentation using the method described by Adeyeye and Afolabi, (2004). The sample were dried to constant weight,

defatted, hydrolyzed, evaporated in a rotary evaporator and loaded into applied phenyl-thiohydantoin (PTH) model 120A(applied biosystem, inc. Foster city, CA, USA) amino acid analyzer.

i.Defatting of sample

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

ii. Nitrogen Determination

A small amount (0.115mg) of ground sample was weighed, wrapped in whatman filter paper (No.1) and put in the Kjelcihal digestion flask. Concentrated sulphuric acid (10ml) was added. Catalyst mixture (0.5g) containing sodium sulphate (Na_2SO_4), copper sulphate ($CuSO_4$) and selenium oxide (SeO_2) in the ratio of 10:5:1 was added into the flask to facilitate digestion. Four pieces of anti-bumping granules were added.The flask was then put in Kjeldhal digestion apparatus for 3 hours until the liquid turned light green. The digested sample was cooled and diluted with distilled water to 100ml in standard volumetric flask. Aliquot (10m1) of the diluted solution with 10ml of 45% sodium hydroxide was put into the Markham distillation apparatus and distilled into 10ml of 2% boric acid containing 4 drops of bromocresol green/methyl red indicator until about 70ml of distillate was collected. The distillate was then titrated with standardize 0.01N hydrochloric acid to grey coloured end point.

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

Where:

a. = Titre value of the digested sample

b. = Titre value of blank sample

v. = Volume after dilution (100ml)

W. = Weight of dried sample (mg)

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

14. = Nitrogen constant in mg.

iii. Hydrolysis of the sample

A known weight of the defatted sample was weighed into glass ampoule. 7ml of 6NHCL was added and oxygen was expelled by passing nitrogen into the ampoule (this is to avoid possible oxidation of some amino acids during hydrolysis e.g. methionine and cystine). The glass ampoule was then sealed with Bunsen burner flame and put in an oven preset at $105^0C \pm 5^0C$ for 22 hours. The ampoule was allowed to cool before broken open at the tip and the content was filtered to remove the humins. It should be noted that tryptophan is destroyed by 6N HCL during hydrolysis**.**The filtrate was then evaporated to dryness using rotary evaporator. The residue was dissolved with 5ml to acetate buffer (pH 2.0) and stored in plastic specimen bottles, which were kept in the freezer.

iv. Loading of the hydrolysate into analyzer

The amount loaded was 60 microlitre. This was dispensed into the cartridge of the analyzer. The analyzer is designed to separate and analyze free acidic, neutral and basic amino acids of the hydrolysate.

v. Method of Calculating Amino Acid Values

An integrator attached to the Analyzer calculates the peak area proportional to the concentration of each of the amino acids.

3.3. Statistical Analysis

All results were obtained in triplicates and expressed as Mean ± Standard deviation (SD). Data were analyzed using analysis of variance (ANOVA). Duncan multiple range test was used for multiple mean comparism to determine differences between the means. $P \leq 0.05$ were considered significant.

CHAPTER FOUR

4.0 RESULTS

4.1 Optimization Studies

The protein enrichment of cassava peel using *Trichoderma viride* and *Sacharomyce cerevisiae* co-cultures were optimized for the following: Substrate particle size, time profile, initial pH of substrate, incubation temperature and inoculum sizes of the microorganisms.

4.1.1 Effect of substrate particle size

Different particle sizes (1mm, 0.5mm and <0.5mm) of cassava peel powder were used to determine the effect of particle size on protein enrichment, it was observed on day five that 1mm particle size gave the highest protein yield (18.88±0.17mg/g cassava peels (CP) while <0.5mm gave the least protein yield (8.21 ± 0.16 mg/g CP) under the same conditions as shown in Table 4.1.

4.1.2: Establishment of time for protein enrichment of cassava peels.

There was a significant increase in the protein content as the days increased and it reached a peak on day five then declined (Table 4.2). Day five had the highest protein yield (20.20 ± 0.80 mg/g CP) when compared with the control; while day one and ten had the lowest yields of 2.27 ± 0.11 and 2.86 ± 0.11 mg/g CP respectively. There was a significant ($p<0.05$) increase from day one to five anda significant ($p<0.05$) decrease as well from day five (5) today ten (10) of the fermentation period.

Table 4.1 Effect of Substrate Particle Size on Protein Enrichment of Cassava Peels (mg/g CP)

Fermentation Period (Days)	**Total Protein (mg/g CP)**		
Particle Sizes	**1mm**	**0.5mm**	**<0.5mm**
1	3.51±0.06[a]	3.56±0.42[a]	3.24±0.67[a]
2	3.62±0.54[b]	4.93±0.04[c]	1.19±0.46[a]
3	7.79±0.42[b]	1.90±0.19[a]	13.84±0.45[c]
5	18.88±0.17[c]	13.42±0.27[b]	8.21±0.16[a]
7	13.61±1.02[a]	13.45±0.31[a]	11.17±1.32[a]

Values are Mean ± SD; Values with different superscript across the rows are significantly different ($P < 0.05$) by Duncan multiple range test

CP= Cassava Peel

Table 4.2TimeProfile for Total Protein Production ofCassava Peelspowder (1mm particle size) using Co-cultures of *Trichoderma viride* and *Sacharomyce cerevisiae*

Incubation period (days)	**Total Protein(mg/g CP)**
1	2.27 ± 0.11^{a}
2	4.97 ± 0.07^{b}
3	10.58 ± 0.09^{c}
4	10.99 ± 0.03^{c}
5	20.20 ± 0.80^{e}
6	16.81 ± 0.42^{d}
7	13.72 ± 0.04^{cd}
8	11.60 ± 0.03^{c}
9	3.29 ± 0.08^{ab}
10	2.86 ± 0.11^{a}

Values are Mean ± SD; Values with different superscripts within the column are significantly different ($P<0.05$) by Duncan multiple range test

CP= Cassava peel

Substrate particle size 1 mm, initial pH 6, incubation Temperature 28^{o}C

4.1.3 Effect of initial pH on fermentation of cassava peels

The result of the effect of initial pH on the concentration of total proteinvarying from pH of 3-9 was presented in Figure 4.1.At pH 5 (36.64 mg/g CP)recorded the highesttotal protein concentration which was significantly ($p<0.05$) higherthan thoseof the pH values of 3, 4, 6, 7 and 9 (19.05, 19.98, 24.41, 17.86 and 18.11 mg/g CP) respectively.

