OPTIMISATION OF THE PARAMETERS FOR BIO-PROCESSING OF CASSAVA PEEL TO INCREASE FERMENTABLE SUGARS AND LYSINE PRODUCTION

BY

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DEDICATION

This thesis is dedicated to my wife Mrs Yvonne Bayitse, my daughter Richelle Edzenunye Bayitse and my son Elorm Bayitse for their support and prayers.

DECLARATION

I declare that except references to works, which I have cited, this dissertation is my original research, and that it has neither in whole nor in part been previously presented for another degree elsewhere.

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ABSTRACT

Bio-processing of cassava peel for Lysine production was done by characterising the cassava peel for carbohydrate types. The process of enzymatic hydrolysis of the cassava peel was optimised to produce fermentable sugars using response surface methodology. The hydrolysed cassava peel was used as a source of carbon to optimise the conditions for lysine production using mutant *Corynebacterium glutamicum* (AHP3) strain.

Cassava peel is a biomass generated as a result of processing cassava tuber by peeling operations. It is a natural resource that shows heterogeneity in structure and chemical composition. Physical and chemical composition analysis was done using standard methods. It was found that nearly 83 % dry matter (DM) composition of the cassava peel was glucose whiles xylose and arabinose have made up only small amount of 2.31 and 2.35 % respectively. The cellulose and hemicellulose were 6.0 % DM and 2.23 % DM respectively and the residual starch content was 47.16 %. The protein was 2.40 % and the cyanide level was 9.3 mg/kg. The lignin and the ash contents were 1.92% and 6.30% respectively. The high level of residual starch and low amount of lignin make the cassava peel very susceptible to enzymatic hydrolysis without laborious pretreatment regimes. Importantly, this study provides a useful base line data for agro-economic evaluation of cassava peel as a feedstock for an integrated biorefinery, because the valorisation of cassava peel is still overlooked and not fully exploited. Additionally, deep understandings of the biomass chemical and physical characteristics need to be known in order to assist in designing safe processing facilities.

Cassava peel is normally considered as waste because of its limited use. Composition analysis revealed that it contained appreciable amount of starch which can be hydrolysed to fermentable sugars. Response Surface Methodology using Central Composite Design (CCD) was applied to optimise the enzymatic hydrolysis of cassava peel in order to produce glucose. Two effective approaches were used in the study. The first one was to optimise the enzymatic hydrolysis process using cellulase, β-glucosidase, amyloglucosidase and α-amylase. The second approach was to optimise enzymatic hydrolysis using the mixture of these enzymes. The effects of enzyme loading, hydrolysis time, substrate concentration, pH and temperature on glucose recovery were investigated. The results were subjected to analysis of variance (ANOVA) to produce polynomial regression model. Mean interaction plot and their effect on glucose recovery were drawn to determine the optimal conditions for enzymatic process. Targeted hydrolysis of specific carbohydrate types in cassava peels with single enzymes showed optimised levels of glucose recovery of over 80% for starch hydrolysing enzymes and about 5% for cellulose hydrolysing enzymes at 0.06 g/ml substrate water ratio at 24 hours of hydrolysis. Single step hydrolysis of cassava peel with mixed enzymes of starch and cellulose hydrolysis enzymes at optimised conditions of celulase (30 FPU/g), β- glucosidase (1.25 U/g), amyloglucosidase (30 U/g), α-amylase (30 U/g), pH 4 and 50 ^{o}C at 24 hours produced recovered glucose of about 100%.

The present study revealed that lysine biosynthesis from *Corynebacterium glutamicum* can be significantly enhanced by optimising the fermentation process. Different ingredients have an essential role in the metabolic pathway of the organism for lysine production. Carbon and nitrogen sources from cassava peel hydrolysate have also been found to have influential role in the amino acid production.

Central Composite Design (CCD) was applied to optimise the amino acid fermentation process in order to produce lysine. The effects of substrate concentration, microbial load and time of fermentation on growth of *C glutamicum*, glucose utilisation and lysine production were investigated. The results were subjected to analysis of variance (ANOVA) to produce polynomial regression model. Mean interaction plot and their effect on microbial growth, glucose consumption and lysine production were drawn to determine the optimal conditions for amino acid fermentation process for lysine production. Glucose utilisation reduced at higher hydrolysate concentration thereby affecting microbial growth. Lysine production was optimum at low initial microbial load of 0.05 nm (OD) and cassava peel hydrolysate concentration not more than 35% (v/v) at 48 hours fermentation.

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LIST OF ABBREVIATIONS

PEM	Protein Energy Malnutrition
FRI	Food Research Institute
USDA	United State Department of Agriculture
USDOE	United States Department of Energy
SRID-MoFA	Statistics, Research and Information Directorate of Ministry of Food and agriculture
FAO	Food and Agricultural Organisation
OECD	Organisation for Economic Co-operation and Development
IEA	International Energy Agency
GAT	Glyphosate N-acetyltransferase
PCR	Polymerase Chain Reaction
DNA	Deoxyribonucleic acid
EA	Enzyme activity
EG	Endoglucanase
CBH	Cellobiohydrolase
CBMs	Carbohydrate Binding Modules
BGs	β-glucosidases
NREL	National Renewable Energy Laboratory
DNS	Dinitrosalicylic
HPLC	High-performance liquid chromatography
PPP	Pentose Phosphate Pathway
NADPH	Nicotinamide adenine dinucleotide phosphate
DM	Dry Mater
RSM	Response Surface Methodology

CCD	Central Composite Design
ANOVA	Analysis of variance
FPU	Filter Paper Unit
SHF	Separate Hydrolysis and Fermentation
DTU	Technical University of Denmark
BHI	Brain Heart Infusion
OD	Optical Density
MM	Minimal Medium

CHAPTER ONE

1.0 Introduction

Bioprocessing of cassava peels to produce the essential amino acid lysine, is critical to the development of local animal feed industry because lysine is an active ingredient in animal feed formulation. In Ghana 53,000 MT of poultry feed is imported annually costing over US $32 million. Lysine is among the 20 standard amino acids found occurring naturally in proteins. It plays a substantial role in formation of collagen and carnitine in addition to calcium absorption, (Ansari & Montazer, 2007; Nelofer et al., 2008). Humans and animals are unable to synthesis this amino acid thus it has to be supplied in food and feed, the absence of which can lead to stunted growth including protein energy malnutrition in humans and reduced tissue growth in farm animals especially poultry and pig.

Protein energy malnutrition (PEM) remains a major public health concern in Ghana and many third world low income nations (Van de Poel et al., 2007). It is a common cause of growth retardation and child mortality in many third world countries. Nearly 54% of all infant deaths were linked to PEM, making it one of the largest cause of child mortality in Ghana (Van de Poel et al., 2007). A major contributor to PEM in Ghana is the low intake of protein from animal sources partly due to the relatively high cost of meat and poultry products among many factors affecting cost of meat production, cost of feed production is of significant importance in reducing cost of animal production in Ghana (Atuahene et al., 2010) It is estimated that Ghana can increase its animal protein (poultry and pig) production by 20 % and decrease cost of production by 30% if feed production costs is reduced by 10% (SRID-MoFA, 2014). This can be achieved by increasing local production of feed. Ghana can increase production of corn which can serve as a base of feed. However, cereals which constitute between 50 - 60% of poultry and animal

feed is very low in protein and amino acids especially lysine. Hence, cereal base poultry and animal feed are traditionally supplemented with protein or essential amino acids from cheaper sources. In 2014, poultry and pig production in Ghana increased only by 7.5% and 7% respectively, but this was accompanied by a 35.5% increase in fishmeal imports to serve as protein supplements for feed production over the same period (SRID-MoFA, 2014). While this resulted in an increased production, the imported fish meal for feed production does not offer a long term solution to the challenges confronting poultry and animal feed production that will lead to sustained availability of meat at low cost to Ghanaians.

Production of lysine for animal feed supplementation is a major undertaking in many countries where low cost production of animal feed has been accomplished (Kjeldsen, 2008) . A 0.5 - 1% lysine supplementation of feed can enhance poultry/pig growth by 20% (Kjeldsen, 2008). Lysine can be produced on industrial scale by microbial fermentation of sugars. A prolific producer of lysine, *Corynebacterium glutamicum*, utilise free fermentable sugars which can be readily obtained from agricultural wastes such as cassava peels, produced in large quantities annually in Ghana (Bayitse et al., 2013), to synthesize products such as serine, glutamate, and lysine.

The processing of cassava, a major crop used in Ghana for various domestic and industrial applications generates 3.8 million tons of waste per year (Bayitse et al., 2013). Cassava is rapidly becoming an important agricultural commodity in Ghana because of deliberate effort by the government of Ghana through the Food Research Institute (FRI), to developing alternate uses for cassava and aggressively promote its industrial use. As this goal of government materialises, it is expected that the challenge of dealing with cassava waste from peels will only worsen. While this waste can and is used to a limited extent as an animal feed, its low protein content, and high cyanogenic glycosides makes it unsuitable as a major

source of feed for animal production. However, due to its high carbohydrate content of 62% (Adegbola & Asaolu, 1986), it can be used as a cheap carbon source for *C. glutamicum* to produce lysine for animal feed production, which will ultimately result in increased production of meat and reduction cost to consumers. However, the carbohydrate composition of cassava peels comprises cellulose, hemicellulose, residual starch and lignin (Kongkiattikajorn & Sornvoraweat, 2011) . The bacteria *C. glutamicum* lacks the hydrolytic enzymes to convert these complex carbohydrates to simple fermentable sugars that it can utilise.

In order that, the large amount of cassava waste generated can be utilised to produce useful amino acids, the complex carbohydrates in cassava peels needs to be hydrolysed to produce simple fermentable sugars for the commercial production of lysine with *C. glutamicum*.

1.1 Project Aim

The aim of this research was to develop a bio-process suitable for optimal conversion of the complex carbohydrates in cassava peels to simple fermentable sugars for the biosynthesis of lysine by *C. glutamicum* fermentation.

1.1.1 Specific Objectives

In pursuit of the aim above, the research reported in this thesis was performed under three specific objectives:

1. To characterise cassava peel for carbohydrate types.
2. To optimise glucose production from cassava peels by mixed enzymatic hydrolysis.

3. To optimise the condition for lysine production using mutant *Corynebacterium glutamicum* (AHP3) strain and hydrolysed cassava peel as carbon source.

1.2 Outline and background for PhD thesis

This thesis emanated from research work performed during Biowaste4SP Project. A European Union Commission sponsored project under Seventh Framework Programme for Research and Technological Development (FP7/2007-2013).

The research was conducted in conjunction with Danish Technological Institute and Technical University of Denmark, National Food Institute, Denmark. The aim of this project was to develop a bio-process suitable for optimal conversion of the complex carbohydrates in cassava peels to simple fermentable sugars for the biosynthesis of lysine by *C. glutamicum* fermentation.

This thesis is alienated into chapters where the first chapter outlines the overview of the research and explains the aim and objectives of the study as well as the methods used whiles the rest of the chapters describes the work undertaken during the PhD study.

Chapter 1 provides an overview to the thesis, and gives information on cassava peel availability and its potential as carbon source for *C. glutamicum* in lysine production. Chapter 2 reviews the literature to support the methods used in this work.

Chapters 3-5 present the scientific findings in the form of manuscripts. Chapter 3 presents characterisation of cassava peel for carbohydrate types. Chapter 4 presents enzymatic hydrolysis process optimisation of cassava peels to produce fermentable sugars using response surface methodology. Chapter 5 presents optimisation of conditions for lysine production, using mutant *Corynebacterium glutamicum* (AHP3) strain and hydrolysed

cassava peel as carbon source. Finally chapter 6 summarises the work and comments on the significance of the results.

CHAPTER TWO

2.0 Literature Review

Waste generated from agricultural activities may provide a cheap source of carbohydrate for microbial fermentation of high value added products especially amino acids (Buzzini & Martini, 1999). Amino acids form the basic building blocks of proteins, and are very essential macromolecules for human and animal functions (Ansari & Montazer, 2007). Lysine is one of the most important amino acids found in most living organisms and is vital for human and animal nutrition. Progressively, its request has been found to increase over the years (Nelofer et al., 2008). Lysine ranked second in large scale industrial amino acid production with several hundred thousand tonnes per annum (Ansari & Montazer, 2007).

Generally, most of the food supply to domestic animals and man contains limited amount of lysine. Cereals and defatted oil seeds used for feeding animals are found to contain only small amount of lysine. Consequently, it must be added to the feed formulation to offer a suitable balanced diet for poultry, cattle and other live stock (Tosaka, 1983). Human nutrition is also enhanced by adding lysine as food supplements. It has been reported in literature that lysine supplementation of cereal based foods enhances their protein quality, thereby stimulating growth and tissue synthesis (Shah, 1998). Additionally, it can be used in the pharmaceutical industries to formulate food supplements with a balanced amino acid composition as well as in the infusion of amino acids.

Lysine production is achieved through variety of ways comprising chemical synthesis, extracting from protein hydrolysate, enzymatic hydrolysis and fermentation processes, technique involving fusion of protoplasm as well as recombinant DNA technology (Nelofer et al., 2008). Fermentation has been found to be more economical and practical method of lysine biosynthesis, because the technology does not demand high temperature, high pressure

and utilises low-cost carbon sources to produce L-lysine (Ekwealor & Obeta, 2005). *Corynebacterium glutamicum* is mostly used in industries to biosynthesis amino acids particularly L-glutamate and L- lysine (Ikeda, 2003). *C. glutamicum* has the capacity to utilise different types of carbons including alcohols and organic acids as a source of energy for growth in addition to the biosynthesis of amino acid (Kircher & Pfefferle, 2001). In fermentations involving large scale processes, the utilisation of different complex sugar substrates from cane and beet has been exploited for a long time. However, glucose from the hydrolysis of corn, wheat and cassava became more prominent in recent times. The type of sugar used for the fermentation depends on its availability and geographical location of the production plant (Hermann, 2003). In addition to carbon and nitrogen sources, other inorganic and organic compounds are also utilised. These compounds include urea, casein hydrolysate, yeast extract and corn steep liquor (Coello et al., 2000).

2.1 Biomass composition and availability

The composition of biomass is found to contain cellulose, hemicellulose and lignin, in addition to little quantities of extractives. Biomass can be defined as any organic matter that can be regenerated. Wood and crops are the two major biomass of importance (e.g. woodlots, cassava, wheat, maize and rice). Another very important type of biomass that is often forgotten is waste from food and manure sources (Deswarte, 2008).

Generally, the proportion of cellulose in biomass is about 38–50% by weight and forms the largest component. Cellulose constitute a polymer of glucose, which is made up of linear chains of (1,4)-D-glucopyranose units with mean molecular weight of about 100,000. It occurs in large quantities in the biosphere and available as a cheap source of carbon for biochemical synthesis (Roewell, 1984). Unlike cellulose, hemicellulose, consists of a mixture

of five carbon sugar primarily xylose, and six carbon sugars. Xylose constitutes the second most copious reducing sugar on the earth. As compared to cellulose, hemicellulose is not adequately used as biochemical feedstock because most microorganisms cannot utilise it as a source of energy. It forms about 20–40% of the material by weight (Roewell, 1984).

Food crops can be used in biorefinery to produce various forms of products including biodiesel, ethanol and lactic acids. Nonetheless, waste and lignocellulosic materials are now considered to provide a much better alternative for biorefinery, because competition from the food sector is avoided. Out of the 170 million tonnes of biomass produced annually through photosynthesis, only 3% comes from cultivation (Sanders et al., 2005). Importantly, United States of America has the capacity to supply over one billion tons of dry biomass per annum by 2030 (USDOE & USDA, 2005).

Agricultural production in sub-Saharan Africa, especially in Ghana, is mostly depended on smallholder farm units. The size of farms cultivated by most farmers is less than 2 hectares. Apart from the small farms, there are other large plantations which are made up of rubber, oil palm and coconut and to a smaller extent, rice, cassava, maize and pineapples. The traditional means of farming using hoe and cutlass is still the major way of farming in Ghana, although there is little mechanised farming, with bullock in some places, especially in the Northern Region. Agricultural production depends to a larger extent on the type of soil, amount and distribution of rainfall in Ghana. Intercropping is mostly practiced by small scale farmers, whiles mono cropping is associated with larger-scale commercial farms (SRID-MoFA, 2010).

Cassava (*Manihot esculenta Cranz*) occupies the sixth position as the most important crop cultivated in the world. It is mostly cultivated in most countries in African, Asian, and Latin America. The root tuber of cassava contains starch and mostly used as food in tropical countries. Cassava as an industrial crop is widely used to produce animal feed, starch and in recent time for bioethanol production. Cassava thrives well on both dry and semidry lands where other crops, like cereals, cannot grow effectively (Lin et al., 2011). Among the tubers grown in Ghana, cassava ranks highest in terms of volume and coverage. About 30% of staple food consume in Ghana daily is cassava (FAO, 2000). Cassava cultivation is widely distributed in Ghana. The majority of cassava produced in the country is located in the southern and middle belts of Ghana, accounting for about 78% of all cassava produced in Ghana (SRID-MoFA, 2014). About 16.5 million metric tonnes of cassava was produced in 2014.

Processing of cassava is normally done in order to improve the storage life as well as to add value to the final products. Majority of cassava processing is done in the rural areas and mostly carried out by women. Traditionally cassava is peeled by hand using knife. Nonetheless, cassava processing is also carried out using small scale industrial processing machines. Out of the total amount of cassava tubers produced in 2014, processing of the tuber yielded about 4.4 million metric tonnes of cassava peels (OECD/IEA, 2010; SRID-MoFA, 2014). These peels are known to contain cyanide (Ofoefule & Uzodinma, 2009). Peels typically are made of the outer thin pericarp and the thicker inner core. Majority of the peeling processes take out the pericarp and the thicker core in addition to some flesh attached to the peels. Chemical composition analysis of cassava peels has been reported in literature as dry matter 86.5–94.5%; crude protein 4.1–6.5%; hemicellulose and cellulose 34.4%; and lignin 8.4% (Kongkiattikajorn & Sornvoraweat, 2011).

2.1.1. Cellulose

Cellulose is a major polymer in plant cell walls and very stable molecule which is known to be recalcitrant (Wolfenden & Snider, 2001; Peciulyte et al., 2014). The principal structure of cellulose consists of chains of glucose linked by β-1,4 linkages. These chains are joined by strong hydrogen bond to form microfibrils, making it crystalline in nature and very difficult to destroy. The amorphous portions of the cellulose is easier to destroy (Van Dyk & Pletschke, 2012). The cellulose is further submerged in a matrix of hemicellulose, pectin and lignin (Eriksson, 2009). Lignin and hemicellulose are embedded in the spaces between cellulose microfibrils in primary and secondary cell walls, together with the middle lamellae (Eriksson, 2009).

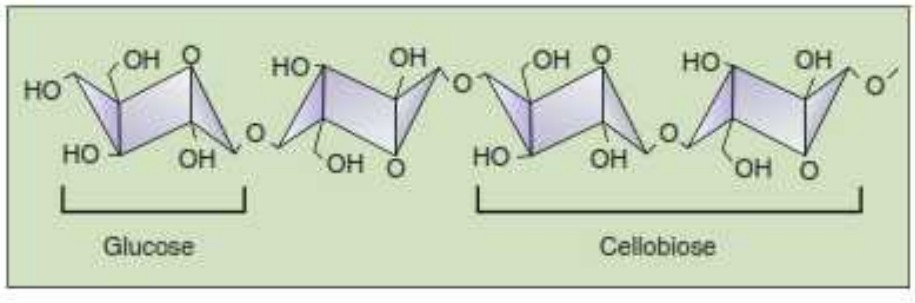

Figure 2.1: Molecular chain structure of cellulose (Zugenmaier, 2001)

The molecular structure of cellulose, has been reported in literature to compose of amorphous regions with chains located at the surface, whereas crystalline components occupy the core of the microfibril (Figure 2.2) (Larsson et al., 1997).

Figure 2.2: Crystalline cellulose is in the core of the microfibril, and it is surrounded by amorphous substrate.

Cellulose exists in seven crystal structures known as celluloses Iα, Iβ, II, III_I, III_{II}, IV_I and IV_{II} (O'Sullivan, 1997). Naturally cellulose Iα and Iß are the most abundant crystal forms. Iα polymorph is meta-stabile, and hence, more reactive than Iß. The percentage of Iα polymorph in crystalline cellulose varies from 70 % in bacterial cellulose (O'Sullivan, 1997), 64 % in II cellulose isolated from algae *Valonia ventricosa*, to 20 % in ramie and cotton cellulose (Yamamoto & Horii, 1994). The co-existence of two polymorphs of native cellulose, which have different stabilities, may imply that the part of the Iα polymorph within the microfibril is most prone to the enzymatic attack (Andersen et al., 2007).