4.1.4 Effect of incubation temperature

The result of the effect of incubation temperature for the optimal total protein concentration was presented in Figure 4.2. There was a regular trend in the increase in protein yield as temperature rose from 25^{o}C (5.98 mg/g CP) to 35^{o}C (28.10 mg/g CP) before a decline at 40^{o}C (17.57 mg/g CP).

4.1.5 Effect of inoculum size of microorganism

The inoculum sizes of the microorganisms (*Trichoderma viride* and *Saccharomyces cerevisiae* co-cultures) for the optimal protein enrichment of cassava peel powder was determined as showed inTable 4.3.Different inoculum sizes of1.5 x10^{8}, 3.0x10^{8}and4.5x10^{8} spores/ml were used respectively. Sample fermented with 3.0x10^{8}spores/mlgave the highest protein yield (27.61 ±1.23mg/g CP) which was significantlyhigher than inoculum sizes of1.5 x10^{8} spores/ml and 4.5x10^{8} spores/ml (14.92 ±1.01 and 14.11± 0.90mg/g CP) respectively.

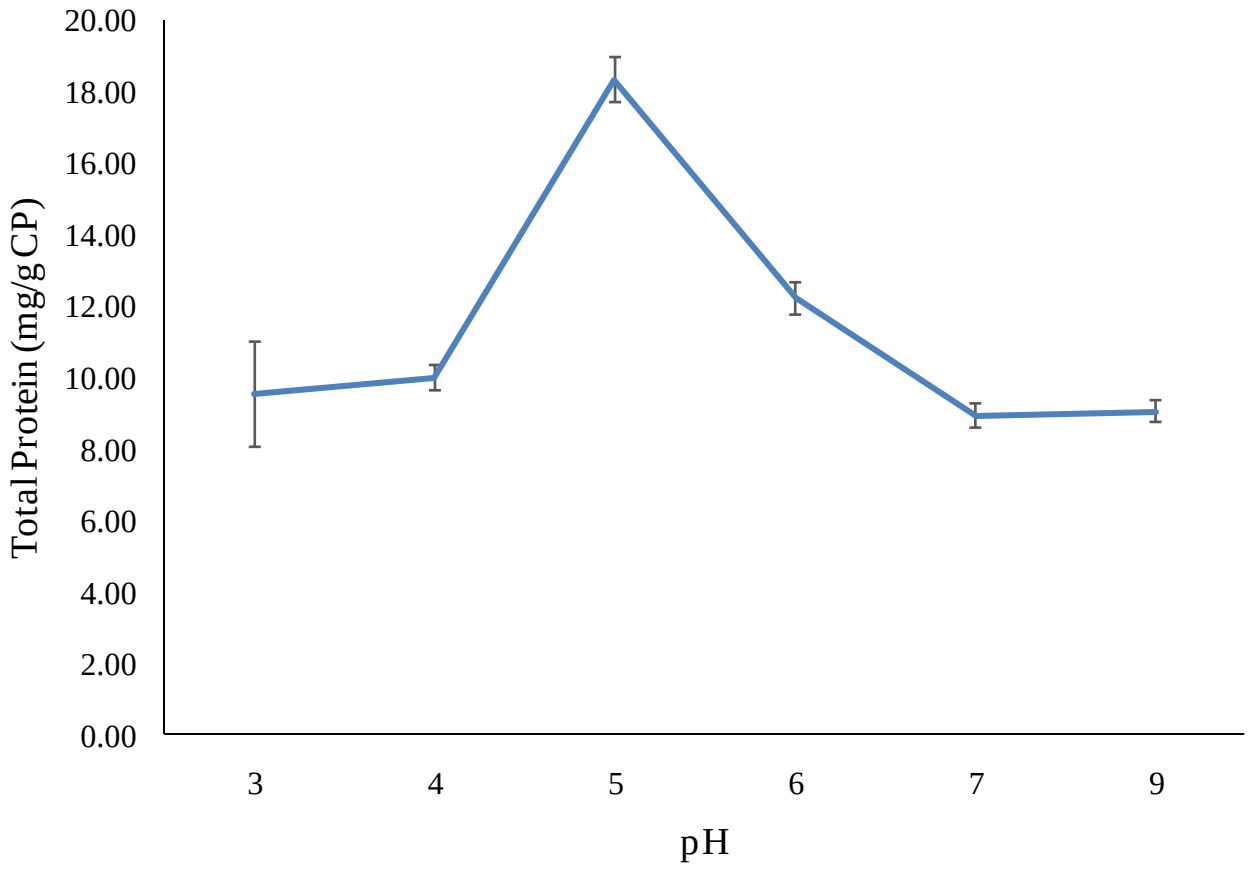


Figure 4.1: Effect of Initial pH on the Protein Enrichment of Cassava Peels Using *Trichoderma viride* and *Sacharomyce cerevisiae*Co-culture.

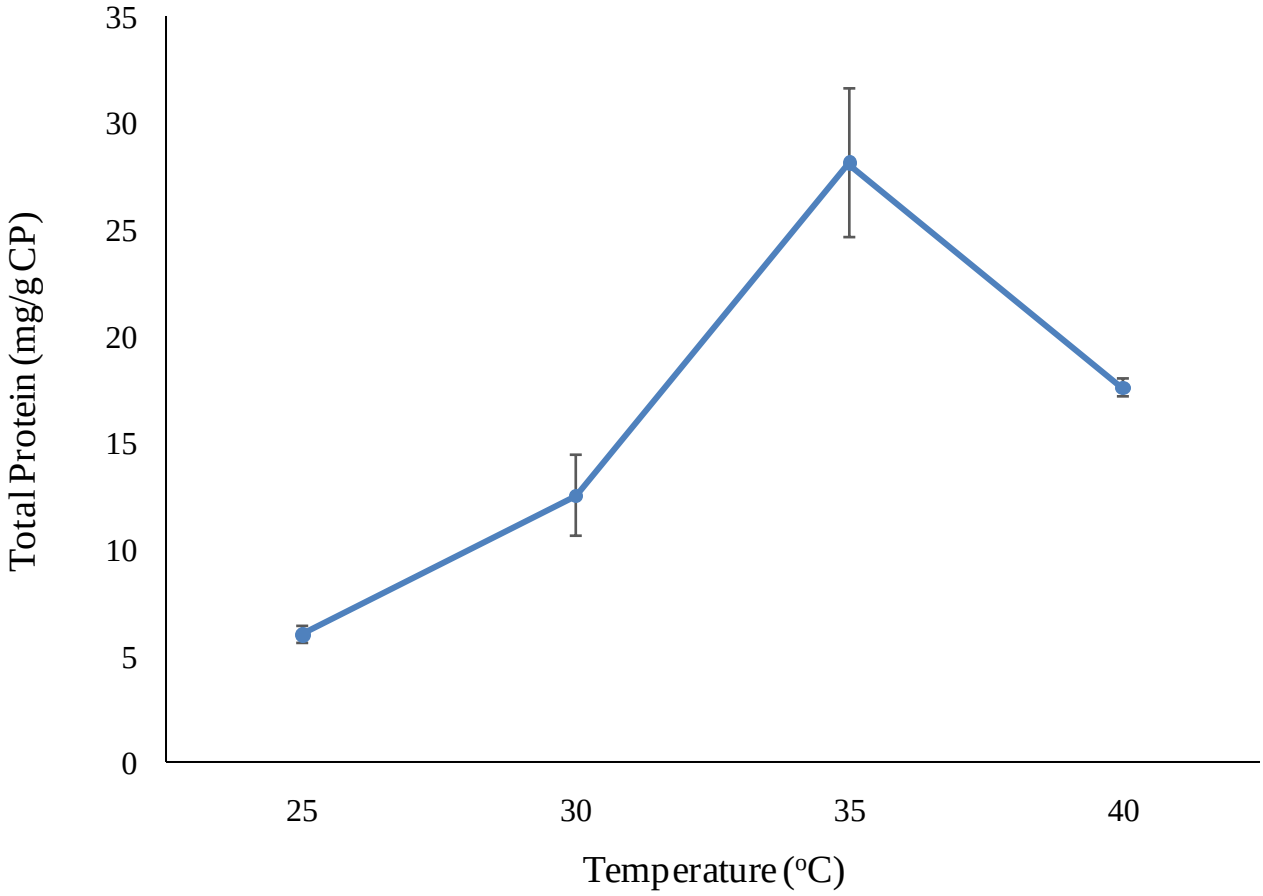


Figure 4.2: Effect of Incubation Temperature on the Protein Enrichment of Cassava Peels Using *Trichoderma viride* and *Saccharomyces cerevisiae* Co-culture.

Table 4.3Effect of Inoculum Size on the Protein Enrichment of Cassava Peel by Co-cultures of *Trichoderma viride* and *Saccharomyces cerevisiae*

Inoculum size	**Total Protein(mg/g CP)**
1ml (1.5×10^8)	14.92 ± 1.01^a
2ml(3.0×10^8)	27.61 ± 1.23^b
3ml (4.5×10^8)	14.11 ± 0.90^a

Values are Mean ± SD; Values with different superscript within the columns are significantly different ($P<0.05$) by Duncan multiple range test

CP= Cassava Peel

4.2 Effect of Fermentation on Protein Enrichment of Cassava Peels

The result showing the level of total protein concentration in the control sample andfermented samplesusing their respective monocultures and co-cultures presented in Table 4.4.The total protein concentration of unfermented sample (4.66 ± 0.40 mg/g CP) increased significantly to (12.85 ± 1.38, 17.88 ± 0.71 and 28.10± 3.49 mg/g CP) in samples fermented with *T viride*, S. *cerevisiae*, *T. viride* and *S. cerevisiae* co-cultures respectively, representing 2.7 fold, 3.84 fold and 6.03 fold increases.Among the fermented samples, the sample fermentedwith co-cultures of *T.viride* and *S. cerevisiae* significantly ($p<0.05$)increased in total protein concentration than the monocultures.

4.3 Effect of Fermentation on the Proximate Composition of Cassava Peels

The result of proximate composition of unfermented sample and fermented samples are shown in Table 4.5. The crude protein content of the control sample (7.01±0.94) increased significantly($p<0.05$) to (16.65±0.78, 18.90±0.94 and 23.49±1.48 %) for samples fermented with *T viride*, S. *cerevisiae*, and *T. viride* and *S. cerevisiae* co-cultures, representing 1.4 fold, 1.7 fold and 2.4 folds increase respectively. The fat of the unfermentedsample (8.49±0.52 %) increased significantly ($p<0.05$) to (8.78±0.46, 9.69±0.94 and 12.89±0.73 %) in samples fermented with *T viride*, S. *cerevisiae*, and *T. viride* and *S. cerevisiae* co-cultures, representing 3.42 %, 14.13% and 51.83% increase respectively. The ash content of the unfermented sample (3.04±0.52 %)increased significantly ($p<0.05$) to (4.80±0.52, 5.80±0.79 and 6.30±0.68 %) in samples fermented with *T viride*, S. *cerevisiae*, and *T. viride* and *S. cerevisiae* co-cultures, representing 57.89%, 90.79% and 107.24% increase respectively. The Moisture content of unfermented sample (4.45±0.73 %)increased significantly ($p<0.05$) to (5.70±0.25, 6.20±0.69 and

6.84±0.98 %) in samples fermented with *T. viride*, S. *cerevisiae*, and *T. viride* and *S. cerevisiae* co-cultures, representing 28.09%, 39.33% and 53.71% increase respectively. Crude fibre content of the unfermented sample (8.45±0.84) decreased significantly($p<0.05$) to (7.74 ± 0.96, 7.85±0.62, and 7.44±0.97 %) in samplesfermented with *T. viride*, S. *cerevisiae*, and *T. viride* and *S. cerevisiae* co-cultures; among the fermented samples there was not difference.Finally, Carbohydrate content forunfermentedsample (68.46 ±1.48 %) decreased significantly ($p<0.05$) to (56.33±0.94, 51.56±1.24, and 43.04±0.82%) in samples fermented with *T viride*, S. *cerevisiae*, and *T. viride* and *S. cerevisiae* co-cultures, representing 26.85%, 33.05% and 44.11% decrease respectively.