2.1.2 Hemicellulose

The compositional structure of hemicellulose is more diverse than cellulose and made up of branched heteropolymer of pentose (D-xylose and D-arabinose) and hexose (D-mannose, D-glucose and D-galactose) sugars (Kumar & Singh, 2008; Beg et al., 2001). Xylan forms the most part of hemicellulose in nature and made up of β-D-xylopyranosyl residues linked by β- 1,4-glycosidic bonds (Beg et al., 2001; Kumar & Singh, 2008). In plants, the xylan forms an overlying layer through hydrogen bonding with the cellulose, and covalently bonded with lignin to form an outside sheath to protect the plant (Beg et al., 2001). 30-35% of total dry weight of plant cell wall is made up of xylan which may differ

between plants. Heteroxylan is the major constituent of xylan , which is made up of xylose residues in the backbone with acetate, arabinose and glucose residues as common substituents (Sunna, 1997; Beg et al., 2001)

Hemicelluloses are more soluble than cellulose because of their branch structure and they can be removed from wood by extraction. They are easily hydrolysed by strong acid or base leaving cellulose and lignin intact (Lloyd & Wyman, 2005; Fan et al., 1982). In many cases diluted sulphuric acid and pretreatment under elevated temperatures will digest most of the hemicellulose to soluble pentose and hexose sugars (Lloyd & Wyman, 2005). Even though this treatment is not intended to degrade lignin, the lignin structure is altered and redistributed leading to much more favourable substrate for enzymatic hydrolysis (Yang & Wyman, 2004).

2.1.3. Lignin

Lignin is considered to be the most complex and the least characterised molecular group among the wood components. Lignin is made up of *p*hydroxyphenoyl, guaiacyl and/or syringyl monomers linked in three dimensions. These three monomers differ in the methoxylation pattern of the aromatic ring (Douglas, 1996). Within the cell wall, lignin has several functions such as changing the permeability and thermal stability, but it has the primary function of providing strength and rigidity to plant tissue (Haghdan et al., 2016). Lignin composition in woody plants is about 20-35% and vary according to species, cell type and stage of development (Fan et al., 1982).

In bioconversion of lignocellulose, lignin can be an obstacle which can prevent enzymatic hydrolysis of substrate (Rahikainen et al., 2013). When lignin is removed, the pretreated substrate becomes more porous , thereby making cellulose and hemicellulose more accessible and open for enzymatic hydrolysis (Taherzadeh & Karimi, 2007). In literature it

has been reported that enzymatic saccharification of lignocellulosic materials can be affected by the structure of lignin (Zhang et al., 2011). Lignin can also adsorb enzymes and then prevent these enzymes from attacking the cellulose and hemicellulose. The interactions involving hydrogen bonding, electrostatic and hydrophobic are thought to be potential reasons for the adsorption of enzyme onto lignin (Rahikainen et al., 2013).

2.1.4 Starch

Starch is stored in most plant as a carbohydrate food which serves as the main source of energy for organisms which cannot photosynthesise their own food. It is copious in nature and mostly found in seeds, roots and tubers, but are also found in stems, leaves, fruits and even pollen. Starch is made up of two discrete polysaccharides, amylose and amylopectin (Gallant et al., 1992; Kessler, 2008). Amylose is fundamentally a linear polymer, soluble in water containing α-1,4-linked D-glucopyranose, whiles amylopectin is extremely branched polymer, and not soluble in water containing amylose chains connected at branch points by α-1,6-bonds (Figure 2.3). Literature report shows that amylopectin forms the major component of starch with about (73-86 %) whiles amylose is the minor (14-27 %) (Kessler, 2008).

Starch normally swells up when the gelatinisation temperature is reached in boiling water. The taking up of water increases the viscosity which can change the chemical and enzymatic degradability of starch. The cooling process of amylose leads to fractional recrystalisation which is referred to as retro-gradation (Kessler, 2008).

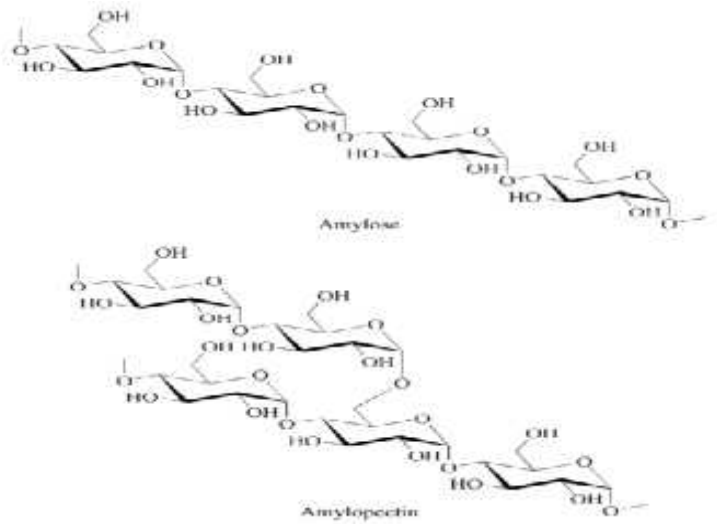

Figure 2.3: Structure of amylose and amylopectin ((Kessler, 2008)

2.2 Enzymes

The word “enzyme”, meaning “from yeast” was reportedly coined by KUHNE in 1887 to denote a catalytic substance derived from yeast. Enzymes can be defined as proteins with catalytic activity (Smith, 2001).

Enzymes have evolved in nature over millions of years to work in an explicit environmental conditions in spite of their wide diversity, although their natural and industrial environments are usually significantly different (Johannes & Zhao, 2006). The role of microbial enzymes in metabolic catalysis cannot be overemphasised, most importantly because they are used in various industrial applications. Enzymes are widely used in industrial applications to produce more than 500 products hence they are in high demand (Adrio & Demain, 2005; Kumar & Singh, 2013). There is an increased desire for industrial enzyme utilisation over the years because of the growing need for finding sustainable solutions to biochemical problems. Microorganisms have been used as one of the major sources for producing many enzymes (Demain & Adrio, 2008). The use of chemicals in industrial applications to produce

chemicals and pharmaceuticals are confronted with many problems including inefficient catalysis, because the processes require extreme pressure and temperature, as well as very low pH (Adrio & Demain, 2014). Enzymes are most appropriate for these applications because they can work under mild reaction conditions to provide the same output. They also accelerate biochemical processes as well as working on unnatural substrates (Hibbert et al., 2005; Johnson, 2013). Additionally, enzymes can be genetically altered to improve upon their vital properties such as stability, substrate specificity and specific activity. However, enzymes have limitations in their applications because certain enzymes require co-factors to function optimally. This challenge can be surmounted through cofactor recycling and use of whole cells (Adrio & Demain, 2014; Hibbert et al., 2005).

The filamentous fungus *Trichoderma reesei* is one of the main microorganisms use for cellulase production for industrial and research use to degrade complex polysaccharides (Diener et al., 2004; Peterson & Nevalainen, 2012). Enzymatic hydrolysis of cellulose is mostly considered as a suitable means of producing simple sugars , which can be used in biorefinery to produce bioethanol through microbial fermentation (Otero et al., 2007). For effective enzymatic hydrolysis of cellulose, a number of enzymes with varying activities are required (Mansfield et al., 1999; Yang et al., 2011).

Literature has shown that efficient hydrolysis of cellulose to glucose demands the use of three different types of enzymes namely, β-1-4- endoglucanase β-1-4- exoglucanase and β-glucosidase (Mansfield et al., 1999). Each one of these components may have glycosidic bonds and may exist in numerous forms, and may have a distinct range of activities on different cellulosic substrates. Work conducted by other researchers showed that additional enzymes such as glucohydrolases (134) and cellobiose dehydrogenase (3, 99) may also play some role in cellulose digestion (Mansfield et al., 1999).

2.2.1 Enzyme mechanism

Experimentally the characteristic feature of an enzyme-catalysed reaction is that it follows saturation or Michaelis-Menten kinetics characteristic (Kirby, 1996).

$$E + S \underset{k_r}{\overset{k_f}{\rightleftharpoons}} ES \xrightarrow{k_{cat}} E + P \qquad (1)$$

Where E and S denote enzyme and substrate molecules, ES is an enzyme substrate complex, P is a product molecule, and k_f, k_r and k_{cat} are the rate constants. The chemical rate equations for the concentrations [ES], [E], [P] are

$$d[ES]/dt = k_f[S][E]-(k_r + k_{cat})[ES] \qquad (2)$$

$$d[E]/dt = -k_f[S][E] + (k_r + k_{cat})[ES] \qquad (3)$$

$$d[P]/dt = k_{cat}[ES] \qquad (4)$$

When S is in excess, [S]>[E], so that its concentration [S] does not depend on time, the steady-state rate v = d[P]/dt of creation of product molecules is

$$v = \frac{V_{max}[S]}{[S] + K_M} \qquad (5)$$

where $V_{max} = k_{cat}[E]_T$ is the maximum enzyme velocity, [E]T = [E] + [ES] is the total enzyme concentration, and KM =$(k_r + k_{cat})/k1$ is the Michaelis–Menten constant. The rate V_{max} is the maximum product generation rate that is reached at high (saturating) substrate concentrations, and K_M is the substrate concentration at which the rate v is half of its maximum V_{max} (Figure 2.4) (Chen et al., 2010).

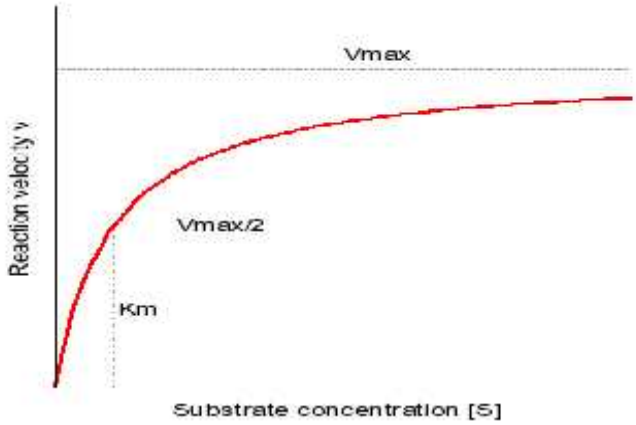

Figure 2.4: Michaelis-Menten saturation curve for an enzyme reaction showing the relation between the substrate concentration and reaction rate.

(https://en.wikipedia.org/wiki/Michaelis–Menten_kinetics)

2.2.2 Enzyme activity

The activity of a naturally occurring enzyme is mostly found to be of less commercial or therapeutic relevance because the activity is hence, it is always important to enhance enzyme activity by genetic manipulation or directed evolution. Literature has shown that eleven rounds of DNA shuffling of the enzyme glyphosate N-acetyltransferase (GAT) could result in an improvement of variant with a 10,000-fold catalytic efficiency compared with that of the parent enzyme (Castle et al., 2004). In addition to the improvement in catalytic efficiency, thermostability was also increased (Siehl et al., 2005).

The use of directed evolution was demonstrated in producing α-amylase from barley, which was used for cold starch hydrolysis to produce corn sweeteners and bioethanol. DNA and epPCR were used in three fold shuffling to engineer α-amylase from barley with an increase in total activity by 1000-fold compared with that of the wild type enzyme (Textor et al., 1998; Wong et al., 2004).

2.2.3 Enzyme stability

Enzyme applications in many industrial settings require that the enzymes must function at high temperatures or in hash environment especially in organic solvents without affecting its activity (Johannes & Zhao, 2006). In order to improve on the stability of these enzymes, many technologies including Diversa's Gene Site Saturation Mutagenesis™ has been employed over the years to enhance the high temperature stability of several different enzymes. Literature reports that melting temperatures of these new enzymes were improved radically as compared to those of the wild type enzymes (Solbak et al., 2005).

Industrial application of enzymes is possible only if the enzymes are stabilised against extreme temperature and pH as well as the presence of salts, alkalis and surfactants. Major applications of enzymes at high temperatures are seen in washing (60-70 °C); starch gelatinization (100 °C); textile desizing (80-90 °C) as well as under high salt concentrations as found in food industry (Iyer & Ananthanarayan, 2008).

2.2.3.1 Temperature stability

The ability of enzymes to withstand high temperatures is clearly advantageous because of a higher reactivity, thus an increase in reaction temperature from 25 to 75 8C will result in about a 100-fold increase in the process rate), fewer diffusional limitations and lower viscosity of the medium, increased solubility of both substrates and product, decreased bacterial contamination (which is very important in food and pharmaceutical industry) and a shift in thermodynamic equilibrium in case of endothermic reactions (Illanes, 1999; Iyer & Ananthanarayan, 2008; Klibanov, 1983). These advantages overrides the infrastructure and processing costs incurred as a result of stringent requirement of materials, harder post-reaction inactivation and restrictions in case of labile substrates or products (Illanes, 1999). Reported cases of temperature related biocatalysed systems for bioconversion include:

conversion of glucose to high fructose at 60-65 °C; α-amylase (85-110 °C) and glucoamylase (55-65 °C) catalysed starch hydrolysis; cellulase hydrolysis of cellulose at 65-70 °C (Iyer & Ananthanarayan, 2008; Klibanov, 1983).

2.2.4 Factors affecting enzyme activity

Enzyme activity is mostly affected by its environmental conditions. Changing the enzyme's environment can affect the rate of reaction of the enzyme. The reaction conditions namely pH, substrate concentration, temperature, ionic strength and the buffering condition affect the rate of enzymatic reactions. Literature reports show that in order to produce a reliable data on enzyme activity, the parameters being considered should be kept constant in evaluating the suitable conditions for assaying the enzyme activity (Shyu et al., 2012).

2.2.4.1 Effect of pH

The pH of a solution plays a vital role in the activity of any biological molecule. A change in pH of a solution has an effect on the charged state of ionisable groups at the surface and/or contained within an enzyme. When the charge is changed it often results in the change of proton donation of residues within an enzyme and can lead to denaturation, non-denaturing structural changes, dissociation of oligomers, and/or changes in activity rate (Grahame et al., 2015). Enzymes will show optimum activity within a narrow range of pH which can vary over a relatively wide range. In experiments involving enzymatic catalysis, the pH of the solution of the reaction mixture must be kept in an optimal condition to prevent pH-induced protein conformational changes, which can lead to reduction or loss of enzyme activity. When a buffered solution has its pH altered with a component of pKa at or near the desired pH level of the reaction mixture, it is only the stability of the environment that can provide the enzyme with optimum catalytic efficiency. Thus, the appropriate pH range of an enzyme must be determined in advance when optimising assay conditions (Shyu et al., 2012).

2.2.4.2 Effect of temperature

Temperature plays a very important role in enzyme activity (EA). Conceptually, this can be seen as the amount of free energy available to assist the reaction in surmounting that transition state energy barrier (Grahame et al., 2015). When reactions occur at room temperature without the involvement of an enzyme, only small proportion of reactants will have enough energy levels to participate in the reaction (Figure 2.5A). However, when the temperature is elevated above room temperature, more reactant molecules will gain enough energy to take part in the reaction (Figure 2.5B). This has not affected EA, but the distribution of energy-sufficient reactants is shifted to a higher average energy level. In an enzyme catalysed reaction, the EA is lowered significantly, and the proportion of reactant molecules at an energy level above the activation energy is also greatly increased (Figure 2.5C) (Shyu et al., 2012).

This is an indication that rate of reaction will be high and will favour the promotion of product formation. In most enzyme-catalysed reactions the rate of reaction is affected when there is an increase in thermal stability of the enzyme couple with temperature increase. When the critical temperature is exceeded, the activity of the enzyme will significantly be reduced. But when the enzyme is maintained within this critical temperature range, the enzyme activity will be constant at a relatively high level, thereby preventing the enzyme from inactivation. The activation energy of the enzyme is lowered when the temperature is increased thereby affecting the rate of reaction, this is expressed by the Arrhenius equation: $k = A \exp(-EA/RT)$, where A is a constant related to collision probability of reactant molecules, R denotes ideal gas constant (1.987 cal/mol -deg), T denotes temperature in degrees Kelvin (K = ◦C + 273.15), and k represents the specific rate constant for any rate, that is, kcat or Vmax. (Grahame et al., 2015; Shyu et al., 2012).

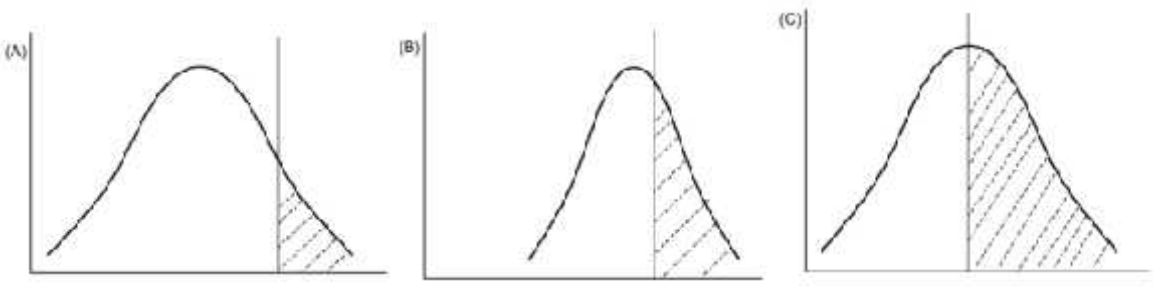

Figure 2.5: Plots showing effect of temperature on the molecular energy of the reactants. (A) Energy distribution of the reactant molecules at room temperature without an enzyme. (B) At a temperature higher than room temperature without enzyme. (C) At a room temperature with enzyme addition. The vertical line indicates the activation energy. The shaded portion shows the amount of reactant molecules that have adequate energy for reactivity. The x-axis shows the energy level of reactant molecules, while the y -axis represents the frequency of reactant molecules at an energy level (Shyu et al., 2012).

2.2.4.3 Effect of substrate concentration and inhibitors

The traditional enzyme has a hyperbolic response to substrate concentration, according to the Michaelis-Menten equation (Scopes, 2002). Generally, substrate concentration should not be kept below that of the enzyme in order to avoid substrate concentration dependency of the reaction rate at low substrate concentrations. The rate of an enzyme-catalysed reaction is

linear to the enzyme concentration at constant substrate concentration. The measurement of the initial rate will be invalid and non linear until the substrate is depleted (Shyu et al., 2012). Initial experiments must be conducted to determine the appropriate range of substrate concentrations over a number of enzyme concentrations to avoid the occurrence of substrate depletion. The presence of inhibitors can affect the activity of enzymes by reducing or masking it thereby reducing the velocity of enzyme catalysed reaction. (Shyu et al., 2012; Smith, 2001).

2.2.5 Cellulase

Cellulases are enzymes that cleave β -1,4 bonds in cellulose chains. They are produced by fungi, bacteria, protozoans, plants, and animals. This enzyme is divided into two classes namely endo-cellulases (endoglucanase,) and exocellulases (cellobiohydrolase,). The ability of cellulases to catalyse a reaction have been classified into numerous families based on their amino acid sequences and crystal structures (Henrissat et al., 1991; Kessler, 2008; Zhang & Zhang, 2013). The EG-type cellulases undergo catalysis of endo-hydrolysis of 1,4-β-D-glycosidic bonds in cellulose, lichenin, and cereal β-D-glucans. CBH-type cellulases can hydrolyse the 1,4-β-D-glycosidic bond in cellulose and cellotetraose, releasing cellobiose from the nonreducing ends of the cellulose chain. Cellulases contain noncatalytic carbohydrate binding modules (CBMs) and/or other functionally known or unknown modules, which may be situated at the N- or C-terminus of a catalytic module. The presence of aCBM is of particular importance for binding of the enzyme on insoluble and crystalline cellulose and for hydrolytic effects (Kessler, 2008; Olsen & Falholt, 1998; Zhang & Zhang, 2013). Both EG and CBH type of cellulases can contain linkers and cellulose binding domains. The cellulase enzyme molecule is made up of three types of functionally different domains, Figure 2.6 (Kessler, 2008).