4.4: Effect of Fermentation on the AntinutrientContent of Cassava Peels

The anti-nutritional factors in unfermented and fermented cassava peel sampleswere shown in Table 4.6. There was a significant ($p<0.05$) difference in the anti-nutritional components of the unfermented and fermented samples, the levels of these compounds reduced significantly.The cyanide level in the control (64.80± 2.49 mg/100g) was reduced significantly ($p<0.05$) to (29.16±1.04, 51.80±1.87, and 16.2±0.89 mg/100g) for samples fermented with *T viride*, S.*cerevisiae*,and *T. viride* and *S. cerevisiae* co-cultures, representing 55%, 20.06% and 75% reduction respectively. Similarly, the concentration of phytate in the unfermented sample (5.47±0.75%) reduced significantly ($p<0.05$) to (3.19±0.58, 3.95±0.68 and 2.80±0.89 %) in samples fermented with *T viride*, S. *cerevisiae*, *T. viride* and *S. cerevisiae* co-cultures, representing 41.68%, 27.79% and 48.81% reduction respectively. Tannin contentin unfermented sample was (0.90±0.05%) which was significantly ($p<0.05$) reduced to (0.65±0.04, 0.52±0.03

and 0.61±0.02%) for samples fermented with *T viride*, S. *cerevisiae*, *T. viride* and *S. cerevisiae* co-cultures,

Table 4.4Effect of Fermentation using Co-culture on the Protein Enrichment of Cassava Peels

Samples	**Total Protein(mg/g CP)**
Unfermented	4.66±0.40[a]
T. viride	12.85±1.38[b]
S. cerevisiae	17.88±0.71[c]
T.viride and S. cerevisiae	28.10±3.49[d]

Values are Mean ± SD; Values with different superscripts within the columns are significantly different (P<0.05) by Duncan multiple range test

CP= Cassava Peel

Table 4.5Effect of Fermentation on the Proximate Composition of Cassava Peels

Proximate composition (%)	**Unfermented**	***T. viride***	***S. cerevisiae***	***T.viride and S. cerevisiae* co-culture**
Moisture	4.45±0.73[a]	5.70±0.25[ab]	6.20±0.69[b]	6.84±0.98[b]
Ash	3.04±0.39[a]	4.80±0.52[b]	5.80±0.79[bc]	6.30±0.68[c]
Fat	8.49±0.52[a]	8.78±0.46[a]	9.69±0.72[b]	12.89±0.73[c]
Protein	7.01±0.94[a]	16.65±0.78[b]	18.90±0.94[c]	23.49±1.48[d]
Fibre	8.45±0.84[b]	7.74±0.96[a]	7.85±0.62[a]	7.44±0.97[a]
Carbohydrate	68.56±1.48[d]	56.33±0.94[c]	51.56±1.24[b]	43.04±0.82[a]

Values are Mean ± SD; Values with different superscripts across rows are significantly different ($P<0.05$)

Table 4.6 Effect of Fermentation on the Antinutrient Content of Cassava Peels

Antinutrient content	**Unfermented**	***T. viride***	***S. cerevisiae***	***T.viride and S. cerevisiae***
Cyanide (mg/100g)	64.80±2.49^{d}	29.16±1.04^{b}	51.80±1.87^{c}	16.20±0.89^{a}
Phytate (%)	5.47±0.75^{b}	3.19±0.58^{a}	3.95±0.68^{a}	2.80±0.89^{a}
Tannin (%)	0.90±0.05^{c}	0.65±0.04^{b}	0.52±0.03^{a}	0.61±0.02^{b}
Oxalate (mg/100g)	125.40±3.25^{d}	41.80±1.24^{b}	52.80±1.31^{c}	37.40±1.84^{a}
Saponin (%)	5.30±0.82^{c}	2.40±0.20^{ab}	3.20±0.12^{b}	1.60±0.46^{a}

Values are Mean ± SD; Values with different superscripts across rows are significantly different ($P<0.05$)

representing 27.78 %, 42.22 % and 32.22 % reduction respectively. The Oxalate concentration in the unfermented sample (125.40 ± 3.25 mg/100g) was also reduced significantly ($p<0.05$) to (41.80±1.24, 52.80 ± 1.31 and 37.40 ± 1.84 mg/100g) in samples fermented with *T viride*, S. *cerevisiae*, and *T. viride* and *S. cerevisiae* co-cultures, representing 66.67%, 57.89% and 70.18% reduction respectively. Finally, Saponin concentration in theunfermented (5.30±0.82%) was reduced significantly ($p<0.05$) to (2.40±0.20, 3.20±0.12, 1.60±0.46 %) in samples fermented with *T viride*, S. *cerevisiae*, and *T. viride* and *S. cerevisiae* co-cultures, representing 54.72%, 39.62% and 69.81% reduction respectively.

4.5 Effect of Fermentation on Amino Acid Concentration of Cassava Peels

The result of the unfermented sample and sample fermented with *T.viride and S. cerevisiae* co-culture are shown in Table 4.7.The total amino acid score of unfermented sample (46.52 g/100g) which was significantly ($p<0.05$) increased to (54.21 g/100g) of the fermented sample. The following essential amino acids increased significantly ($p<0.05$) by more than 25% after fermentation: histidine (39.21%), threonine (34.68%), lysine (34.16%), methione (30.19%), and Valine (25.98%) respectively.

Table 4.7 Effect of Fermentation with co-culture of *T.viride* and *S. cerevisiae*on Amino Acid Concentration of Cassava Peels

Amino acids	**Concentration (g/100g Protein)**		**Percentage increase (%)**
	Unfermented	**Fermented**	
Leucine	4.14	4.96	19.81
Lysine	2.43	3.26	**34.16**
Isoleucine	3.07	3.30	7.49
Phenylalanine	2.66	2.84	6.77
Tryptophan	0.68	0.79	16.18
Valine	3.04	3.83	**25.98**
Methionine	0.53	0.69	**30.19**
Proline	1.42	1.82	28.17
Arginine	3.09	3.78	22.33
Tyrosine	1.89	2.41	27.51
Histidine	1.02	1.24	**39.21**
Cystine	0.54	0.48	-11.11
Alanine	3.30	3.90	18.18
Glutamic acid	6.51	7.34	12.75
Glycine	2.44	2.40	-1.64
Threonine	2.22	2.99	**34.68**
Serine	1.56	1.64	5.13
Aspartic acid	5.98	6.54	9.36
Total Amino Acid	**46.52**	**54.21**	**16.53**

CHAPTER FIVE

5.0 DISCUSSION

The optimization of substrate particle size suitable for protein enrichment of cassava peel was evaluated in this study. Lower particle sizes (0.5mm and <0.5mm) of cassava peel provided a greater saturated surface area with lower inter-particular porosity than larger particle size (Camacho-Ruiz *et al.,* 2003) which poses difficulty in aeration/respiration. Larger particle size provide better aeration opportunities but provide lesser surface area (Pandey, 2003) which does not support the growth of filamentous organisms. The optimal particle size in this study was 1mm,Membrillo*et al.* (2008) reported t increase of protein content (48% on particle size of 1.68mm when compared to 0.92mm) in the production of lignocellulosic enzymes from wheat straw substrate by *Pleurotusostreatus.* The surface area to volume ratio increases as the particle size decreases, hence smaller particle sizes would provide a larger surface area per unit volume on which the fungus can grow. Also, the smaller the particle sizes the better the nutritional availability for fungal cultures by making it easier for the fungi to utilize nutrients from the substrate and possibly avoiding metabolite intermediates into the particles (Wan and Li, 2010). However, smaller particle sizes will likely negatively affect mass transfer and could result in substrate agglomeration and poor aeration (Krishna, 2005).

Protein production with respect duration of fermentation closely followed the pattern of fungal growth. The production started after day one and increased progressively until the 5th day and began to decline thereafter; hencethe optimum protein yield was observed on the 5th day of fermentation. Ezekiel and Aworh, (2013) also reported Optimal proteinyield on the 8th day of fermentation using a single culture of*Trichoderma viride*, but in this studies optimal protein

production was observed on the 5th day of fermentation using *Trichoderma viride* and *Sacharomyce Cerevisiae* co-culture this could be as a result of the synergistic effect of both microorganisms activity on the substrate. *T. viride* secretes hemicelulolytic enzymes such as, cellulase, hemicelulase and lignase to break down the cellulose and hemicellulose cell wall to cellobiose and glucose unit and *S.cerevisiae* utilizes it and they both increase the biomass content in the substrate (Raimbault, 1998).

The initial pH optimization for fermentation with *Trichoderma viride* and *Saccharomycescerevisiae* co-culture yielded the highest amount of protein at pH 5 in cassava peels. The protein content increased gradually from pH 4 to 5 and then decreased rapidly (Figure 4.1). At pH less than 5 mycelia growth would have been inhibited by the high acidity resulting in reduced bioconversion of sugars to protein. In a study with *Aspergilusterreus* pH 5.5 was found to be the best suitable for maximum protein production (Jaganmohan*et al.,* 2013).

Protein production was highest at 35^{o}C relative to 25, 30 and 40^{o}C (Figure 4.2), this could be as a result of the active growth phase, temperature increase from 34.5 ^{o}C to 37.4^{o}C due to high oxygen requirement and heat generation (Bathe *etal..,* 2012). Jaganmohan*et al.*(2013) also reported maximum protein yield at 35^{o}C, in the production of single cell protein by *Aspergillusterreus*using banana peels as substrate.Inoculum size of the fungal culture has an important effect on protein production. Total protein was highest in inoculum size 3.0 x10^{8} compared to 1.5 x10^{8} and 4.5 x10^{8} spores/ml (Table 4.3) which could imply that lower protein production at lower inoculum size is probably due to less conidial cells which are insufficient to use the fermentation medium for maximal protein production via substrate utilization, while the

decreased yield at higher inoculum size is probably due to saturation of the substrate thereby the microorganism are crowded and cannot survive.

Protein production was significantly high using *Trichoderma viride* and *Saccharomycescerevisiae*co-cultures compared to their respective single cultures, the increase in protein yield could be as a result of the substrate utilization and the growth of the organisms thereby increasing the rate of bioconversion into protein.Okpako*et al.* (2008) reported significant increase in protein yield when cassava peels were fermented with a mixture of *A.niger* and *L.rhamnosus*when compared to their single cultures.

The proximate composition of unfermented and fermented cassava peel with both single and co-culture of*Trichoderma viride* and *Saccharomycescerevisiae*showed an increase in moisture, ash, protein, fat and fibre content and a decrease in carbohydrate. It has been observed that moisture has a stable effect on livestock, with respect to this cassava peels with high moisture content will be of essential value in livestock feeds (Oboh, 2006). From the results shown in Table 4.5, the cassava peel fermented with co-cultures of organisms had the highestmoisture content of 6.84±0.98%.This increase might be as a result of the combined activities of the organisms which had utilized the substrate during respiration to release more water during the breakdown process. Another possibility could be the activities of the microorganisms may have caused the pulp to clump together thereby reducing the process of evaporation.This result is in line with Okpako*et al.*(2008) who reported increase in moisture content in cassava peel using mixed culture of *Aspergillusniger* and *Lactobacillus rhamnosus*.Ash content of food samples is essential in determining their mineral element content; it is always a rough estimate of the inorganic compound present in the sample (Olaniyi*et al.*, 2010). The increase in the ash content of the

fermented sample as compared with the unfermented could be as a result of the growth and multiplication of the microorganisms in the fermentation medium (Ahaotu*et al.,* 2013). This findings was in agreement with those of Oboh and Akindahunsi (2003), who reported an increase in ash content in cassava products fermented with *Saccharomycescerevisiae.*The fat content of the fermented cassava peel increased significantly compared with the unfermented; this increase could be due to the increase in the microbial mass, secretion of microbial oil into the fermenting medium and other products from the metabolism (Oboh*et al.,* 2002).Protein is required for normal body growth, repairs and maintenance. Hence, protein is needed for the growth of livestock. In this study there was a significant increase in the crude protein content level in cassavapeelsfermented with the microorganisms (Table 4.5). These increase could be attributed to the secretion of someextracellular enzymes (proteins) such as cellulase, linamerase, hemicellulases and amylase into the cassava mash byfermenting microorganisms in an attempt to utilize the cassava starch as a carbon source (Raimbault,1998). The growth and proliferation of the microorganism growth complex in the form of single cell protein may possibly account for the apparent increase in the protein content of the peels fermented with the microorganisms (Oboh*et al.,* 2002;Oboh, 2006). Crude fibre content reduced over the period of fermentation which may be due to the hydrolytic nature of fungi used for the biodegradation. This is similar to the report of Ezekiel and Aworh, (2013) in solid state fermentation of cassava peel with *Trichoderma viride.*Carbohydrate content in the fermented cassava peels were significantly lower than in the unfermented peels. This low level could be an indication that the microorganisms hydrolysed the starch into glucose and used it as a source of carbon for their growth (Oboh, 2006). It could also be possible that an increase in the protein level of the

fermented cassava peel resulted in the decrease in carbohydrate, through the bioconversion of starch to protein.