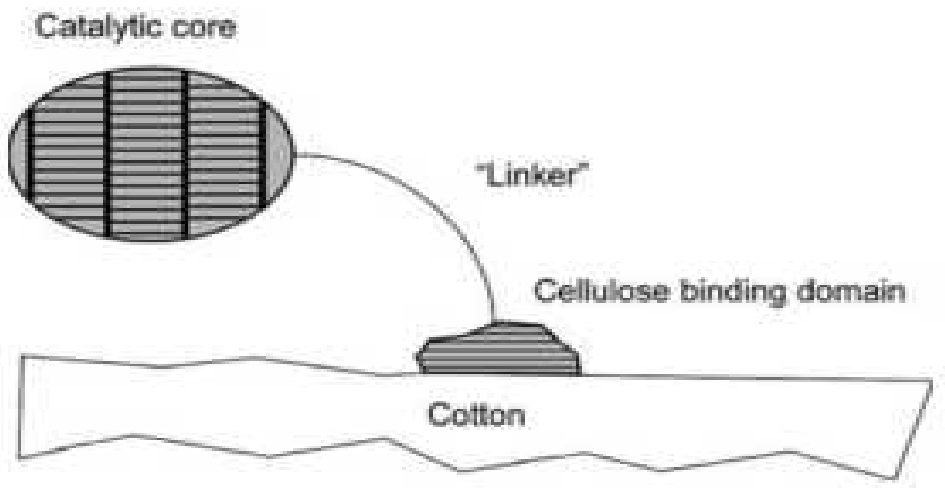

Figure 2.6: Schematic presentation of a multi-domain cellulase adsorbed to cellulose substrate. The enzyme having a catalytic core, a linker, and a cellulose-binding domain (Kessler, 2008; Olsen & Falholt, 1998).

2.2.6 β-Glucosidase

β-glucosidases (BG) belong to cellulose enzyme complex and are promising candidates in biotechnological applications. They are classified as glycoside hydrolases based on the type of reaction they catalyse. β-Glucosidases that do not contain a CBM hydrolyse soluble cellodextrins and cellobiose to glucose. The activity of BG on insoluble cellulose is negligible. BGs degrade cellobiose, which is a known inhibitor of cellobiohydrolases (CBHs) and endoglucanase (Bairoch, 2000; Tiwari et al., 2013; X. Zhang & Zhang, 2013).

2.2.7 Amylases

Amylases are enzymes which cleave starch at specific points . They digest starch and related compounds by hydrolysing the α-1,4 and/or α-1,6 glucosidic linkages in an endo or an exo acting manner (Upadek & Kottwitz, 1997). The α-amylases are calcium metalloenzymes, and cannot function in the absence of calcium. α-amylase is an endoenzyme which breaks down long-chain carbohydrates by cleaving randomly α-1,4 glycosidic linkages (Upadek &

Kottwitz, 1997) (Patil & Patil, 2000) at positions along the starch molecule, thereby producing maltotriose and maltose from amylose, or maltose, glucose and "limit dextrin" from amylopectin. The action of α-amylase on starch tends to be fasterthan β-amylase because its hydrolitic action is not restricted along the substrate (Das et al., 2011; Kessler, 2008).

2.2.8 Amyloglucosidase

Amyloglucosidase (EC 3.2.1.3) is exo-enzyme which catalyses in a stepwise manner the hydrolysis of α (1,4) glycosidic bonds from the non reducing ends to release glucose. The hydrolysis of α (1,6) branch links are relatively slow forming glucose syrups with glucose content of 97% (w/w) (Patil & Patil, 2000).

2.3 Enzymatic Hydrolysis

Cellulose is a linear condensation polymer which is made up of D-anhydroglucopyranose linked by β-1,4-glycosidic bonds (Zhang & Lynd, 2004). Cellulose hydrolysis using enzymes is normally defined as a heterogeneous reaction system, because both the highly and less ordered regions of the cellulose are involved in the enzymatic reaction (Arantes & Saddler, 2010a). The rate catalysed reaction of fungal cellulase in cellulose hydrolysis is classically 3-30 times faster in amorphous cellulose than high crystalline cellulose (Lynd et al., 2002).

The core role of enzyme is to speed up the rate of enzymatic reaction. However, literature shows that the final reducing sugar concentration in lignocellulosic biomass hydrolysis reaction is considerably affected at constant substrate concentration when the enzyme load is varied (Bommarius et al., 2008). This occurrence is normally seen in both the hydrolysis of lignocellulosic biomass and pure cellulosic substrate, such as filter paper although filter paper has no lignin to inhibit the reaction. The rate of reaction in any catalysed system can change

but not the equilibrium. Consequently, in thermodynamics the enzyme loading affects only the rate of reaction, but not the final sugar concentration when the substrate concentration is kept constant. The phenomenon change in the behaviour of enzymes in lignocellulosic hydrolysis is attributed enzymes not stable to heat, inhibition from product, and substrate transformation into a form which is more difficult to digest. Additionally, enzymes can also be denatured when excessive force is applied during agitation (Taneda et al., 2012).

Compounds which contained β-d-glucosidic bonds are usually hydrolysed with β-glucosidase enzyme (Shewale, 1982). Literature shows that cellulase enzyme complex comprising exo β-1,4-glucanase, endo-β-1,4-glucanase and β-glucosidase, are responsible for the hydrolysis of crystalline cellulose. Endo-glucanase hydrolyses cellulose by breaking the internal bonds of β-1,4-glucosides along the cellulose link, to release more reactive ends for exo-glucanase to cleave. The two ends of the shorter cellulose chain containing cellobiose are hydrolysed by exoglucanase (Ogeda et al., 2012). The amorphous regions of the cellulose are mostly attacked during the hydrolysis reaction (Shewale, 1982). Prolong use of endo-glucanase normally results in the production of cellobiose and higher cellodextrins. β-glucosidase then hydrolyses these products to glucose. β-glucosidase is important in cellulose hydrolysis because it plays a significant role in removing cellobiose which is known inhibitor of exo and endo glucanases (Shewale, 1982).

Cassava has a high level of starch, which can be used as food and also in industries as raw materials. Literature shows that amyloglucosidase and α-amylase is mostly used to hydrolyse cassava starch to achieve more than 90% hydrolysis efficiency (Collares et al., 2012). However, organic acid pretreatment before hydrolysis of cassava peel into monosaccharides was also reported (Olanbiwoninu & Odunfa, 2015).

2.3.1 Efficiency of hydrolysis and product yield

The efficiency of substrate hydrolysis with enzymes can be determined through various methods deppending on the objectives of a particular study. In biotechnology the focus is placed on the efficiency of the bioconversion to monomer sugars as well as the yield (Van Dyk & Pletschke, 2012). In most cases, the efficiency of the hydrolysis is measured by the amount of glucose yield. Sometimes, the yield of other sugar monomers such as xylose, mannose and arabinose may also be determined . However in order to determine the yield of glucose, variety of enzymes in different proportions are needed during the hydrolysis (Banerjee et al., 2010c). The interactive effect of more that one enzyme on substrate hydrolysis to understant their mechanism and synergy can be studied using other methods. Dinitrosalicylic acid (DNS) assay or the Somogyi-Neslson assay are used to reduce sugar formation by measuring the rate of enzyme activity. This methods have their limitations because they cannot measure reducing sugars therefore cannot give the true picture of product yield (Van Dyk & Pletschke, 2012).

2.3.1.1 Yield determination of sugar monomers

Yield determination of sugar monomers can be done in two stages. Firstly, initial substrate composition must be determined to know the overall carbohydrate content. This is followed by quantification of the total cellulose and hemicellulose. Secondly, the resultant sugar yield through enzymatic hydrolysis into the aqueous phase must be appropriately quantified (Van Dyk & Pletschke, 2012). Overestimation of yield has been reported in literature especially at high solid loadings (Kristensen et al., 2009). The substrate composition is always determined first before the yield is calculated. The protocol for determining lignin and carbohydrate content of lignocellulose is published in literature (Sluiter et al., 2008). Although pretreatment degrades the polysaccharide fraction of the biomass, yield determination is

always done after pretreatment. Consequently, the loss of sugars during pretreatment must be accounted for in the final calculations (Kumar & Wyman, 2009).

There are different methods used in yield determination of sugar after enzymatic hydrolysis. One method is to use DNS assay. This assay cannot determine the activity of the enzyme hence it is always necessary to measure enzyme activity by different method before using it to determine reducing sugars. Such assays also respond differently to different sugars depending on the type of sugar used as standard. Hence, yield determination should be done based on the reducing sugars produced during enzymatic hydrolysis (Van Dyk & Pletschke, 2012). The monomer sugars are mostly measured using High Powered Liquid Chromatography (HPLC) and various columns and methods (Sluiter et al., 2008). The yield is normally calculated in percentages based on the original substrate composition after quantifying the monomer sugars. The estimated yield of sugars in a hydrolysate can be affected by lignin, proteins and ash together with hydrated substrate. These combine factors may cause significant underestimation of the yield. Consequently, National Renewable Energy Laboratory (NREL) proposed a standard calculation for yield.

$$\text{Percentage Hydrolysis} = \frac{\text{Glc} + 1.0526 \times \text{Cel}}{1.111 \times F_{cellulose} \times \text{Ini. Sol.} \times 100}$$

Glc - glucose concentration in the hydrolysate

Cel - cellobiose concentration in the hydrolysate

$F_{cellulose}$ - cellulose concentration in the substrate

Ini. Sol - initial solid concentration in g/L

The assumption for the equation is based on fixed specific gravity of 1.000 g/ml, at constant reaction volume. This equation is only used to measure glucose and cellobiose yields. Nonetheless, it can be modified to determine carbon five sugars (Kumar & Wyman, 2009).

However, Garcia-Aparicio et al (2007) used a simplified method to estimate percentage hydrolysis yield

$$\text{Hydrolysis Yield (\%)} = \frac{\text{glucose (g)} \times 0.9}{\text{Polysaccharides in substrate (g)} \times 100}$$

0.9 represents the correction factor for the water hydration during hydrolysis. For xylan the value is 0.88 whiles 0.95 is for cellobiose.

In literature these calculations are accepted as the standard method for yield determination. Nonetheless, many writers have criticised the flaws in these methods as a result of overestimation of yield, specifically when economic yield is dependent on high substrate loading (Kristensen et al., 2009a). They further reiterated that the assumptions in the standard calculation are the main shortcoming. Because there are variations in initial and final solid water ratio, when using high substrate loadings (Kristensen et al., 2009a). Zhu et al. (2011) in their work proposed liquid density determination followed by solid concentration measurement as the best way of calculating yield, although this could be time consuming and quite difficult. Consequently, Kristensen et al. (2009) suggested a 10 fold sample slurry dilution, preceding the removal of solids and measurement of sugars in the filtrate based on the diluted volume. This accounts for error reduction from 30% to 35%, providing more accuracy for yield estimation.

2.3.2 Factors affecting enzymatic hydrolysis

The hydrolysis of polysaccharides using enzymes is a heterogeneous process which is influenced by the interaction between the enzymes and the polysaccharide and divided into four main steps (Azevedo & Reis, 2005): during the first step enzymes diffuse to the solid surface from the bulk solution; in the second stage enzymes are adsorbed on the substrate, to form the enzyme-substrate complex; the third stage involved the catalysis of the hydrolysis reaction; and the fourth stage deals with the formation of hydrolysed product. The adsorption and rate of hydrolysis reaction is influenced by both the physical and chemical properties of the substrate as well as the innate characteristics of a specific enzyme. pH and temperature also play important roles because they have effect on the activity of enzymes and the property of the substrate (Azevedo & Reis, 2005).

2.3.2.1 Accessibility of cellulose structure to reactivity

Cellulose is a non-soluble polymer which consists of glucose units linked together with β-(1-4) glucosidic bonds. Substantial amount of research has been conducted over the years to bring out new ideas to help understand its molecular structure (Ding & Himmel, 2006; Somerville et al., 2004). In literature, it has been established that higher degree of fibrillar aggregation produces a more compact fibre structure, which contains smaller interstices therefore affecting accessibility of the internal surface area (Krässing, 1993). The permeability of the highly ordered regions of cellulose is difficult because the cellulose molecules are closely packed thereby preventing water molecules from passing through easily (Krässing, 1993). The limited accessibility to these regions affects their ability to swell and also impede the actions of cellulases. Structurally, it is clear that degrading enzymes can only have access to molecules on the surface of the cellulose (Arantes & Saddler, 2010b).

Because enzyme catalysed reactions of cellulosic materials mostly occur on the surface, the rate of hydrolysis is influenced by the available surface area. In literature it has been shown that the resistance of cellulose to cellulase hydrolysis is influenced by the closely packed cellulose structure (Laureano-Perez et al., 2005).

2.3.2.2 Effect of temperature

Enzymatic hydrolysis employs enzymes as biocatalysts in a biochemical reaction to increase the rate of hydrolysis without being used up in the reaction. Enzyme activity is affected by temperature. The rate of the enzyme reaction increases with increasing temperature and reduces at low temperature. At very high or low temperatures enzymes can be denatured thereby making them ineffective. All enzymes have optimum temperature at which they are most effective although they can also work within a specific range of temperatures. Hydrolysis rate of pretreated soybean straw is found to increase with increasing temperature, but decreases when temperature exceeds 50 ^{o}C. This has been attributed to the denaturing of endoglucanase and cellobiohydrolase by heat (Xu et al., 2007). The optimum temperature for hydrolysing different lignocellulosic materials was also found to be 50 ^{o}C (Srinorakutara et al., 2014; Xiao et al., 2012).

2.3.2.3 Effect of pH

Changes in pH can cause the ionic bonds that hold the tertiary structure of the enzyme to break. This can cause the enzyme to denature, especially by changing the shape of the active site, thereby preventing the substrate from fitting into it. It can also affect the charges on the amino acids within the active site thereby preventing the formation of enzyme-substrate complex (Grahame et al., 2015).

An optimised pH of 5 was reported for enzymatic hydrolysis of sugarcane trashes with cellulase (Srinorakutara et al., 2014).

2.3.2.4 Effect of time

The time for enzymatic hydrolysis is important to allow adequate time for enzyme substrate interaction to produce hydrolysate. The balance between time of hydrolysis and product formation provides the basis for optimisation. The hydrolysis of pretreated soybean straw over 48 hours was reported in literature to have reduction in the rate of hydrolysis and reducing sugar after optimum time is reached. This behaviour might be due to the inhibition of the enzyme action by the accumulated hydrolysis products (Xu et al., 2007).

2.3.2.5 Effect of substrate concentration

Bioconversion of lignocellulosic material into value added products, demands the use of biomass within the locality rather than sourcing from other places which required transportation over long distances. This may affect the economic benefits of the project. The effect of substrate concentration on enzymatic hydrolysis varies with the substrate type and solid loading. For pretreated soybean straw increasing the substrate loading beyond 5% reduced the rate of hydrolysis due to stirring difficulties. However, glucose concentration increased with increasing solid loadings of olive tree pruning biomass up to 30% (w/v) (Xu et al., 2007;Cara et al., 2007; Kristensen et al., 2009b). This result is in agreement with literature, as most studies performed at high-solids loadings sacrifice conversion for a more concentrated glucose product (Jørgensen et al., 2007).

2.4 Amino acids

Out of the over 700 amino acids found in nature, most of them exist as α-amino acids. These α-amino acids are basically provided by bacteria, fungi, algae and other plants, which may occur either as constituents of peptides and proteins and other types of amide, and of alkylated and esterified structures (Barrett & Elmore, 2004).

The twenty amino acids that are responsible for protein synthesis in living cells under the influence of genes are considered special because they are fundamental to all life forms as building blocks for peptides and proteins. Peptides and proteins play a wide variety of roles in living organisms and display a range of properties which include the potent hormonal activity of some small peptides to the structural support and protection for the organism shown by insoluble proteins as well as animal nutrition and maintenance of health (Barrett & Elmore, 2004; Leuchtenberger et al., 2005).

2.4.1 Protein amino acids

The twenty L-amino acids are made up of nineteen α-amino acids and one α-imino acid (Figure 2.7). They are normally linked in vivo through their carboxy group to tRNA to form α-aminoacyl-tRNAs, and are organised by ribosomal action into specific sequences in accordance with the genetic code. Out of the twenty standard protein amino acids, nine are referred to as essential. These are L-valine, L-leucine, L-isoleucine, L-lysine, L-threonine, L-methionine, L-histidine, L-phenylalanine, and L-tryptophan. They are essential because they cannot be synthesised by both animals and humans but must be ingested with feed or food (Barrett & Elmore, 2004; Leuchtenberger et al., 2005).

Conventionally, the twenty amino acids occurring naturally can be categorised into different groups based on their chemical structure and properties (Baynes & Dominiczak, 2010). Among the twenty amino acids, the acidic or basic side chains at neutral pH forms the

majority. Five of the essential amino acids (glutamic acid, aspartic acid, arginine, histidine and lysine) are among the majority of amino acids which are acidic or basic side chain at neutral pH. These safe, biodegradable acids were utilised in functionalising some of the ionic liquids (Kagimoto et al., 2006; Ohno & Fukumoto, 2007).

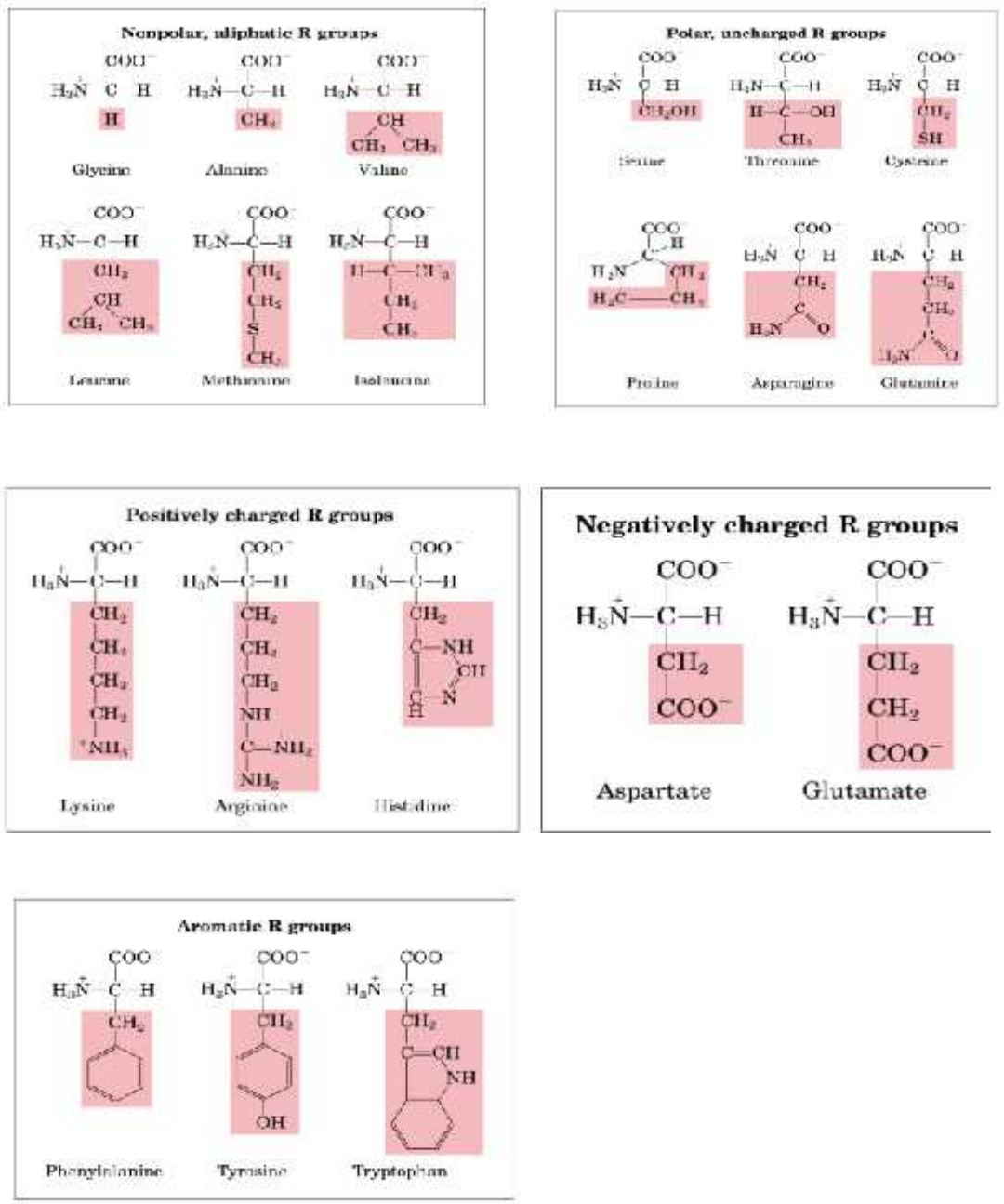

Figure 2.7: Conventional molecular structures of the twenty amino acids (Nelson & Cox, 2000).