Fermentation had a significant effect in the reduction of antinutritional factors, but the use of co-culture had the highest reductive properties except for Tannin where *S.cerevisiae*had the highest reduction compared to the unfermented cassava peel. Several authors have reported that cassava peels usually have higher concentration of cyanogenic glucosides than other root tubers which makes the peel unsuitable as animal feed.Processing of cassava peels by fermentation has been shown to significantly reduce the cyanide content of the cassava peels (Larsen and Amaning-Kwarteng, 1976;Tweyongyere and Katongole, 2002).In this study, cassava peels fermented with microorganisms showed significant reduction in the cyanide level especially in peels inoculated with*Trichorderma viride* and *Saccharomyces cerevisiae* co-culture compared to their respective monocultures.This significant low cyanide value recorded in the inoculated peels suggests that the microorganisms were capable of partially degrading the cyanogenic glycosides in the peels, confirming earlier reports by Obo*het al.* (2002);Obohand Akindahunsi(2003);Belewu and Musa (2003). The unfermented cassava peel phytate content reduced significantly by fermentation, which could be attributed to the possible secretion of the enzyme phyatase by the microorganisms. This enzyme is capable of hydrolyzing phytate thereby decreasing the phytate content in the fermented cassava peel.Oboh(2006)reported a decrease in phytate content in the study of nutrient enrichment of cassava peels using mixed culture of *Saccharomyces cerevisiae* and *Lactobacillus spp*. Oxalates are antinutrient that negatively affect calcium and magnesium bioavailability. The observed reduction of oxalate content in unfermented cassava peel caused by fermentation may be due to the effect of leaching and enzyme/acid hydrolysis of the starch

granule during fermentation (Oke and Bolarinwa, 2012). In this study 70.18 % oxalate reduction was observed by co-culture of *T.viride* and *S. cerevisiae* on cassava peel, this is similar to 65% reduction reported by Oke and Bolarinwa, (2012) in the effect of fermentation on oxalate content of cocoyam flour. Tannin content wasreduced significantly by 42.22%, 32.22% and 27.78% in sample fermented with *S.cerevisiae*, co-culture of *T.viride* and *S. cerevisiae*and*T.viride*respectively. Etsyankpa*et al.* (2015) reported 33.86% reduction in *S. cerevisiae*inoculated "garri", and 30.33% reduction in *L.bulgaricus*- treated garri. This reduction could be as a result of the degradation of polyphenols as a result of the release of microbial enzymes during fermentation (Ebuchi*et al.,* 2005). Saponin reduced significantly by 54.72%, 39.62% and 69.81% in sample fermented with *T.viride,S.cerevisiae*, and co-culture of *T.viride* and *S. cerevisiae*respectively.Etsyankpa*et al.* (2015) also reported 70.11% reduction in *S. cerevisiae*inoculated "fufu" and 2.43% reduction in*L.bulgaricus*- treated "garri".

Sample fermented with *T.viride and S. cerevisiae* co-culture showed a significant improvement in the amino acid profile when compared with the unfermented sample. The following essential amino acids; [histidine (39.21%), threonine (34.68%), lysine (34.16%), methionine (30.19%) and valine (25.98%)] were improved with over 25%. This is indicative that fermentation led to a significant improvement of the protein quality of these waste. Aro and Aletor, 2012 reported increase in amino acid profile from naturally fermented starch residueand Ezekiel *et al.* (2010) alsoreported similar enhancement of cassava peels subjected to submerged fermentation with *Trichoderma viride*.

CHAPTER SIX

6.0 SUMMARY, CONCLUSION AND RECOMMENDATIONS

6.1 Summary

1. This study revealed that fermentingcassava peel waste using co-cultures of *Trichoderma viride* and *Saccharomyces cerevisiae* had greater potentials for increasing the protein contents when compared to their respective monocultures.

2.Fermentation using co-culture of microorganisms was suitable and effective in reducing the cyanogenic glycosides present in cassava peels as well as other antinutritional factors such as phytate, tannin, oxalate and saponins, thereby making it valuable nutritionally.

3. The essential amino acids component of the cassava peel was improved with over 25% for histidine, threonine, lysine, methionine and valine.

4.Hence, cassava peel which currently is an agro-waste could become a valuable resource which could be included in animal feeds, thereby reducing the level of environmental pollution it currently possess and it could become a means of economic empowerment to cassava farmers.

6.2 Conclusion

The optimal conditions for protein enrichment of cassava peel using solid state fermentation with *T. viride and S. cerevisiae* co-culture was established to be 1mm particle size, pH 5.0, Inoculum size 3.0×10^8 spore/ml, incubation temperature of 35^oC at day five(5) of fermentation.Protein enrichment of cassava peel was achieved by 2.4 fold when fermented with *T. viride and S. cerevisiae* co-culture as compared to 1.7 fold with*S. cerevisiae* and 1.4 fold with *T. viride*

respectively. Fermentation using co-culture of fermenting organisms significantly reduced antinutritional content compared to their monoculture (20.06% to 75% reduction (cyanide), 27.79% to 48.8% reduction (Phytate), 57.89% to 70.8% reduction (oxalate)). The following essential amino acids; (histidine, threonine, lysine, methionine and valine) were improved with over 25%.Hence, protein enrichment using SSF with co-culture of fermenting organisms is better than their monocultures. Alsofermentation of cassava peel could enhance the decontaminationof waste disposed into the environment for a valuable products formation in the animal feed processing industry in Nigeria.

6.3Recommendations

Solid state fermentation of cassava peel usingco-culture of *Trichoderma viride* and *Sacharomyce cerevisiae* has great potential for bio-utilizing the waste for producing protein enriched component for inclusion in animal feed, thereby improving the economic value of cassava peel and at the same time solving the pollution problems arising from this waste and is recommended.

Protein enrichment of cassava peel with *T. viride* and *S. cerevisiae* suggests that it can be considered as constituents of animal feeds rather than other edible grains such as maize.

Further work could be done on the fermented cassava peel feed formulation and their growth performance in different livestock animals.

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APPENDICES

Appendix 1: Preparation of Standards and Reagents

Preparation of cyanide standard curve

Different concentrations of KCN solution containing 0.2 to 1.0µg/ml cyanide were prepared from a stock solution of KCN prepared by dissolving 0.2g of KCN salt in 1000ml distilled water. To 1ml of the aqueous extract and standard cyanide solution in test tubes, 4ml of alkaline picrate (obtained by dissolving 1g of picrate and 5g of Na_2CO_3 in 200ml of distilled water) was added and the whole setup was incubated in a water bath at a temperature of 50^oC for 30 min. The formation of a dark red colour was read spectrophotometrically at 490nm against a reagent blank whose content was 1ml of distilled water and 4ml of alkaline picrate solution. The cyanide content of the flours was subsequently extrapolated from the cyanide standard curve.

Preparation of protein standard curve

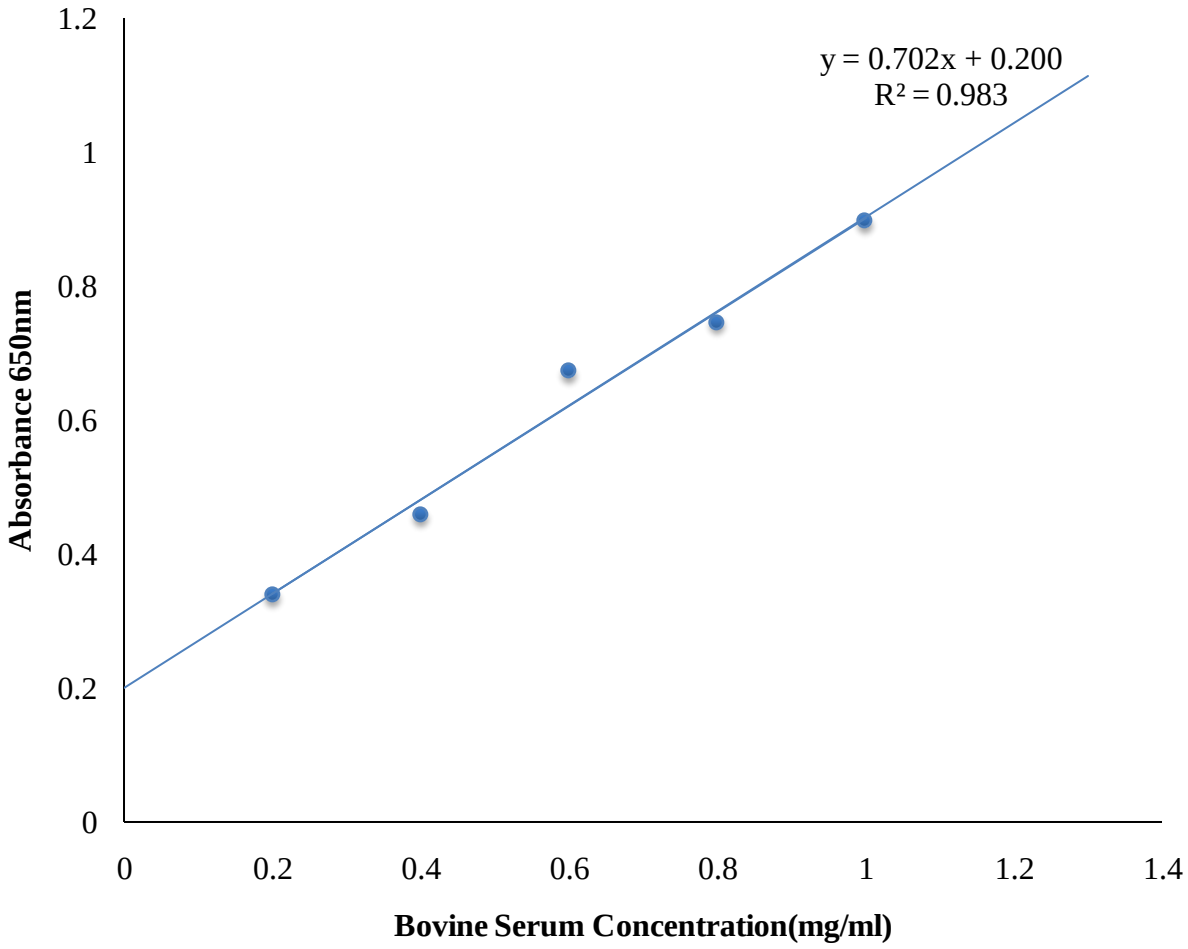
In preparing the standard curve of protein determination using Folin-ciocalteu reagent, different concentrations of BSA stock solution (1 mg/ml) was prepared as shown below and following the procedure of Hatree (1972), absorbance of each concentration was taken at 650 nm.