The amino acid has a terminal NH_3^+ group which serves as an important site on proteins for many post-translational modifications. The terminal amino group of lysine side chain also makes this amino acid very reactive and involved in many reactions including carbonyl-amine interactions. Thus, lysine can become unavailable in different food systems because it takes part in different interactions with amino acid side chains or with other food components including carbohydrates or lipids (Tomé & Bos, 2007).

2.4.1.1 Lysine

Lysine is mostly known to be the most lacking amino acid in the food supply of both human and domestic animals for meat production. In humans, it helps in calcium absorption, muscle building, production of enzymes and hormones. It is normally added to cereals and oil seeds in animal feed formulation, because they have only a small amount of lysine. Poultry, pigs and other livestock cannot synthesise this amino acid. Consequently, it must be formulated into the animal feed to provide a balance diet. It can also be used to fortify human foods which do not have this vital amino acid (Ansari & Montazer, 2007; Tosaka et al., 1983; Nollet & Toldrá, 2012).

L-lysine (Figure 2.8) occupies the second position in the amino acid production industry with its largest application in animal feeds. It is the limiting amino acid while optimising the growth and production of lean meat in cattle and poultry. L-lysine is used as a supplement or mixed with low protein diets to enhance the available amino acid concentration. Extensive studies were done on the dietary requirements and the subsequent effects of L-lysine on poultry, cattle and fish. Dietary L-lysine supplementation is reported to improve the breast meat yield, feed to gain ratio and weight gain in broilers (Bulbul et al., 2015; Corzo et al., 2012; Siqueira et al., 2013).

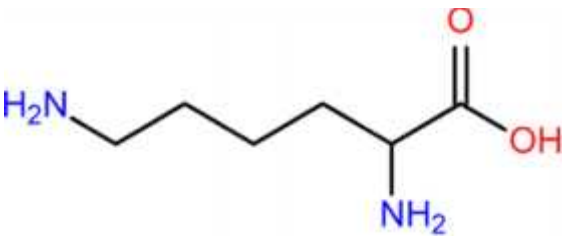

Figure 2.8: Molecular structure of Lysine

2.4.2 Microbial amino acid fermentation

The use of hydrolysate from protein as a starting material for synthesising L-amino acids is becoming less important, although it still has some limited use for the production of L-proline, L-hydroxy-proline, and L-tyrosine. This method is not good for amino acid production in large-scale (Wolfgang Leuchtenberger et al., 2005). The extraction method for producing L-glutamate was replaced almost half a century years ago by fermentation, as a result of sharp increase in demand for the flavour enhancer momosodium glutamate. The unearthing of the soil bacterium, *Corynebacterium glutamicum*, which has the ability to producing L-glutamic acid from monosaccharide, provided the platform for successful amino acid production by fermentation process (Kinoshita et al, 1957). The use of the wild strain of *C. glutamicum* in industrial scale fermentation creates suitable environment for large scale production of glutamate. A lot of research has gone into the improvement of strains for production of glutamate over the years (Kimura, 2003). In principle fermentation process is simple. It involves the inoculation of a sterilised fermentation tank with a culture medium containing a suitable fermentable sugar such as glucose. Additionally, required amount of nitrogen, sulfur, phosphorus, some trace elements and vitamins are added. A starter culture of the production strain is prepared in a prefermenter and added to the fermentation tank and stirred under a required condition of temperature, pH and oxygen (Wolfgang Leuchtenberger et al., 2005).

2.4.2.1 Lysine fermentation

Microbial fermentation is the most preferred methods of L-lysine production as it gives higher yields than chemical and enzymatic methods. Additionally, it produces biologically active form which has food and feed applications (Anusree & Nampoothiri, 2015; Leuchtenberger, 1996). Notwithstanding the strain improvement studies which has helped in the utilisation of different substrates including inexpensive carbon and nitrogen sources, research interests have also focussed on lowering the production cost, increasing the productivity and decreasing co-product formation (Gopinath et al., 2012; Nielsen, 2001). The use of cheap and easily available substrates which has low competing value as food can provide an alternative carbon source. In industrial fermentations, substrates are selected based on availability and geographical locations (Anusree & Nampoothiri, 2015). Molasses are used in Europe, China and South America, corn syrup is used in North America whereas complex substrates like cane molasses, beet molasses and starch hydrolysates of corn, wheat or cassava have also been used (Kelle et al., 2005; Kimura, 2005).

2.4.2.1.1 Importance of Corynebacterium glutamicum in lysine fermentation

C. glutamicum is a Gram-positive, rod-like, none spore forming and soil dwelling microorganism. It belongs to the suborder *Corynebacterianeae*. Additionally, *C. diphtheria* and *Mycobacterium tuberculosis* are pathogenic, belonging to the family of *Corynebacteriaceae* and *Mycobacteriaceae* respectively (Stackebrandt et al., 1997). *C. glutamicum* is commonly used in biotechnological processes to produce over 41.2 million tons of L-glutamate per annum, 4.5 million tons of L-lysine per annum as well as numerous other amino acids (Hermann, 2003). A number of molecular targets important for effective lysine production have been recognised in the lysine biosynthesis pathway. This pathway can lead to side products formation which is attributed to carbon precursor supply (Pfefferle et al.,

2003; Hermann, 2003). Although lysine and glutamate can be biosynthesised using different media containing glucose, fructose or sucrose , glucose is mostly used as a source of carbon for the evaluation of molecular targets (Georgi et al., 2005). Literature shows that Aspartokinase (lysC) is the major enzyme that shows cumulative feedback inhibition by threonine and lysine and can split lysine biosynthesis pathway (Schrumpf et al., 1991; Kalinowski et al., 1991). Overexpression of lysC especially the lysC alleles which codes for types of aspartokinase that are not affected by feedback inhibition improves lysine synthesis (Thierbach et al., 1990). Additionally, lysine synthesis can be improved by overexpression of dapA, which codes for dihydrodipicolinate synthase. More importantly, identification of the lysine export system LysE provides another target for strain enhancement (Eggeling et al., 1998; Vrljic et al., 1996).

For effective utilisation of glucose for lysine synthesis, carbon precursor has to be increased by over-expression of pyc, which encodes for pyruvate carboxylase (Peters-Wendisch et al., 2001) (Figure 2.8). Besides the two anaplerotic C3 carboxylating enzymes PEP carboxylase and pyruvate carboxylase, *C. glutamicum* possesses the C4 decarboxylating enzymes PEP carboxykinase and malic enzyme encoded by pck and malE (Eikmanns et al., 1989; Gourdon et al., 2000; Peters-wendisch et al., 1997; Riedel et al., 2001). Lysine production using glucose can also be enhanced in *C. glutamicum* if pck gene is removed to change the flux distribution at the PEP/ pyruvate and oxaloacetate/malate nodes (Petersen et al., 2001; Riedel et al., 2001; Stephanopoulos & Vallino, 1991). Application of genetic engineering to improve lysine synthesis using glucose from the wild strain type was achieved by introduction of mutant strain in the aspartokinase gene lysC, in the homoserine dehydrogenase gene hom and also in the pyruvate carboxylase gene pyc. These mutant strains were discovered in a classically obtained lysine production strain (Ohnishi et al., 2002).

Importantly, optimisation of lysine production in *C. glutamicum* has a strong linkage to the close relationship between central metabolism and lysine biosynthetic pathway. Previous metabolic flux studies done by Kiefer et al. (2004) with *C. glutamicum* mutant showed a strong relationship between lysine synthesis and carbon flux through the pentose phosphate pathway (PPP).

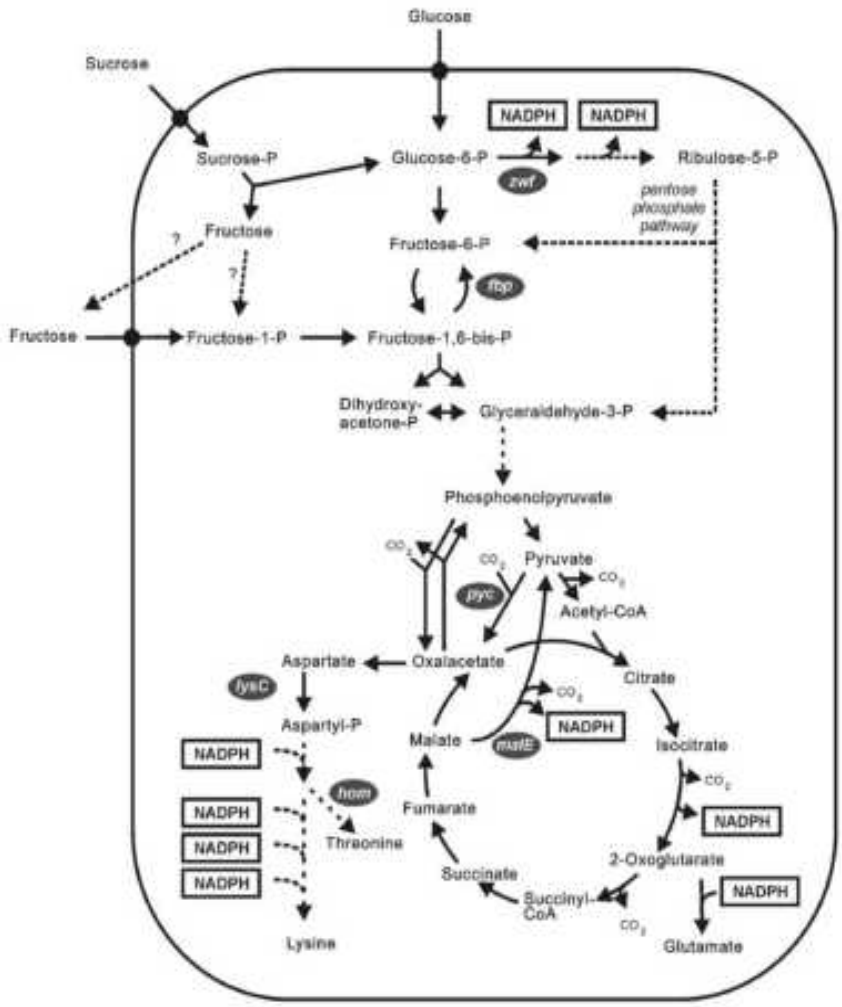

Figure 2.9: Schematic diagram of the central carbon metabolism of *C. glutamicum*. (Georgi et al., 2005)

The generation of the cofactor NADPH is very important in lysine formation. Carbon flux analysis showed that 4 mol of NADPH is needed to produce 1 mol of lysine through pentose phosphate pathway during microbial growth and lysine synthesis on glucose. Literature on metabolic flux genealogy study of different lysine-producing strains also confirmed the importance of the pentose phosphate pathway (Marx et al., 1996; Wittmann et al., 2002).

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CHAPTER THREE

Characterisation of cassava peel for carbohydrate types

Abstract

Cassava peel is a biomass generated as a result of processing cassava tuber by peeling operations. It is a natural resource that shows heterogeneity in structure and chemical composition. The aim of this study was to characterise cassava peel for carbohydrate types and to suggest appropriate technology options for its efficient utilisation. The reducing sugar concentrations were determined using High Performance Liquid Chromatography (HPLC) following acid hydrolysis to release the sugars. Cellulose and hemicellulose content were determined by enzyme hydrolysis. The hydrolysed sugars were quantified by HPLC. Total starch content was determined using a Megazyme starch assay kit. Protein content was measured following Kjeldahl digestion and cyanide content by gas chromatograph after enzyme hydrolysis, followed by acid and basic distillation. The results from the analysis showed that nearly 83 % dry matter (DM) composition of the cassava peel was glucose whiles arabinose and xylose have made up only small amount of 2.35 and 2.31 % respectively. The cellulose and hemicellulose were 6.0 % DM and 2.23 % DM respectively and the residual starch content was 47.16 %. The protein was 2.40 % and the cyanide level was 9.3 mg/kg. The lignin and the ash contents were 1.92% and 6.30% respectively. The high level of residual starch and low amount of lignin make the cassava peel very susceptible to enzymatic hydrolysis without laborious pretreatment regimes.

Key words: Cassava peel, Cellulose, Hemicellulose, residual starch

3.0 Introduction

Cassava peel is a biomass generated during cassava processing and is known to compose of cellulose, hemicellulose, starch, lignin and some extractives (Gerber et al., 2013; Kongkiattikajorn & Sornvoraweat, 2011). The percentage composition of cassava peel for carbohydrate types varies because of the difference in the processing. Agricultural residues may be useful resource as low-cost carbohydrate in biorefinery to produce high value-added products such as amino acids (Buzzini & Martini, 1999). The availability of cassava peel in large volumes results from the cultivation of cassava across almost all the geographical regions of Ghana.

Cassava (*Manihot esculenta Cranz*) occupies the sixth position as the most important crop cultivated in the world. It is mostly cultivated in most African countries as well as some countries in Asia, and Latin America. The cassava tuber contains starch which is mostly used as food in tropical countries. Cassava as an industrial crop is widely used to produce animal feed, starch and in recent times for bioethanol production. Cassava thrives well on both dry and semidry lands where other crops, like cereals, cannot grow effectively (Lin et al., 2011). Among the tubers grown in Ghana, cassava ranks highest in terms of volume and coverage. Consumption of cassava as a staple food in Ghana is high and accounts for a daily carbohydrate intake of about 30% (FAO, 2000). Cassava cultivation is widely distributed in Ghana. The majority of cassava produced in the country is located in the southern and middle belts of Ghana, accounting for about 78% of all cassava produced in Ghana (SRID-MoFA, 2014). About 16.5 million metric tonnes of cassava was produced in 2014.

Processing of cassava is normally done in order to improve the storage life as well as to add value to the final products. Majority of cassava processing is done in the rural areas and mostly carried out by women. Traditionally cassava is peeled by hand using a knife. Nonetheless, cassava processing is also carried out using small scale industrial processing machines. Out of the total amount of cassava tubers produced in 2014, processing of the tuber yielded about 4.4 million metric tonnes of cassava peels (OECD/IEA, 2010; SRID-MoFA, 2014). These peels are known to contain cyanide (Ofoefule & Uzodinma, 2009). Peels typically are made of the outer thin pericarp and the thicker inner core. Majority of the peeling processes take out the pericarp and the thicker core in addition to some flesh attached to the peels. Chemical composition analysis of cassava peels has been reported in literature as dry matter 86.5–94.5%; crude protein 4.1–6.5%; hemicellulose and cellulose 34.4%; and lignin 8.4% (Kongkiattikajorn & Sornvoraweat, 2011).

Composition analysis of cassava peels as reported by Aderemi & Nworgu (2007); Moshi et al. (2014) showed starch 45%, cellulose 5.4-8.4%, lignin 15-16, protein 5.5-8.1. Akpabio et al. (2012) in their work reported that fresh cassava peels of sweet and bitter varieties have 17.40% and 18.86% carbohydrate respectively. Adegbola et al. (1992) also reported cassava peels to contain 23.0% carbohydrate. However, Adegbola and Asaolu (1986) reported that cassava peel is high in soluble carbohydrate content of 62.0%. Physicochemical characterisation of cassava peels by Gerber et al. (2013) showed starch content of 56.5% and 2.27% ash. Additionally, Obueh & Ikenebomeh (2014) also reported carbohydrate content of cassava peels to be 77.67%, ash 1.29% and protein 4.11%.

Over the years, research focus has been on cassava peels because of its availability as process residues at small and medium scale cassava processing units because of the potential as a renewable feedstock for biorefinery. One of the most important considerations for cassava

peel utilisation is to look for economic and viable ways of exploiting this inevitable process residue (Reis et al., 2011). Residues may have several substances of high value which can be transformed into commercial products or raw materials for secondary processes by means of appropriate technology (Laufenberg et al., 2003). Data from composition analysis of cassava peels from various researchers revealed the complexity of the carbohydrate types indicating varying carbohydrate percentages.

The complexity of carbohydrate composition in cassava peel can be attributed to the variety of cassava as well as the methods used in processing the tuber into various products. In Ghana traditional processing of cassava by hand peeling is dominant in almost all the small and medium scale cassava processing facilities.

The aim of this study was to characterise cassava peel for carbohydrate types and to suggest appropriate technology options for its efficient utilisation.

3.1 Materials and Methods

Materials used for the research were sourced both from Ghana and Denmark. Standard methods were used to prepare and analyse the results.

3.1.1 Materials

Materials used for the research were acquired locally in Ghana as well as internationally from Denmark. Cassava peel was sampled locally from Ghana at Bawjiase in the Central Region, Enzymes from Sigma Aldrich, Denmark.

3.1.2 Feedstock preparation

Cassava peels were sampled from a small scale cassava processing facility at Bawjiase, in the central region of Ghana. The peels were soaked in water at room temperature (about 28^{o}C) for 30 min to allow for easy removal of the brown skin by peeling with the finger. The peels were then dried in a hot air oven at a temperature of 60 °C overnight, and milled to 0.25 mm particle size in a hammer mill. This sample was stored in air tight bottles till ready to use.

Dry matter of the sample was determined by drying the milled samples to a constant weight in hot air oven at a temperature of 105 ^{o}C overnight. Ash content was determined by taking the sample weight before and after ashing at a temperature of 550 °C for 2 hours in Muffle Furnace.

3.2 Determination of the chemical and physical composition of the cassava peel

The milled cassava peel samples were analysed to determine the total carbohydrate content of the peels and to characterise the carbohydrate composition by type. The hemicellulose, cellulose, starch, reducing sugars, cyanide and protein content were determined by standard methods.

Reducing sugar content was analysed using the protocol A0003 from Enzyme Laboratory of Danish Technological Institute (Denmark) as previously described by Saeman et al. (1954). In brief, the milled cassava peel biomass was acid hydrolysed into reducing sugars by a two-step acid hydrolysis. The primary hydrolysis was performed by first suspending 0.16 g of the milled samples in 1.5 ml of 72 % (w/w) sulphuric acid at a temperature of 30°C for 60 minutes with intermittent stirring at 20 minutes intervals followed by dilution of the slurry to 4 % (w/w) sulphuric acid for further hydrolysis at a temperature of 121°C for 60 minute. The reducing sugars in the hydrolysed samples were then quantified with an HPLC equipped with an RI detector and an Aminex HPX-87H column maintained at a temperature of 63°C with 4 mM sulphuric acid as mobile phase at a flow rate of 0.6 ml/min. Klason lignin content was estimated as the ash free residue after the two step acid hydrolysis.

Total starch content was determined using a Megazyme starch assay kit according to the manufacturer's instructions. In principle, the kit consists of a thermostable α-amylase, amyloglucosidase, glucose oxidase and peroxidase which catalyse the conversion of starch to quinoneimine (Mcdonald, 1977; Megazyme International, 2014, McCleary et al., 1997); the absorbance of which is measured at 510 nm on a (Shimadzu UV Min 1240), UV-VIS Spectrophotometer, Tokyo, Japan).

Cellulose was analysed using the method described by Updegraff (1953) as modified in Kulic and Radojicic (2011) The cassava peel material was hydrolysed to sugars with cellulase (50 U/g) and β-glucosidase (50 U/g) at 50°C. In brief, about 0.2000 g of the milled cassava peel samples were weighed into 15 ml falcon tubes containing 10 ml of 0.1 M acetate buffer (pH 5.0). To prevent microbial contamination, the reaction mixture was amended with 150 µg/mL Ampicillin. Hydrolysis of samples was run for 48 hours in Enviro Geni incubator (Scientific

Industries Inc. Bohemia, New York) at vertical agitation speed of 30 cycles per 60 seconds. The hydrolysates were analysed for glucose concentrations using ultimate 3000 HPLC system (Dionex, Sunnyvale, USA) equipped with an Aminex HPX-87H column (Bio-Rad, Hercules, USA) and a Shodex RI-101 detector (Showa Denko KK, Tokyo, Japan). The column oven temperature was set to a temperature of 60°C, and the mobile phase consisted of 5 mM sulphuric acid with a flow rate of 0.5 mL/min.

The hemicellulose content was determined according to the method of Ververis et al. (2007) from the equation:

$$\% \text{ Hemicellulose} = (0.88/0.93) \times C \times (V/M) \times K \times 100$$

where

0.88 is the coefficient that results from the molecular weight ratio of the polymer and the monomer pentose, 0.93 is the saccharification yield of xylan to xylose,

C is the measured xylose concentration (g/L),

V is the total volume of glucose solution (L),

M is the dry weight of the cassava peel sample (g) and

K is the dilution of the sample.

Protein content was measured following Kjeldahl digestion as previously described by (AOAC, 2006) in Eurofins Steins Laboratorium A/S (Denmark). Briefly, 1.0 g of sample was digested in 20 ml of H_2SO_4 and nitrogen content quantified using titration. The protein content was determined by multiplying the nitrogen content of the sample with a factor of 6.25.