Preparation of Folin-Ciocalteu Reagent

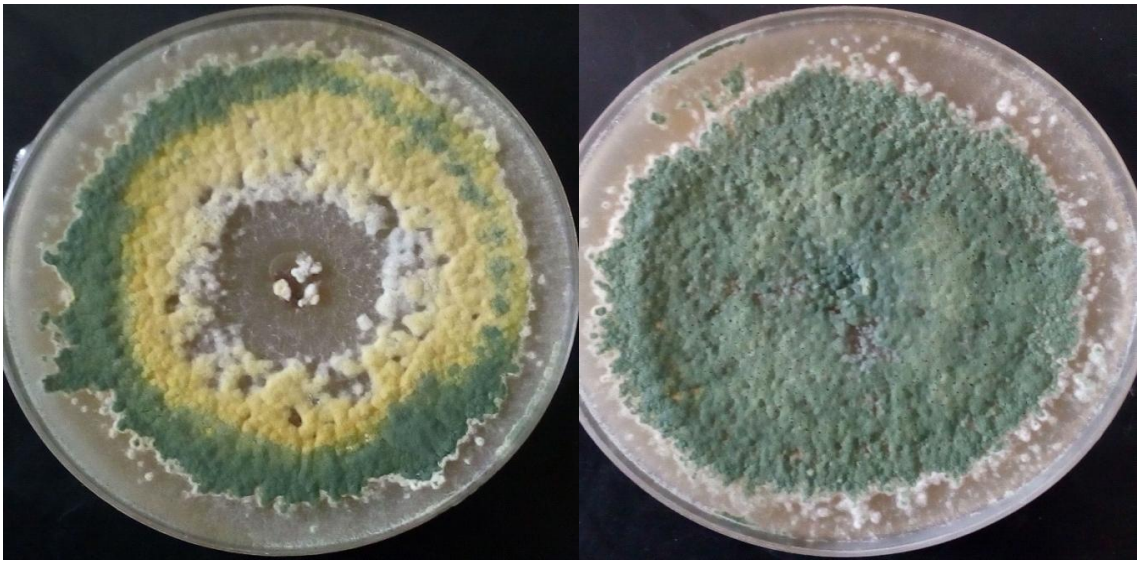
To prepare 100 ml Folin-ciocalteu reagent, 10 grams sodium tungstate and 2.5 gram sodium molybdate were dissolve in 70 ml distilled water, 5 ml 85% phosphoric acid and 10 ml concentrated hydrogen chloride were then added to the solution. The solution was hence refluxed for 10 hours after which 15 gram lithium sulphate, 5 ml distil water and one drop of bromine was added. The resulting solution was again refluxed for 15 minutes, cool to room temperature and

brought to 100 ml with distil water. A clear yellowish solution was obtained which store away from light.

Appendix 2Protein Standard Curve



Appendix 3: Cultures of *Trichoderma viride* Strain on PotatoeDestrose Agar (PDA) Plates.

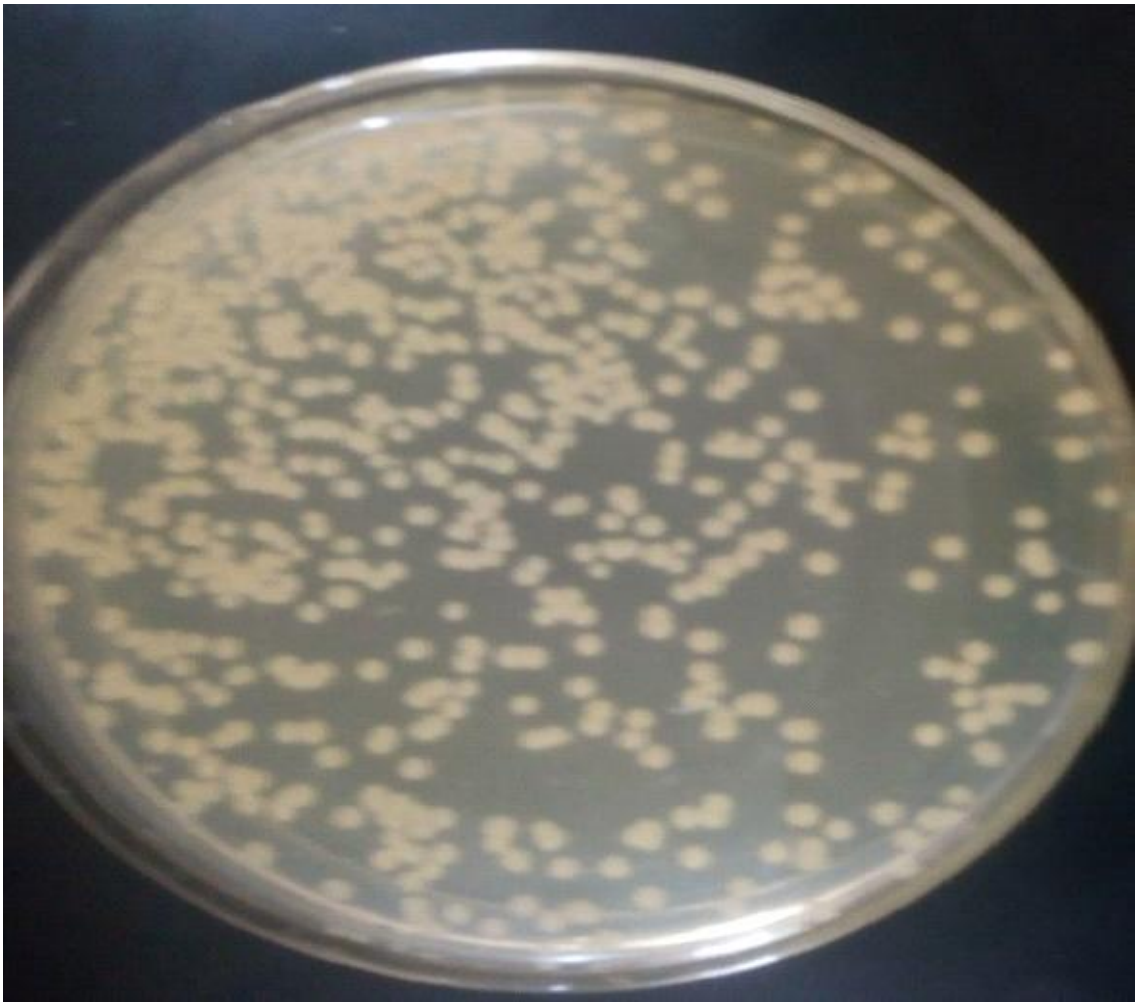


A: At day four B: At day five



C: At day seven D: At day six

Appendix 4Culture of *Saccharomyces cerevisiae* on Potatoes Dextrose Agar (PDA) Plates



Appendix 5Cassava Peel Product Fermented with *Saccharomyces cerevisiae*



Appendix 6: Cassava Peel Product Fermented with*Trichoderma viride*



Appendix 7 Cassava Peel Product Fermented with *Trichoderma viride* and *Saccharomyces cerevisiae* Co-culture.



Appendix 8: Effect of Fermentation on Amino Acids Concentration of Cassava Peel