Cyanide content in the cassava peel biomass was measured in Eurofins Steins Laboratory A/S (Denmark), by using appropriate enzymes to hydrolyse the substrate, followed by distillation in an acidic environment. The Hydrogen cyanide was then distilled into a basic medium and measured by using Eurofins' method No DS 204 mod, Denmark with gas chromatograph (GC).

3.3 Results

3.3.1 Characterisation of Cassava Peel

The inorganic and the mineral matter of the cassava peel biomass (ash), was determined to constitute 6.30% of the dry matter with lignin accounting for 1.92% (Table 3.1). Lignin can be an obstacle which prevents carbohydrate substrates from being acted upon by hydrolytic enzyme (Rahikainen et al. 2013). The 1.92% lignin content of the cassava peel (Table 3.1) compared with previous findings of about 8.4% -16% suggests that peels generated at Bawjiase constitute a better biomass for bioprocessing than comparable substrates (Aderemi and Nworgu 2007; Kongkiattikajorn & Sornvoraweat 2011; Moshi et al. 2014).

To determine the potential value of the peel as a substrate for fermentation, the total reducing sugar content was determined by HPLC (Table 3.2). The hexose sugar, glucose was the most abundant and accounted for as much as 83% of total reducing sugar composition with the pentose sugars, xylose and arabinose constituting only 2.31% and 2.35 % respectively. The residual starch could have contributed to the high hexose glucose content (Table 3.1 and 3.2).

The knowledge of the composition of starch, cellulose and hemicellulose in cassava peel is important because their monomeric sugars after hydrolysis provide a source of carbon in fermentation process to produce a range of products. The residual starch content was 47.16 %, whiles the cellulose and hemicellulose were 6.00 % and 2.23 % of dry matter respectively (Table 3.1). The protein was 2.40 % and 9.3 mg/kg cyanide was recorded. The protein content of the cassava peel is low (Table 3.1) and consistent with previous findings. Low protein levels of (1.40% - 6.5%) in cassava peel have been reported by Akpabio et al. (2012); Devendra (1977); Kongkiattikajorn and Sornvoraweat (2011) and Obueh and Ikenebomeh (2014).

The environmental condition and the cassava variety can influence the level of cyanide in the peels. Cyanide levels of between 1-1500 ppm and 1600 mg/kg in untreated cassava peel have been reported in literature (Bokanga, 1994); (Tivana, 2012).

Table 3.1: Chemical and Physical Composition of Cassava Peel

DM (%)	**Ash (%DM)**	**Lignin (%DM)**	**Starch (%DM)**
89.7±0.6	6.3±0.34	1.92±0.07	47.16±3.19
Protein (%DM)	**Cyanide (mg/Kg**	**Cellulose (%DM)**	**Hemicellulose (%DM)**
2.4±0.28	9.3±0.42	6.0±0.02	2.23±0.02

Table 3.2: Reducing Sugar Composition of Cassava Peel

Glucose (%)	**Xylose (%)**	**Arabinose (%)**
83.41±0.82	2.31±0.08	2.35±0.08

3.4 Discussions

Cassava production and processing in Ghana and most of West Africa has an associated high percentage (approximately 26%) of waste amounting to about 3.8 million MT per year. This is projected to keep increasing as more farmers are encouraged to increase production due to increasing utilisation of the cassava starch and chips by local industries and a growing export market. To derive optimum value from cassava as an economic commodity, additional high-value products ought to be developed from less used parts of the crop; such as development of biofuels, bioplastics and animal feed products from cassava peels. The goal of this work was to determine the potential of cassava peels generated in Ghana as a source of fermentable sugars for the production of animal feed and/or biofuel.

To this end, the chemical and physical composition of cassava peel was analysed to determine the composition of total fermentable sugars, protein, cyanide and ash content on as is basis in the cassava processing industry at Bawjiase. Ash and lignin content are important components in the consideration of biomass choices for industrial scale fermentation. Ash content of biomass has been extensively studied but poorly understood (Gandolfi et al., 2013). For industrial application, the ash content of biomass forms part of the residue that is left in a reaction vessel after hydrolysis of the biomass. This causes lots of operational problems such as the wear out of the blades of the size reduction unit (Vamvuka, 2010) and the fouling of heat exchangers and slagging in the bottom of the furnace. These affect the smooth operations due to regular shutting down of the production units, contributing to operating time reduction of the production units, as well as increasing the cost of maintenance.

The 6.3% ash content recorded in this study is significantly higher than the 1% - 3% of dry matter that had previously been reported for cassava peels (Gerber et al., 2013; Obueh &

Ikenebomeh, 2014). High lignin content of the biomass on the other hand can be an obstacle which prevents carbohydrate substrates from being acted upon by cellulases during hydrolysis (Rahikainen et al., 2013) due to the close association of lignin with cellulose and hemicellulose microfibers in a manner that limits free access to hydrolytic enzymes. Adsorption of cellulases and hemicellulases onto lignin further increases the cost of enzyme requirement for hydrolysis. It is estimated that between 60% - 70% of cellulases and hemicellulases used in hydrolysis of lignocellulose substrates remain bound to lignin after complete hydrolysis of the carbohydrates ((Berlin et al., 2005; Jørgensen et al., 2006; Lu et al., 2002). The 1.92% lignin content of the cassava peel compared with previous findings of about 8.4% -16% suggests that peels generated at Bawjiase constitute a better biomass for bioprocessing than comparable substrates (Aderemi and Nworgu, 2007; Kongkiattikajorn & Sornvoraweat, 2011; Moshi et al., 2014). The low lignin content recorded in this study compared with previous findings could be explained by three factors. Firstly, the brownish outer skin of the peel was removed before drying and milling. Secondly, the traditional process of generating the peels at Bawjiase leaves significant portions of the starch storage pulp with the peel. Thirdly, the variety of cassava (Afisiafi) processed. This traditional method of peeling is practiced in all processing facilities across the country. I therefore believe that peels collected from other locations in the country may have comparably low levels of percentage lignin content after pre-treatment.

Of all the reducing sugar, glucose is the most fermentable as it is the biochemically preferred monosaccharide utilised by fungi and bacterial fermenters for ethanol and amino acid production. The fermentation of xylose remains a key problem in cellulose ethanol fermentation. Although, xylose can be fermented to ethanol by many bacteria and yeasts, by-product formation or slow xylose conversion limits its economical application for ethanol

production (Zhang et al., 2014). Thus the low level of the pentose sugars relative to glucose, makes cassava peels an ideal substrate for producing high glucose fermentation material for ethanol and amino acid biosynthesis. The results of the residual starch and cellulose composition are in agreement with the findings of Aderemi and Nworgu (2007) and Moshi et al. (2014). Enzymatic hydrolysis is preferred to acid hydrolysis because acid hydrolysis requires the use of corrosion resistant materials, giving rise to high colour and salt ash and requires more energy for heating (Roberto do Nascimento et al., 2010). Cassava peel is more a suitable substrate for producing fermentable sugars from enzymatic hydrolysis without acid and heat pretreatment as compared to other lignocellulosic materials. For example to convert sugarcane bagasse to glucose, Ramos et al. (2015) Braga et al. (2014) achieved 50.8% conversion of cellulose to glucose from the hydrothermally pretreated bagasse. Ramos et al. (2015) also hydrolysed phosphoric-catalysed steam-exploded sugar cane bagasse to release 69.2% glucose. Thus compared with biomass from wood and grasses (Hayn et al., 1993; Wiselogel et al. 1996), cassava peel produced at Bawjiase in Ghana is an extremely rich source of fermentable carbohydrates for industrial exploitation.

The low protein content is not surprising given that, the cassava tuber is principally a starch storage organ with often, very thin peels. Starch storage organs are generally low in protein composition (Shewry, 2003). Depending on the end use, low or high protein content may be desired. For incorporation into animal feed production or direct use as an animal feed, high protein content may be desired. The low protein content of cassava peel makes it an unsuitable substrate for animal feed. To enhance its utilisation in animal production, various workers have demonstrated through biotechnology interventions that the protein content can be significantly augmented through solid state fermentation. (Bayitse et al., 2015; Aderemi & Nworgu, 2007). However, in other applications such as chemical and enzymatic hydrolysis

of complex carbohydrates in the peels to produce simple sugars, low protein content may not affect the hydrolysis process. This assertion is confirmed by Abidin et al., (2014; 2015)and Elechi et al. (2016) as they hydrolysed cassava peel to simple sugars without protein supplement.

The low cyanide content (Table 3.3) could be attributed to the cassava cultivar (Afisiafi) in addition to the pre-treatment process which involves soaking the peels in water before removing the brownish skin, followed by drying at a temperature of 60 °C for 24 hours before milling. Literature reports show reduction in cyanide levels of cassava peel from 268 to 140 mg, 364.2 to 814.7 ppm to 264.3 to 321.5 ppm by drying. In addition, the peels of "bitter" cassava variety contain higher levels of cyanide (650 ppm) than the "sweet" variety (200 ppm) (Attahdaniel et al., 2013; Tewe & Iyayi, 1989). The reduction of cyanide content by soaking in water and drying at a temperature of 60 °C was substantial. Because cyanogenic glucoside is toxic to most microorganisms it can inhibit microbial growth in fermentation processes. This modest pretreatment of the cassava peel is considered to be an effective means for removing the toxin before further bioprocessing to especially bioethanol, biobutanol and amino acid by fermentation. Low cyanide level reduces the risk associated with inhibitors in fermentation especially when hydrolysate from cassava peel is used in fermentation process because it is known to have high levels of cyanide (Ofoefule & Uzodinma, 2009). Although the level of cyanide in the cassava peel might not inhibit hydrolysis or fermentation, fresh cassava peels might have levels toxic to animals when used continuously as animal feed.

3.5 Conclusions

Cassava peel is a biomass generated as a result of processing cassava tuber by peeling operations. It is a natural resource that shows heterogeneity in structure and chemical composition. Physical and chemical composition analyses were done using standard methods. The monosaccharide composition of the cassava peel showed glucose levels of 83 % DM, with xylose and arabinose taking only small amount of 2.31 and 2.35 % respectively. The cellulose and hemicellulose were 6.0 % DM and 2.23 % DM respectively and the residual starch content was 47.16 %. The protein was 2.40 % whiles the cyanide content was 9.3 mg/kg. The lignin and the ash contents were 1.92% and 6.30% respectively. The high level of residual starch and low amount of lignin make the cassava peel more susceptible to enzymatic hydrolysis without laborious pretreatment regimes. Importantly, this study provides a useful base line data for agro-economic evaluation of cassava peel as a feedstock for an integrated biorefinery, because the valorisation of cassava peel is still overlooked and not fully exploited. Additionally, a deep understanding of the biomass chemical and physical properties, are required for the design and safe operation of processing facilities.

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CHAPTER FOUR

Optimisation of enzymatic hydrolysis of cassava peels to produce fermentable sugars using response surface methodology

Abstract

A Central Composite Design (CCD) was applied to optimise the enzymatic hydrolysis of cassava peel in order to produce glucose. Two effective approaches were used in the study. The first one was to optimise the enzymatic hydrolysis process using cellulase, β-glucosidase, amyloglucosidase and α-amylase. The second approach was to optimise enzymatic hydrolysis using the mixture of these enzymes. The effects of enzyme loading, hydrolysis time, substrate concentration, pH and temperature on glucose recovery were investigated. The results were subjected to analysis of variance to produce polynomial regression model. Mean interaction plot and their effect on glucose recovery were drawn to determine the optimal conditions for enzymatic process. Targeted hydrolysis of specific carbohydrate types of cassava peels with single enzymes showed optimised levels of glucose recovery of over 80% for starch hydrolysing enzymes and about 5% for cellulose hydrolysing enzymes at 0.06 g/ml substrate water ratio at 24 hours of hydrolysis. Single step hydrolysis of cassava peels with mixed enzymes of starch and cellulose hydrolysis enzymes at optimised conditions of celulase (30 FPU/g). β glucosidase (1.25 U/g), amyloglucosidase (30 U/g), α-amylase (30 U/g), pH 4 and 50 ^{o}C at 24 hours produced recovered glucose of about 100%.

Keywords: Glucose recovery, enzymatic hydrolysis, cassava peel, optimisation

4.0 Introduction

Currently the most effective method of producing reducing sugars from cellulose and pretreated lignocellulosic biomasses is enzymatic hydrolysis. The shift towards the use of more effective and sustainable material for biotechnological conversion into valuable product became prominent in recent years because of the availability and utilisation of agro-industrial by-products. Additionally, biotechnological conversion of these residues can help minimise environmental implications of their disposal (Pandey et al., 2000). The use of state of the art, effective biological processes for underutilised biomasses is the way forward for sustainable industrial application (Rattanachomsri et al., 2009).

Cassava (*Manihot esculenta Cranz*) is a starchy root crop which is an essential food eaten mainly in developing countries in Africa, Southern America and Asia. Cassava is the fourth most cultivated food crop in these developing countries, next to rice, maize and wheat (FAO, 2002). The importance of cassava as an important crop in developing countries stems from its drought tolerance, resistance to plant diseases, and flexibility in its cultivation, management requirements and harvesting cycles (FAO, 2002; Meridian Institute, 2009). Cassava is said to be the highest producer of carbohydrates when it comes to staple crops. Ghana produces an average of about 16 million MT of cassava per year. In 2015, Ghana was the sixth largest producer of cassava (15,113,000 MT) in the world in terms of value and volume, and the second and third respectively among producers of fresh cassava roots in West African region and Africa (FAO, 2015). Currently, cassava occupies an important position in Ghana's agricultural economy and contributes about 46% of agricultural Gross Domestic Product (GDP). At the current level of production, cassava accounts for about 30% of a daily carbohydrate intake in Ghana and is cultivated by almost every farming family (FAO, 2000). Recent effort by the Ministry of Agriculture, through the Crop Research

Institute of Ghana to promote cassava production and alternate industrial utilisation as a major economic activity is anticipated to increase the amount of cassava produced and by extension, the amount of cassava residue. In 2014, an estimated 4.4 million MT of cassava peels was generated from the 16.5 million MT of cassava produced and processed (OECD/IEA, 2010; SRID-MoFA, 2014). The residue generated from cassava processing has a huge potential in biorefinery because it can easily be hydrolysed by enzymes into fermentable sugars (Olanbiwoninu & Odunfa, 2015).

Agro-processing activities at the rural level undertaken mainly by women who employ very old and reliable traditional methods to peel the cassava generates residue that is composed of peels and trimmings. Majority of cassava processes take off both the pericarp and the thicker core together with some pulp attached to the peels. Composition analysis of cassava peels as reported by Aderemi & Nworgu, (2007); Moshi et al. (2014) showed starch 45%, cellulose 5.4-8.4%, lignin 15-16, protein 5.5-8.1. (Kongkiattikajorn & Sornvoraweat, 2011) also indicates that cassava peel composed of 86.5–94.5% dry matter; organic matter 81.9–93.9%; crude protein 4.1–6.5%; hemicellulose and cellulose 34.4%; and lignin 8.4%. As the chemical composition of cassava peels vary between different varieties and primary processing techniques, the optimal enzyme mixture required for the efficient and cost-effective hydrolysis of the carbohydrates starch, cellulose and hemicellulose into fermentable sugars has to be optimised for each source material.

Cellulose is an insoluble polymer comprising glucose units joined by β-1,4 linkages. (Zhang & Lynd, 2004). These chains are joined by strong hydrogen bonds to form microfibrils, making it crystalline in nature and very difficult to destroy (Krässing, 1993). The limited accessibility to these regions affects their reactivity to swelling and reactive agents such as cellulases. Despite extensive research, hydrolysis of biomass using enzymes remains a major

challenge in the process of bioconversion of the cellulosic substrates into reducing sugars (Ioelovich & Morag, 2012).

Literature shows that organic acid pretreatment of cassava peel is carried out before enzymatic hydrolysis with cellulase (Olanbiwoninu & Odunfa, 2015). However, much attention is not given to starch hydrolysing enzymes and other variables such as substrate loading, pH and temperature and their interactions. Moreover, the potential of utilising enzyme cocktails without prior treatment with acid before converting cassava peel to reducing sugars especially glucose has not been adequately investigated.

In literature it has been reported that the effectiveness of the enzymatic hydrolysis depends on several parameters such as enzyme loading, substrate concentration, reaction time, and addition of surfactant (Zaghloul et al., 2011). These factors normally interrelate with each another; hence, optimisation of the enzymatic hydrolysis process plays a key role in improving the performance of the procedure. Unlike conventional optimisation, statistical optimiation methods can take into account the interactions of variables in generating process responses (Bas & Boyacı, 2007).

Response Surface Methodology (RSM) is a statistical procedure, based on the fundamental principles of statistics, randomisation, replication and, duplication. It is an effective statistical procedure for optimisation of multiple variables in order to predict the best performance conditions with a minimum number of experiments. The procedure has been widely applied in chemical and biochemical processes to analyse the effect of independent variables and optimise the process responses using appropriate values of the factors (Qi et al., 2009). These designs are used to find improved or optimal process settings, troubleshoot process problems and weak points and make a product or process more robust against external and non-controllable influences (Adinarayana & Suren, 2005).

The objective of this research was to examine the influence of enzyme loading, substrate concentration, pH, temperature and hydrolysis time, on the hydrolysis of cassava peel polysaccharides. Accordingly, response surface methodology based on Central Composite Design (CCD) was implemented to determine the significant factors that affect the response and assessing the response in terms of glucose yield from hydrolysis and also to determine the optimum conditions for glucose yield.

4.1 Materials and Method

4.1.1 Feedstock Preparation

The cassava peel sample was prepared according to procedure used in Chapter (3.1.2).

4.1.2 Enzymes

In order to investigate the influence of enzyme concentration on the enzymatic hydrolysis of cassava peel, four commercial enzymes; Cellulase from *Trichoderma Reesei* (≥ 700 U/g), Beta glucosidase (10-30 U/mg), Amyloglucosidase from *Aspergillus niger* (≥ 300 U/ml), and α-amylase from *Aspergillus oryzae* (30 U/mg) obtained from Sigma Aldrich, Denmark were used.

4.2 Optimisation of enzymatic hydrolysis by response surface methodology

The enzymatic hydrolysis of polysaccharide is a heterogeneous process that is affected by the mode of interaction between the enzymes and the polysaccharide chains (Azevedo & Reis, 2005). Generally, enzyme action on substrates is influenced by a combination of factors including, substrate-enzyme concentrations ratio, substrate-water ratio, incubation time for enzyme action, pH and temperature of enzyme reaction mixture, enzyme cofactors, and allosteric effectors, among others. The effects of five factors, including hydrolysis time, incubation temperature, enzyme concentration, substrate-water ratio and pH of reaction medium on glucose recovery from cassava peel biomass were investigated using the response surface methodology (central composite design).

4.3 Design of Experiment

To determine the optimal values of significant factors affecting the hydrolysis of cassava peel polysaccharides, the central composite rotatable design for three and six independent variables were employed for single and mixed enzyme hydrolysis assays. The experimental design for the single enzyme hydrolysis had the recommended number of tests at the centre

point of six and the total number of experiments was 20 for each enzyme. The values of the variables were coded to lie at ±1 for factorial points, 0 for the centre points in axial, 6 for axial points, 8 for cube points with alpha at 1. For the mixed enzymes, number of tests at the centre point was fourteen (14) and the total number of experiments was ninety (90). The Values of the variables were coded to lie at ±1 for factorial points, 0 for the centre points in axial, 12 for axial points, 64 for cube points and alpha as 1.

The quadratic equation for predicting the optimal point for enzymatic hydrolysis of cassava peel was expressed as $Y = \beta_0 + \sum \beta_i X_i + \sum \beta_{ii} X_i^2 + \sum \beta_{ij} X_i X_j$

$i = 1,2,\ldots k, j = 1,2,\ldots k; i \neq j$

where: Y is the predicted response (glucose yield, g/L),

β_0 is the interception coefficient,

β_i is the linear term,

β_{ii} is the quadratic term, and

β_{ij} is the interaction term.

The experimental factors and corresponding levels as well as the experimental design is presented in (Tables 4.1 and 4.2).

Table 4.1: Process variables in coded and actual units for single enzyme hydrolysis

	Coded level		
Variables	-1	0	+1
Substrate water ratio (g/ml), Z	0.02	0.06	0.10
Time (h), Y	0	24	48
Cellulase concentration (FPU/g), X_1	10	30	50
β – glucosidase concentration (U/g), X_2	0.50	1.25	2.00
Amyloglucosidase concentration (U/g), X_3	10	30	50
α – amylase concentration (U/g), X_4	10	30	50

Table 4.2: Process variables in coded and actual units for mixed enzyme hydrolysis

	Coded level		
Variables	-1	0	+1
Cellulase concentration (FPU/g), X_1	10	30	50
β – glucosidase concentration (U/g), X_2	0.50	1.25	2.00
Amyloglucosidase concentration (U/g), X_3	10	30	50
α – amylase concentration (U/g), X_4	10	30	50
Temperature (^oC), X_5	40	50	60
pH, X_6	4	5	6

The optimisation study was carried out in two stages. The first stage involved the hydrolysis of the milled cassava peel with each of the four enzymes (cellulase, β-glucosidase, amyloglucosidase and α-amylase) in single enzyme catalysed reactions to determine the optima for each enzyme's hydrolysis time, substrate water ratio and enzyme concentration. For each of the enzymes, hydrolysis was optimised by varying the three factors (enzyme concentration (0.5-50 U/g), substrate water ratio (0.02-0.1 g/ml) and hydrolysis time (0–48 hrs), within a range as previously determined by Bayitse et al., (2015). The medium pH and temperature for each enzyme hydrolysis was maintained at the optimum temperature and pH for the individual enzymes as per the manufacturer's instruction. (Hydrolysis with cellulase was done at pH 5 and temperature 50 ^oC; β-glucosidase - was at pH 5 and 45 ^oC; amyloglucosidase was at pH 4.5 and 55 ^oC and hydrolysis with α amylase was done at pH 6 and temperature of 40 ^oC). In the second stage of the optimisation process involving mixed enzyme hydrolysis, the optimum enzyme concentration (0.5-50 U/g), medium pH (4-6) and temperature (40-60 ^oC) were determined within the specified ranges with substrate water ratio and hydrolysis time maintained at 0.06 g/ml and 24 hours respectively, based on the optimised condition for the single enzyme hydrolysis.

4.4 Enzymatic hydrolysis of cassava peel

Cassava peel samples (0.2-1.0 g) were weighed into 15 ml falcon tubes. A total of 10 ml solution was added containing 4 ml of 0.2 M acetate buffer (pH 5.0) and 6 ml of distilled water. To prevent bacterial contamination during the hydrolysis process, 150 µg/mL Ampicillin was added and the pH appropriately adjusted with 0.1 M NaOH or 0.1M HCl. The required enzyme was then added to the samples in the buffer solution. Hydrolysis was run at the appropriate temperature (40-60 ^{o}C) at vertical agitation speed of 30 cycles per minute in incubator for 48 hours. The freed sugars were then quantified by HPLC.

4.5 Measurement of glucose concentration and yield of hydrolysis

The concentrations of glucose were determined using an Ultimate 3000 HPLC system (Dionex, Sunnyvale, USA) equipped with an Aminex HPX-87H column (Bio-Rad, Hercules, USA) and a Shodex RI-101 detector (Showa Denko KK, Tokyo, Japan). The column oven temperature was set to 60°C, and the mobile phase consisted of 5 mM H_2SO_4 with a rate of flow at 0.5 mL/min.

The effectiveness of the hydrolysis process using enzymes was estimated by the recovery of glucose which is defined as the total amount of glucose in the hydrolysate as compared to the total hydrolysable glucans in biomass, following the equation:

Glucose recovery = [glucose concentration in hydrolysate (g/L) x volume of hydrolysate (L)] / [Glucose concentration (%DM) x sample for hydrolysis (g) x DM of the sample] x 100%

4.6 Data analysis

The results of each CCD were analysed using Minitab software version 14, USA. Both linear and quadratic effects of the six variables under study were calculated, in addition to their possible interactions, on recovery of glucose. Variance analysis (ANOVA) was used to

evaluate their significance. Interactive and main effect plots were drawn to illustrate the effects of the independent variables on the dependent variable, being described by a quadratic polynomial equation, fitted to the experimental data. The fit of the models was evaluated by the determination of R-squared coefficient and adjusted R-squared coefficient. The student's t-test and p-value were performed to determine significance of the regression coefficients. The size of the regression coefficients for each independent variable gives an indication of the effect that variable has on the response. Coefficients with negative signs suggest that there is a negative effect on the recovery. The larger the t- value and the smaller the p-value ($p<0.05$) give an indication of the significance of the coefficient and their effects on the hydrolysis process. A p-value of less than 0.05 was used as the model criteria.

4.7 Results

4.7.1 Optimisation of enzymatic hydrolysis

The choice of enzymes for the hydrolysis of the cassava peel biomass was informed by the carbohydrate composition analysis of the milled cassava peel (Chapter 3, Table 3.1). Four polysaccharide hydrolysis enzymes, cellulase, β-glucosidase, amyloglucosidase and α-amylase were used in the optimisation of enzymatic hydrolysis independently or in combination. In the hydrolysis of the cassava peel substrate in single enzyme reactions, the highest glucose recovery of 6.9% was achieved from (run 3) of cellulase enzymatic hydrolysis of the cassava peel at high enzyme concentration, substrate water ratio and time (Table 4.6). However, from the interactive analysis of the variables, hydrolysis of cassava peel with 30 FPU/g of cellulase at 50 ^{o}C and pH 5 for 24 hours with water-substrate ratio of 0.06 g/ml provides mean optimum glucose recovery of 5 % (Figure 4.1a and 4.1b). Glucose recovery for hydrolysis using β-glucosidase ranged from 1.9% - 5.5% for the 20 runs (Table 4.8). β-glucosidase catalyses the conversion of cellobiose content of cellulose to glucose. The mean optimum glucose recovery of 4.2 % (Figure 4.2a and 4.2b) from the hydrolysis of cassava peel was recorded with 2.0 U/g of β-glucosidase at 45 ^{o}C and pH 5 for 48 hours with water-substrate ratio of 0.06 g/ml.

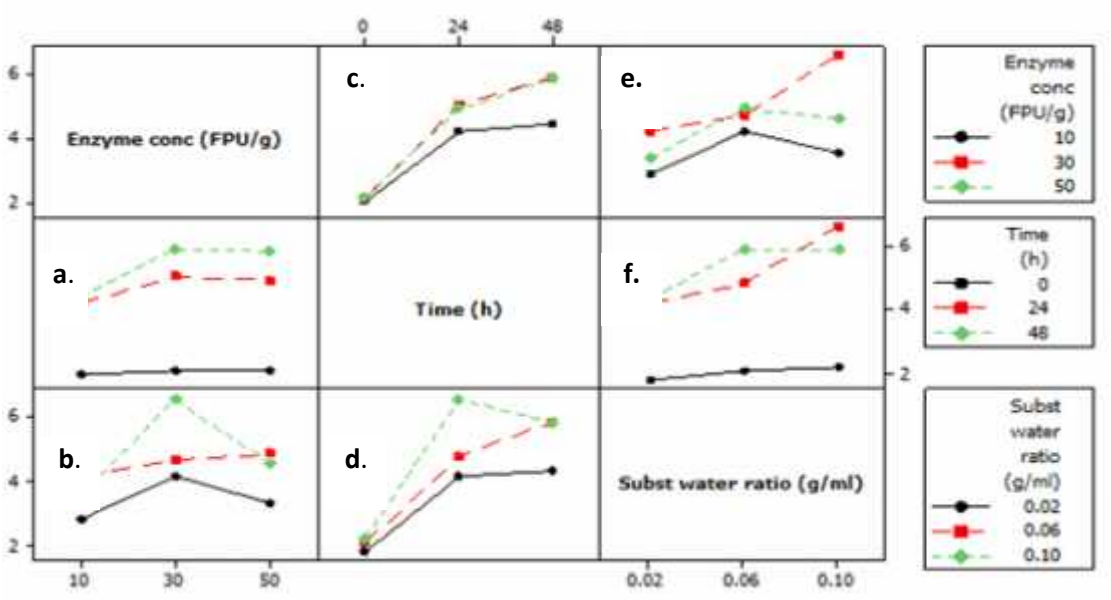

Figure 4.1a: The effect of interaction between cellulase concentration, substrate-water ratio and hydrolysis time on the percentage recovery of glucose. Data plotted are the means of glucose recovery (%) resulting from; (a and c) Enzyme concentration and hydrolysis time; (b and e) Enzyme concentration and substrate water ratio; (d and f) Substrate water ratio and hydrolysis time.

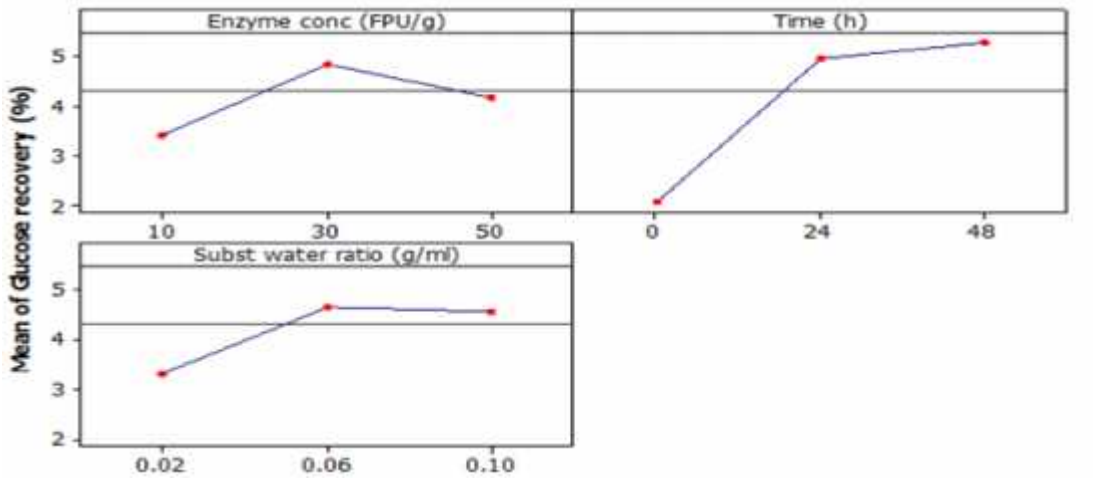

Figure 4.1b: Optimum effect of the interactions between cellulase concentration, substrate water ratio and hydrolysis time on glucose recovery (%)

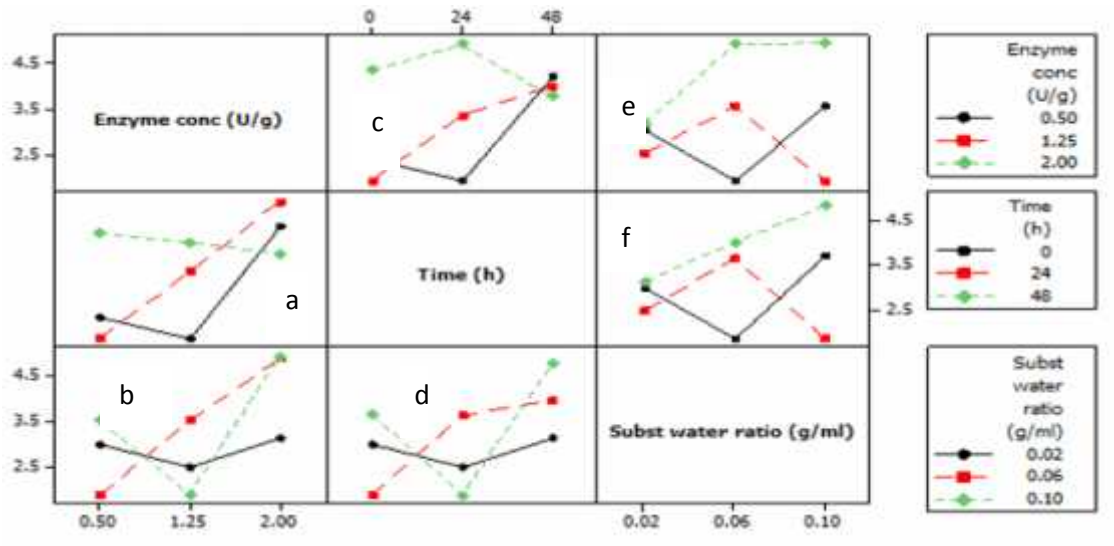

Figure 4.2a: The effect of interaction between β-glucosidase concentration, substrate-water ratio and hydrolysis time on the percentage recovery of glucose. Data plotted are the means of glucose recovery (%) resulting from; (a and c) Enzyme concentration and hydrolysis time; (b and e) Enzyme concentration and substrate water ratio; (d and f) Substrate water ratio and hydrolysis time.

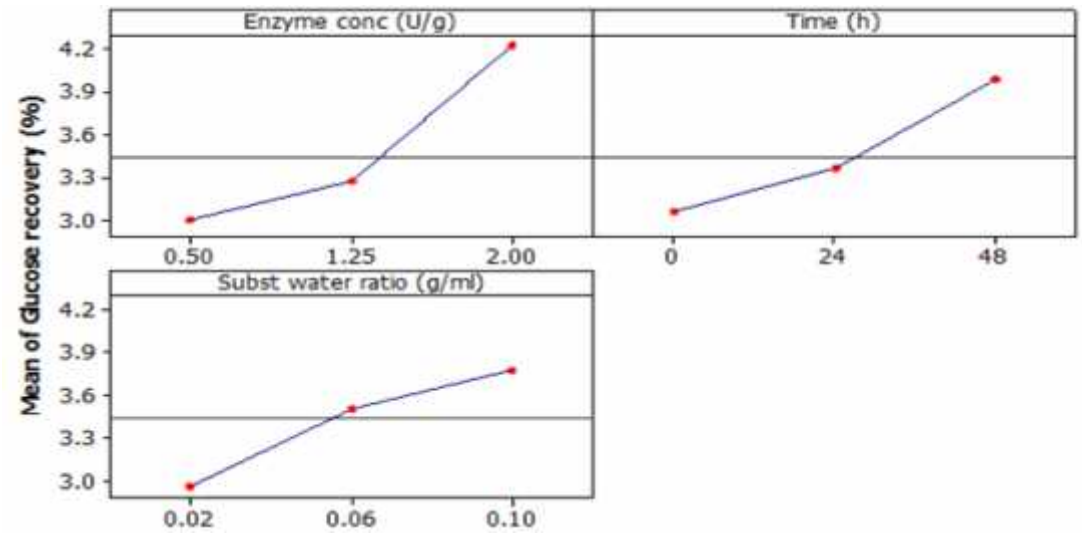

Figure 4.2b: Optimum effect of the interactions between β-glucosidase concentration, substrate water ratio and hydrolysis time on glucose recovery (%).

Starch constitutes 47.16% of the cassava peel biomass (Table 3.1) Starch was hydrolysed by the enzymes, α-amylase and amyloglucosidase (Table 4.3 and 4.5). Glucose recovery from amyloglucosidase enzyme hydrolysis from the experimental runs ranged from 6.6% - 130% whiles that of α-amylase enzyme hydrolysis ranged from 0.6% - 4.4%. Glucose recovery reduced from 80% to 50% when substrate water ratio was increased from 0.06 g/ml to 0.1 g/ml (Figure 4.3a and 4.3b). The model coefficient of substrate water ratio (331.72) was higher than the coefficient of time (4.23) and enzyme concentration (-0.45), signifying the importance of substrate water ratio rather than enzyme concentration and time of hydrolysis in amyloglucosidase hydrolysis process (Table 4.4). The interactive analysis of the independent variable showed that hydrolysis of cassava peel with 30 U/g of amyloglucosidase at 55 ^{o}C and pH 4.5 for 48 hours with water-substrate ratio of 0.06 g/ml provides mean optimum glucose recovery of 100 % (Figure 4.3a and 4.3b). Increasing α-amylase enzyme activity from 10 (U/g) to 30 (U/g) did not increase glucose recovery. However, glucose recovery only increased slightly to 2.5% at 50 (U/g). Generally, glucose recovery reduced from 3% intitially to 1.5% after 24 hours and rose to 1.8% at 48 hours (Figure 4.4a and 4.4b). Hydrolysis of cassava residue with 30 Units of α amylase at 40 ^{o}C, pH 6 for 48 hours with water-substrate ratio of 0.06 g/ml provides mean optimum glucose recovery of 1.5 % (Figure 4.4a and 4.4b).

Table 4.3: Central Composite Design Matrix for Three Independent Variables on Glucose Yield.

Run Order	X_3 (U/g)	Y (hrs)	Z (g/ml)	Glucose (g/L)	Glucose recovery (%)
1	10	24	0.06	36.4325	79.9
2	50	24	0.06	41.9871	93.5
3	50	48	0.10	82.8364	110.4
4	30	24	0.06	39.3220	86.9
5	10	48	0.02	20.1034	130.9
6	30	24	0.06	41.4296	91.6
7	50	48	0.02	20.0347	125.7
8	10	0	0.10	5.1008	6.8
9	30	24	0.06	36.0184	78.4
10	30	24	0.06	38.4489	84.8
11	30	48	0.06	54.9229	121.3
12	10	0	0.02	1.0623	6.6
13	50	0	0.02	2.0232	13.4
14	30	0	0.06	3.0277	6.7
15	30	24	0.10	51.1959	68.4
16	30	24	0.06	40.0190	87.5
17	30	24	0.02	13.6912	87.1
18	10	48	0.10	71.7190	95.2
19	50	0	0.10	5.2738	6.9
20	30	24	0.06	36.1972	80.5

Table 4.4: Estimated Regression Coefficients for Glucose recovery (%), Amyloglucosidase

Term	Coef	SE Coef	T	P
Constant	6.68	8.85	0.755	0.468
X_3	-0.45	0.51	-0.867	0.406
Y	4.23	0.32	13.199	0.000
Z	331.72	256.87	1.291	0.226
X_3^2	0.01	0.01	0.941	0.369
Y^2	-0.03	0.01	-6.181	0.000
Z^2	-3718.75	1992.08	-1.867	0.091
X_3Y	0.00	0.00	0.207	0.840
X_3Z	2.14	2.34	0.916	0.381
YZ	-5.82	1.95	-2.990	0.014

S = 5.286 R-Sq = 99.2% R-Sq(adj) = 98.4%

X_3- Amyloglucosidase enzyme concentration (U/g), Y- Time (h), Z- Substrate water ratio (g/ml)

Table 4.5: Central Composite Design Matrix for Three Independent Variables on Glucose Yield**.**

Run Order	X_4 (U/g)	Y (hrs)	Z (g/ml)	Glucose (g/L)	Glucose recovery (%)
1	10	24	0.06	0.2833	0.6
2	50	24	0.06	0.4743	1.0
3	50	48	0.10	0.0966	0.1
4	30	24	0.06	0.6187	1.4
5	10	48	0.02	0.4677	3.1
6	30	24	0.06	0.6428	1.4
7	50	48	0.02	0.6855	4.4
8	10	0	0.10	2.2731	2.9
9	30	24	0.06	0.6585	1.5
10	30	24	0.06	0.5991	1.3
11	30	48	0.06	0.5997	1.3
12	10	0	0.02	0.3909	2.5
13	50	0	0.02	0.4073	2.6

14	30	0	0.06	1.4365	3.1
15	30	24	0.10	1.4526	1.9
16	30	24	0.06	0.4712	1.0
17	30	24	0.02	0.6081	3.7
18	10	48	0.10	0.0342	0.0
19	50	0	0.10	2.7778	3.6
20	30	24	0.06	0.3564	0.8

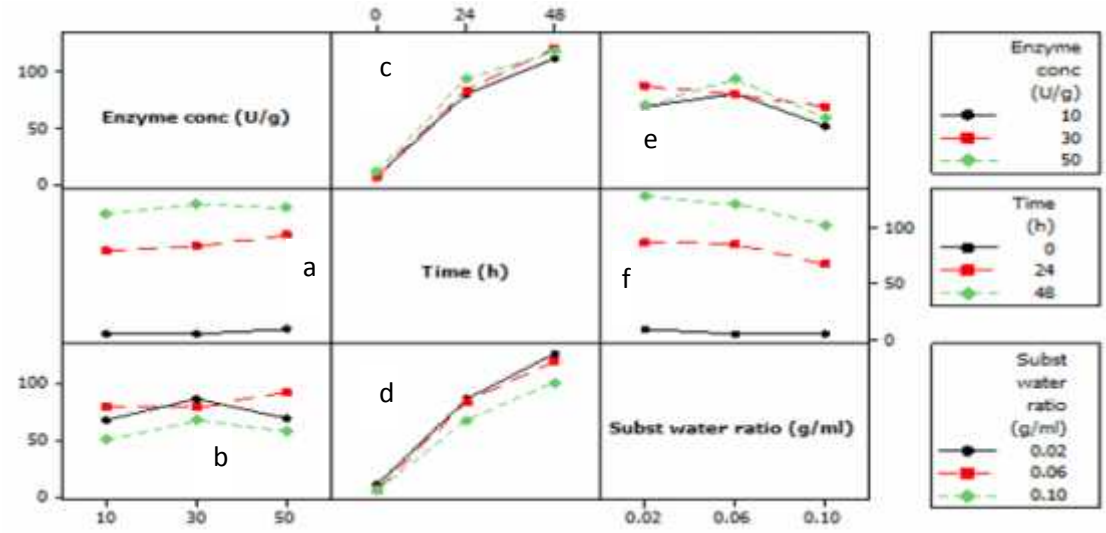

Figure 4.3a: The effect of interaction between amyloglucosidase concentration, substrate-water ratio and hydrolysis time on the percentage recovery of glucose. Data plotted are the means of glucose recovery (%) resulting from; (a and c) Enzyme concentration and hydrolysis time; (b and e) Enzyme concentration and substrate water ratio; (d and f) Substrate water ratio and hydrolysis time.

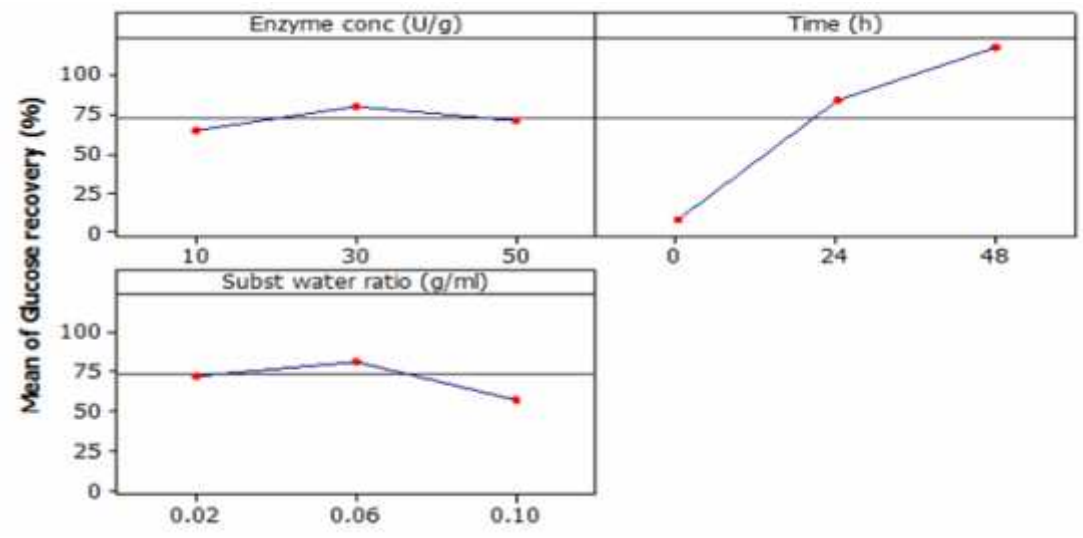

Figure 4.3b: Optimum effect of the interactions between amyloglucosidase concentration, substrate water ratio and hydrolysis time on glucose recovery (%)

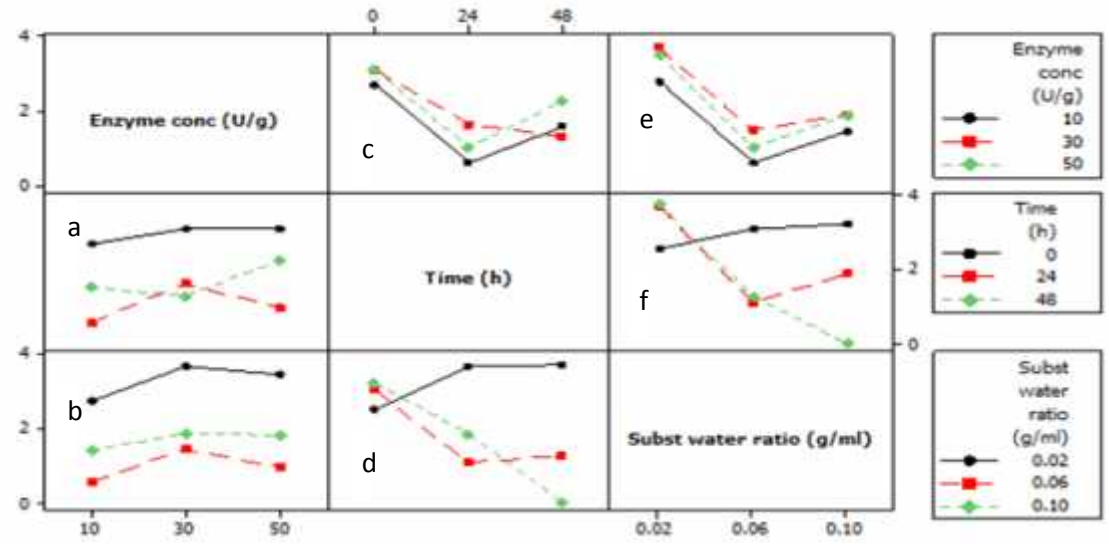

Figure 4.4a: The effect of interaction between amylase concentration, substrate-water ratio and hydrolysis time on the percentage recovery of glucose. Data plotted are the means of glucose recovery (%) resulting from; (a and c) Enzyme concentration and hydrolysis time; (b and e) Enzyme concentration and substrate water ratio; (d and f) Substrate water ratio and hydrolysis time.

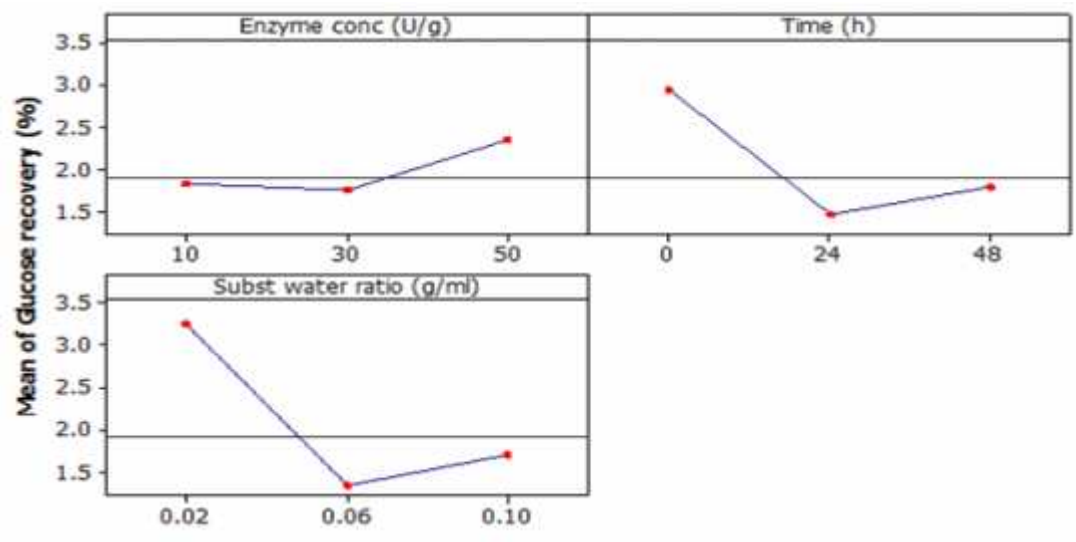

Figure 4.4b: Optimum effect of the interactions between amylase, substrate water ratio and hydrolysis time interactions on glucose recovery (%)

The experimental values of glucose recovery in the single enzyme catalysed hydrolysis reactions (Table 4.6, 4.8, 4.3 and 4.5) were subjected to multiple regression analysis using Minitab 14 software. Positive coefficient for enzyme concentration and time were respectively observed for cellulase (0.076, 0.120), and β-glucosidase (0.610, 0.460) enzymes used in the hydrolysis except amyloglucosidase which showed negative coefficient (-0.45) for enzyme concentration. Substrate water ratio was also positive for β-glucosidase and amyloglucosidase in the enzymatic hydrolysis except cellulase and amylase (Tables 4.7, 4.9, 4.4 and 4.10). None of the independent variables were significant in β-glucosidase hydrolysis (Table 4.9). The hydrolysis time (Y) was significant for cellulase ($p = 0.001$) and amyloglucosidase ($p = 0.000$) hydrolysis whiles enzyme concentration (X_4) was significant for α-amylase ($p = 0.006$) hydrolysis and were found to have significant effects on glucose recovery (Table 4.7, 4.4 and 4.10).

Table 4.6: Central Composite Design Matrix for Three Independent Variables on Glucose Yield.

Run Order	X_1 (U/g)	Y (hrs)	Z (g/ml)	Glucose (g/L)	Glucose recovery (%)
1	10	24	0.06	1.9242	4.2
2	50	24	0.06	2.2419	4.9
3	50	48	0.10	5.2850	6.9
4	30	24	0.06	2.2607	5.0
5	10	48	0.02	0.6053	3.9
6	30	24	0.06	1.8967	4.2
7	50	48	0.02	0.7582	4.8
8	10	0	0.10	1.6825	2.2
9	30	24	0.06	2.2458	5.0
10	30	24	0.06	2.3126	5.1
11	30	48	0.06	2.6879	5.9
12	10	0	0.02	0.2874	1.8
13	50	0	0.02	0.2966	1.9
14	30	0	0.06	0.9644	2.1
15	30	24	0.10	4.9511	6.6
16	30	24	0.06	2.3157	5.1
17	30	24	0.02	0.6312	4.2
18	10	48	0.10	3.7476	4.9
19	50	0	0.10	1.7607	2.3
20	30	24	0.06	2.3233	5.1

Table 4.7: Estimated Regression Coefficients for Glucose recovery (%), Cellulase

Term	Coef	SE Coef	T	P
Constant	1.403	0.735	1.910	0.085
X_1	0.076	0.043	1.774	0.106
Y	0.120	0.027	4.526	0.001
Z	-18.412	21.331	-0.863	0.408
X_1^2	-0.001	0.001	-2.112	0.061
Y^2	-0.002	0.000	-4.190	0.002
Z^2	181.818	165.425	1.099	0.297
X_1Y	0.001	0.000	2.175	0.055
X_1Z	0.172	0.194	0.886	0.396
YZ	0.299	0.162	1.853	0.094

S = 0.4389 R-Sq = 95.6% R-Sq(adj) = 91.7%

X_1- Cellulase enzyme concentration (FPU/g), Y- Time (h), Z- Substrate water ratio (g/ml)

Table 4.8: Central Composite Design Matrix for Three Independent Variables on Glucose Yield.

Run Order	X_2 (U/g)	Y (hrs)	Z (g/ml)	Glucose (g/L)	Glucose recovery (%)
1	1.25	24	0.06	2.1470	4.7
2	2.00	0	0.10	4.1640	5.5
3	1.25	24	0.06	2.1724	4.7
4	1.25	24	0.06	1.2008	2.6
5	2.00	24	0.06	2.2012	4.9
6	1.25	24	0.06	1.9406	4.2
7	0.50	48	0.10	4.0292	5.2
8	1.25	24	0.06	1.6669	3.7
9	1.25	48	0.06	1.8350	4.0
10	1.25	24	0.02	0.3952	2.5
11	0.50	48	0.02	0.4901	3.2
12	1.25	24	0.10	1.4277	1.9
13	2.00	0	0.02	0.4894	3.2
14	0.50	0	0.02	0.4291	2.8

15	0.50	24	0.06	0.8507	1.9
16	2.00	48	0.10	3.3685	4.4
17	0.50	0	0.10	1.4774	1.9
18	1.25	24	0.06	1.1115	2.5
19	2.00	48	0.02	0.4782	3.1
20	1.25	0	0.06	0.8900	1.9

Table 4.9: Estimated Regression Coefficients for Glucose recovery (%), β-glucosidase

Term	**Coef**	**SE Coef**	**T**	**P**
Constant	3.3582	0.4040	8.313	0.000
X_2	0.6100	0.3716	1.642	0.132
Y	0.4600	0.3716	1.238	0.244
Z	0.4100	0.3716	1.103	0.296
X_2^2	0.6045	0.7086	0.853	0.414
Y^2	0.1545	0.7086	0.218	0.832
Z^2	-0.5955	0.7086	-0.840	0.420
X_2Y	-0.6125	0.4155	-1.474	0.171
X_2Z	0.3125	0.4155	0.752	0.469
YZ	0.2375	0.4155	0.572	0.580

S = 1.175 R-Sq = 49.1% R-Sq(adj) = 3.3%

X_2- Enzyme concentration (U/g), Y- Time (h), Z- Substrate water ratio (g/ml)

Given that the substrate consisted of mixed carbohydrate polymers, we investigated the effect of mixed enzyme hydrolysis of the cassava peel biomass in one reaction vessel. A total of ninety (90) hydrolysis experiments of variable factor combinations were undertaken (Table 4.11). The experimental results of the ninety (90) runs from a mixture of the four enzymes at varied concentrations, temperature and time as six independent variables resulted in 31 out of the 90 runs yielding glucose recovery of more than 80% (Table 4.11). The model coefficient of temperature (21.164) was higher than the coefficient of the other variables suggesting the importance of temperature in mixed enzymatic hydrolysis of the cassava peel (Table 4.12).

Table 4.10: Estimated Regression Coefficients for Glucose recovery (%), Amylase

Term	Coef	SE Coef	T	P
Constant	2.673	0.639	4.180	0.002
X_4	0.131	0.037	3.522	0.006
Y	-0.012	0.023	-0.534	0.605
Z	-81.233	18.566	-4.375	0.001
X_4^2	-0.002	0.001	-3.354	0.007
Y^2	0.001	0.000	2.723	0.021
Z^2	767.045	143.982	5.327	0.000
X_4Y	0.000	0.000	0.555	0.591
X_4Z	-0.094	0.169	-0.555	0.591
YZ	-1.146	0.141	-8.144	0.000

S = 0.3820 R-Sq = 95.1% R-Sq(adj) = 90.7%

X_4- Amylase enzyme concentration (U/g), Y- Time (h), Z- Substrate water ratio (g/ml)

Table 4.11: Central Composite Design Matrix for Six Independent Variables on Glucose Yield.

Run Order	X_1 (FPU/g)	X_2 (U/g)	X_3 (U/g)	X_4 (U/g)	X_5	X_6	Glucose (g/L)	Glucose Recovery (%)
1	50	2.00	50	10	40	4	49.1210	107.4
2	50	0.50	10	50	60	4	69.1921	152.9
3	30	1.25	30	30	50	5	42.9363	95.1
4	50	0.50	50	50	60	6	10.7165	23.5
5	10	0.50	50	50	60	4	74.7468	164.6
6	50	0.50	10	50	40	6	5.7578	12.7
7	10	0.50	10	50	60	6	7.8990	17.4
8	30	1.25	30	30	50	5	35.1171	77.6
9	50	0.50	50	50	40	4	44.5113	98.7
10	10	2.00	50	50	60	6	6.7031	14.9
11	50	0.50	10	10	40	4	40.9370	90.3
12	30	1.25	30	30	50	5	35.5443	78.5
13	30	1.25	30	30	50	5	40.3106	88.9

14	10	2.00	50	10	60	6	6.5342	14.3
15	30	1.25	30	30	50	5	38.5009	85.2
16	30	1.25	30	30	50	5	36.5155	81.2
17	30	1.25	30	30	50	5	36.2493	80.5
18	10	2.00	50	50	40	6	3.5745	7.9
19	10	0.50	10	50	60	4	55.8886	123.7
20	50	1.25	30	30	50	5	35.4380	78.5
21	50	0.50	10	50	40	4	34.6163	76.5
22	10	0.50	10	50	40	4	38.8366	85.8
23	50	2.00	50	50	40	4	41.5228	91.6
24	10	2.00	10	50	60	6	14.9875	33.0
25	10	2.00	10	10	60	4	48.6858	108.0
26	50	2.00	50	10	40	6	3.2152	7.1
27	30	0.50	30	30	50	5	35.4484	78.0
28	10	2.00	50	50	40	4	38.343	84.8
29	30	1.25	30	30	50	4	57.3197	126.8
30	10	1.25	30	30	50	5	31.0419	68.6
31	30	1.25	30	30	60	5	26.9587	59.5
32	10	2.00	10	10	60	6	4.3287	9.5
33	10	0.50	50	10	40	4	36.9384	81.3
34	30	1.25	30	30	50	5	33.8879	75.4
35	50	0.50	10	10	60	4	41.3387	91.7
36	10	2.00	10	50	40	4	31.568	70.2
36	10	0.50	50	50	60	6	4.4277	9.8
38	50	0.50	50	50	40	6	3.6969	8.2
39	10	0.50	50	10	40	6	3.1448	7.0
40	50	0.50	10	10	60	6	4.6209	10.2
41	50	0.50	50	10	60	6	5.3478	11.8

42	10	2.00	50	10	60	4	52.0764	114.4
43	50	0.50	50	10	60	4	54.9117	120.8
44	30	1.25	50	30	50	5	29.7251	66.0
45	30	1.25	30	30	50	5	29.2344	64.2
46	10	0.50	10	10	60	6	4.2224	9.3
47	30	1.25	10	30	50	5	28.2534	62.6
48	10	2.00	50	10	40	4	38.433	84.5
49	50	2.00	50	10	60	4	51.9492	114.1
50	30	2.00	30	30	50	5	28.6986	63.6
51	30	1.25	30	30	40	5	21.4417	47.2
52	50	2.00	10	50	60	6	4.5604	10.1
53	30	1.25	30	50	50	5	27.9972	61.7
54	10	2.00	10	50	60	4	46.6661	103.1
55	50	2.00	50	50	60	6	6.8746	15.2
56	50	2.00	50	50	40	6	2.3281	5.2
57	50	0.50	50	50	60	4	52.7802	116.2
58	50	2.00	10	50	40	6	2.6202	5.8
59	30	1.25	30	30	50	5	29.8704	65.7
60	50	2.00	50	10	60	6	7.1585	15.8
61	50	2.00	10	10	40	4	32.6876	72.0
62	10	0.50	10	50	40	6	4.4058	9.7
63	50	2.00	10	10	60	6	3.9949	8.8
64	50	2.00	10	50	40	4	34.5967	76.5
65	30	1.25	30	30	50	5	31.0588	68.7
66	30	1.25	30	30	50	5	30.1778	66.2
67	10	0.50	10	10	60	4	39.8262	87.4
68	50	2.00	10	10	40	6	2.7445	6.0
69	10	0.50	50	10	60	6	4.1862	9.2

70	30	1.25	30	30	50	5	31.0644	69.0
71	10	0.50	10	10	40	4	34.0116	75.3
72	10	0.50	50	10	60	4	49.4293	109.6
73	50	0.50	50	10	40	6	3.8313	8.4
74	30	1.25	30	30	50	5	31.3561	69.4
75	10	0.50	10	10	40	6	2.9991	6.6
76	50	2.00	50	50	60	4	48.625	107.4
77	50	0.50	50	10	40	4	37.2966	82.1
78	10	2.00	10	10	40	4	30.8892	68.5
79	50	0.50	10	10	40	6	2.4564	5.4
80	30	1.25	30	10	50	5	29.7811	65.4
81	50	2.00	10	50	60	4	37.2907	82.9
82	10	2.00	50	10	40	6	3.4991	7.7
83	10	2.00	10	50	40	6	4.3667	9.7
84	30	1.25	30	30	50	6	13.9322	30.7
85	50	2.00	10	10	60	4	38.7941	85.9
86	50	0.50	10	50	60	6	4.5931	10.2
87	10	0.50	50	50	40	6	3.8483	8.5
88	10	2.00	10	10	40	6	3.5135	7.8
89	10	0.50	50	50	40	4	35.8205	79.0
90	10	2.00	50	50	60	4	47.4749	104.2

Table 4.12: Estimated Regression Coefficients for Glucose Recovery (%) (Mixed Enzymes)

Term	Coef	SE Coef	T	P
Constant	-187.854	198.455	-0.947	0.348
X_1	0.289	1.123	0.257	0.798
X_2	-4.467	32.627	-0.137	0.892
X_3	1.769	1.123	1.576	0.120
X_4	1.320	1.123	1.176	0.244
X_5	21.164	6.763	3.130	0.003
X_6	-92.487	67.626	-1.368	0.176
X_1^2	0.007	0.017	0.424	0.673
X_2^2	0.170	11.943	0.014	0.989
X_3^2	-0.016	0.017	-0.953	0.344
X_4^2	-0.018	0.017	-1.065	0.291
X_5^2	-0.174	0.067	-2.583	0.012
X_6^2	8.046	6.718	1.198	0.236
X_1X_2	-0.069	0.087	-0.796	0.429
X_1X_3	0.002	0.003	0.709	0.481
X_1X_4	-0.003	0.003	-0.844	0.402
X_1X_5	-0.009	0.006	-1.378	0.173
X_1X_6	-0.032	0.065	-0.487	0.628
X_2X_3	0.068	0.087	0.784	0.436
X_2X_4	-0.209	0.087	-2.411	0.019
X_2X_5	-0.216	0.173	-1.250	0.216
X_2X_6	3.569	1.731	2.062	0.043
X_3X_4	-0.004	0.003	-1.123	0.266
X_3X_5	0.002	0.006	0.374	0.709
X_3X_6	-0.162	0.065	-2.500	0.015
X_4X_5	0.012	0.006	1.864	0.067
X_4X_6	-0.053	0.065	-0.817	0.417
X_5X_6	-0.567	0.130	-4.370	0.000

X_1 - Cellulase (FPU/g), X_2 - β glucosidase (U/g), X_3 - Amyloglucosidase (U/g), X_4 - Amylase (U/g), X_5- Temperature ^oC, X_6 - pH

S = 10.39 R-Sq = 95.5% R-Sq(adj) = 93.6%

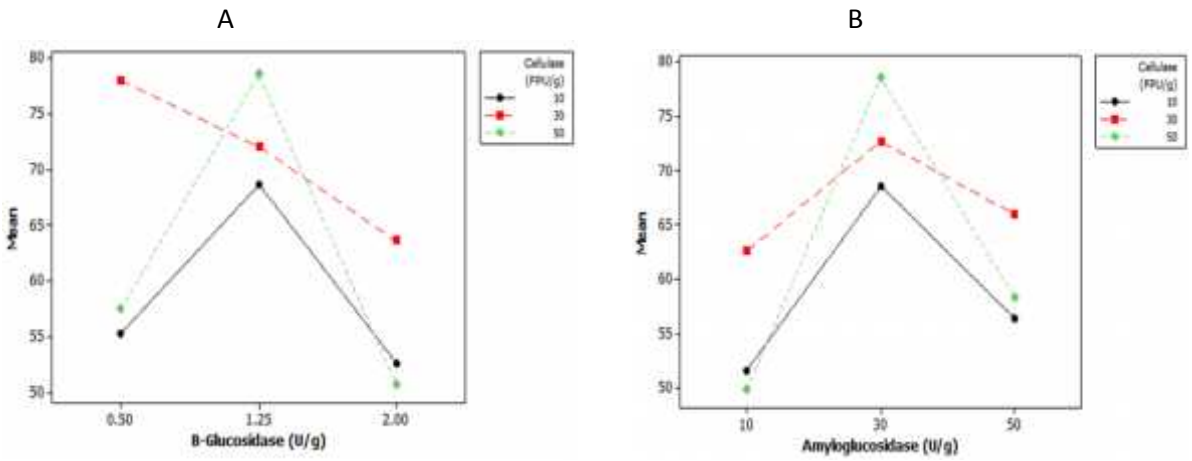

Figure 4.5: Mean plot of interactions of cellulase with β-glucosidase (A) and amyloglucosidase (B) on glucose recovery (%)

High cellulase activity of 50 (FPU) was needed in combination with starch hydrolysis enzymes to produce 75% glucose recovery. However, a low β-glucosidase activity (0.5 U/g) was needed when used with starch hydrolysing enzymes. This is due to low concentration of cellobiose in the cassava peel for β-glucosidase to convert to glucose (Figures 4.5, 4.6 and 4.7). The activity units of β-glucosidase and cellulase were lower when used together because they supplement each other in cellulose hydrolysis to produce glucose (Figure 4.5). Equal activity units of amyloglucosidase and α-amylase were adequate for optimum glucose recovery in the mixture (Figure 4.7). Temperature of 50 ^{o}C provided the optimum conditions for all the enzymes in the mixture to produce glucose recovery of over 70% while pH 4 provided the optimum conditions for all the enzymes in the mixture to produce glucose recovery of over 100% (Figures 4.8, 4.9, 4.10 and 4.11).

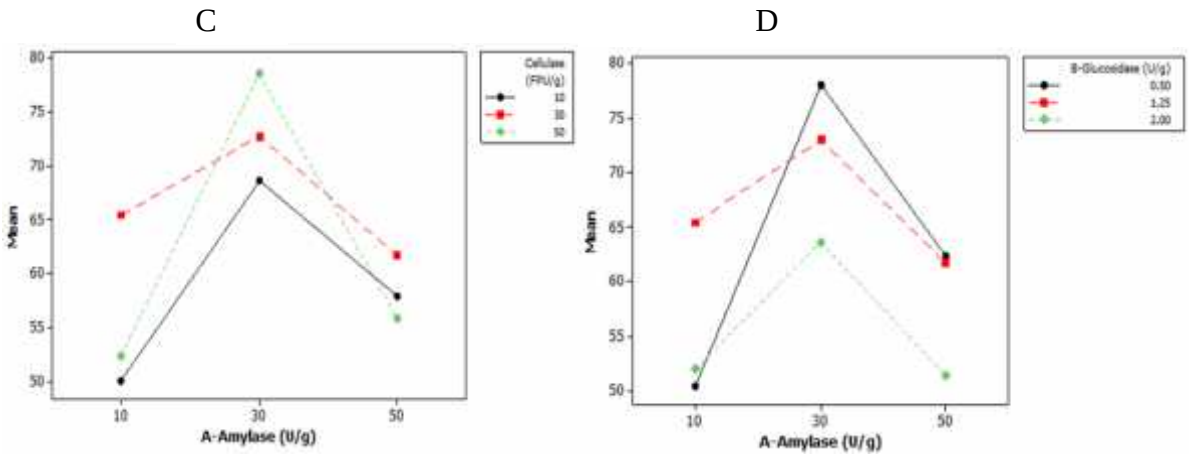

Figure 4.6: Mean plot of interactions of cellulase with α-amylase (C), β-glucosidase and α-amylase (D) on glucose recovery (%)

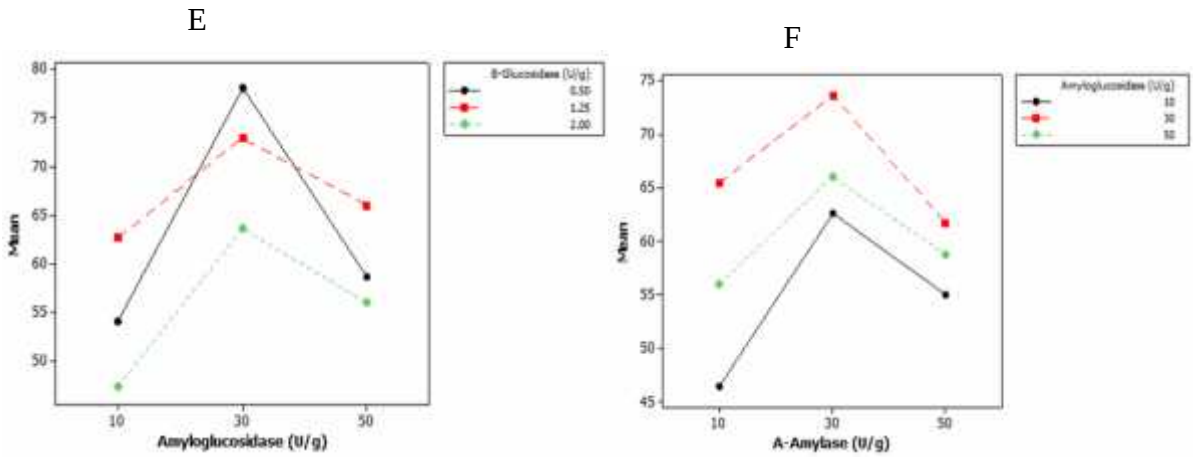

Figure 4.7: Mean plot of interactions of β-glucosidase and amyloglucosidase (E), amyloglucosidase and α-amylase (F) on glucose recovery (%)

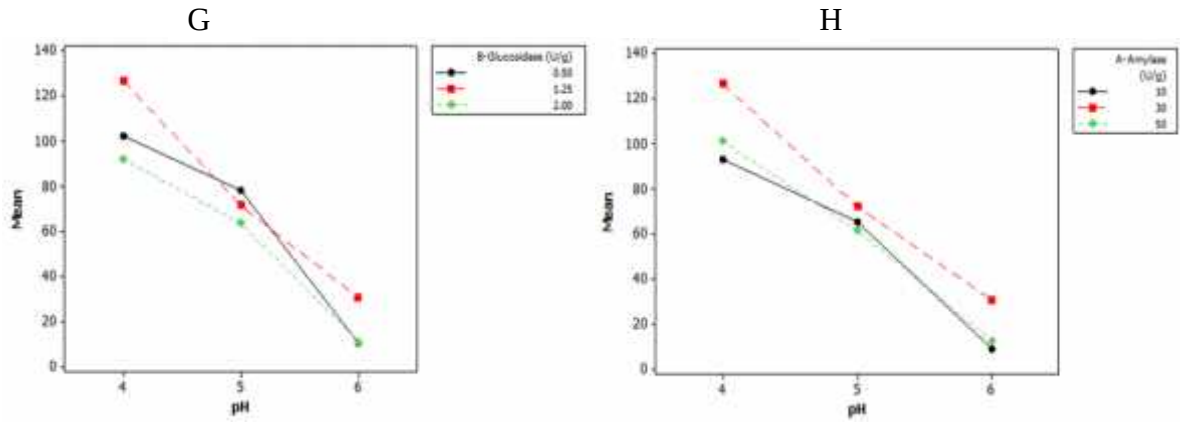

Figure 4.8: Mean plot of interactions of β-glucosidase and pH (G), α-amylase and pH (H) on glucose recovery (%)

All the other variables (X_1, X_2, X_3, X_4 and X_6) in the mixture were not statistically significant for glucose recovery except X_5 (temperature) with p-value 0.003. Although, X_6 (pH) and X_2 (β-glucosidase) were not significant they have negative effect on glucose recovery. However, significant negative effect on glucose recovery were observed in the interactions between β-glucosidase concentration and α-amylase concentration (X_2X_4, p = 0.019), β-glucosidase concentration and pH (X_2X_6, p = 0.043), amyloglucosidase concentration and pH (X_3X_6, p = 0.015), temperature and pH (X_5X_6, p = 0.000) (Table 4.12, Figure 4.6, 4.9 and 4.12).

I J

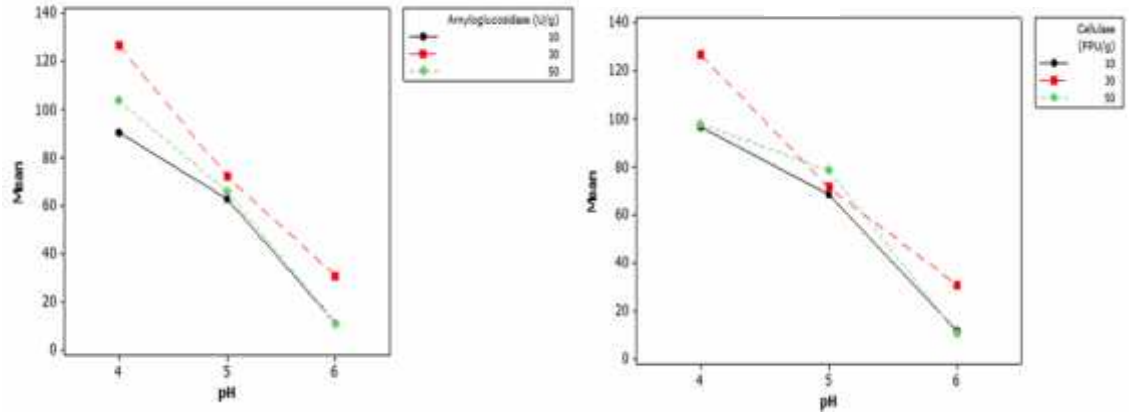

Figure 4.9: Mean plot of interactions of amyloglucosidase and pH (I), cellulase and pH (J) on glucose recovery (%)

K L

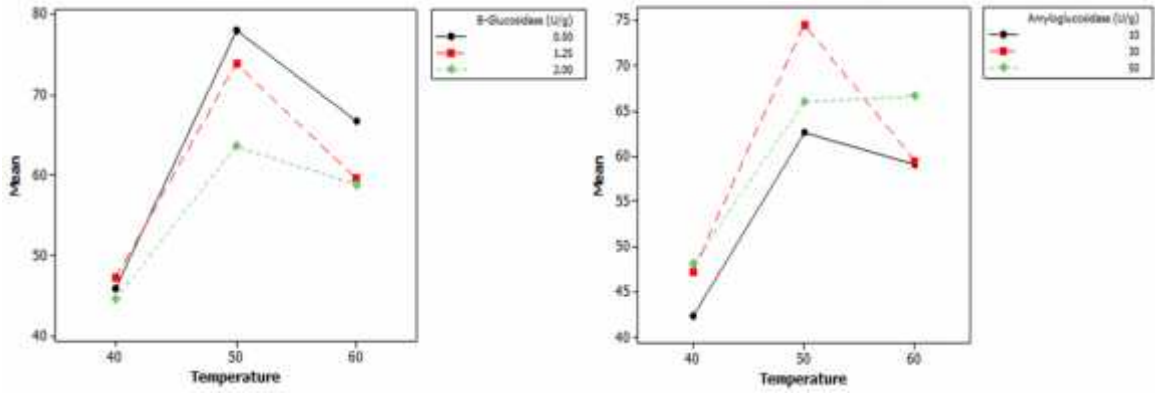

Figure 4.10: Mean plot of interactions of β-glucosidase and temperature (K), amyloglucosidase and temperature (L) on glucose recovery (%)

M N

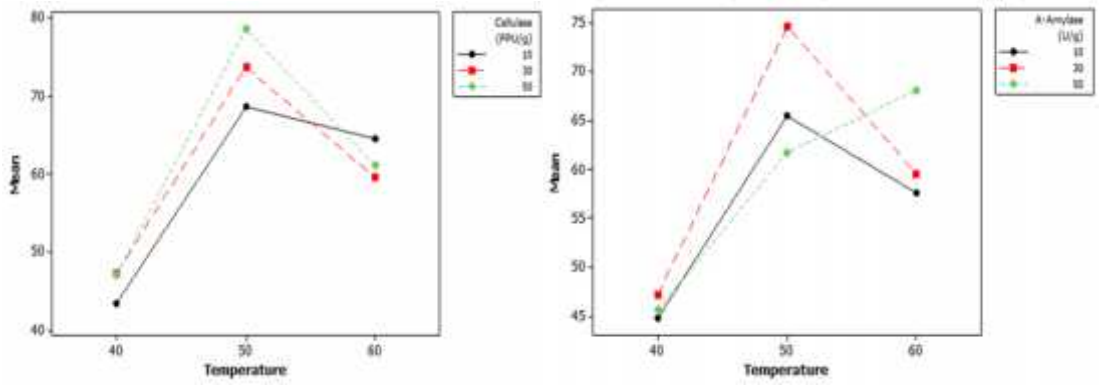

Figure 4.11: Mean plot of interactions of cellulase and temperature (M), α-amylase and temperature (L) on glucose recovery (%)

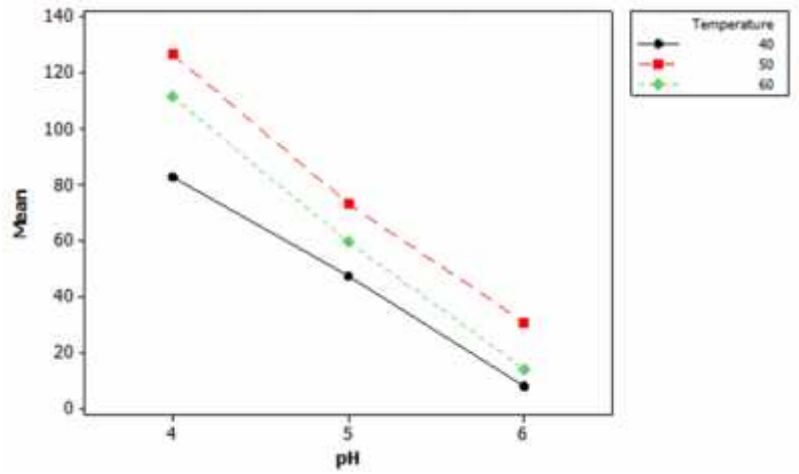

Figure 4.12: Mean plot of interactions of temperature and pH on glucose recovery (%)

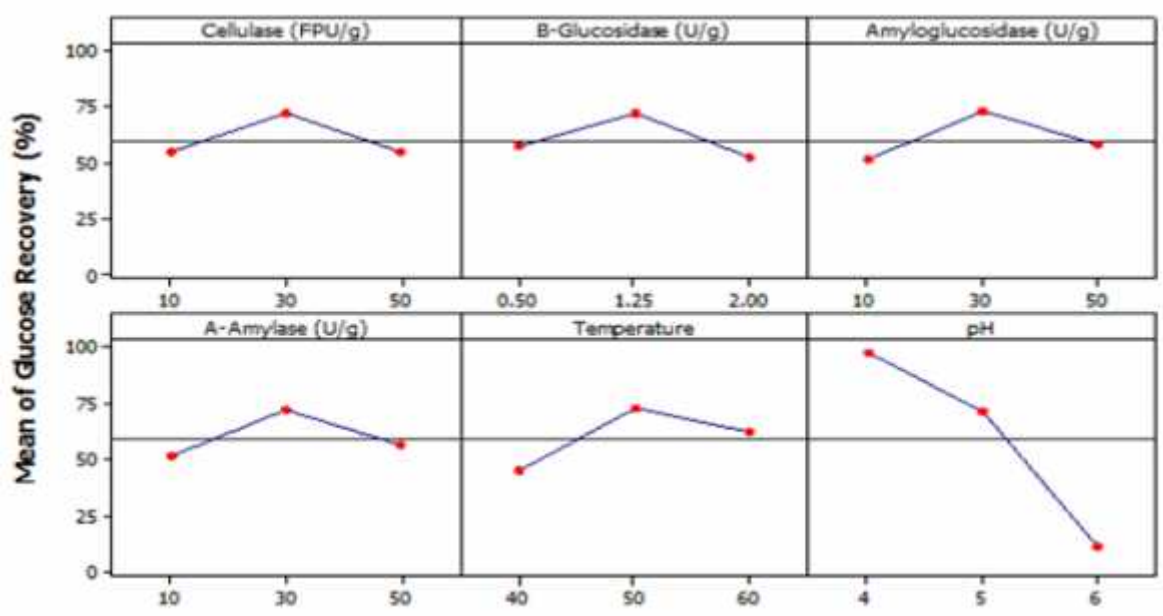

Figure 4.13: Optimum effect of the interactions between cellulase, β-glucosidase, amyloglucosidase, amylase, temperature and hydrolysis time on glucose recovery (%)

4.8 Discussions

4.8.1 Optimisation of enzymatic hydrolysis

The principal structure of cellulose consists of glucose units joined by β-1,4 linkages. These units are joined by strong hydrogen bonding to form microfibrils, making it crystalline in nature and very difficult to degrade. However, some portions of the cellulose structure may be amorphous in nature, which is easier to digest (Van Dyk & Pletschke, 2012). The low hydrolysed glucose concentration released by cellulase in the hydrolysis process could be ascribed to the carbohydrate content of the cassava peel which was high in residual starch, with low content of cellulose, hemicellulose and lignin. The cellulase is only able to convert cellulose to beta-glucose and oligosaccharides by cleaving the 1, 4-beta-d-glycosidic bonds which resulted in less than 6 % of glucose recovery. Increasing the cellulase activity beyond 30 FPU/g could not increase glucose recovery (Figure 4.1a and 4.1b). This phenomenon could be ascribed to the release of cellobiose which is known to inhibit the action of exo and endo glucanases (Shewale, 1982). This confirms that the cellulase enzyme activity has reached its climax and increasing its loading could not be useful.

Unlike the cellulase, increasing activity of β-glucosidase increased glucose recovery (Figure 11a and 11b). The increase in β-glucosidase load in the mixture enhanced its activity thereby stimulating the hydrolysis of cellobiose and cellodextrins to increase glucose recovery. β-glucosidase is very important in cellulose hydrolysis because it removes cellobiose which inhibits the action of exo and endo glucanases (Bairoch, 2000; Tiwari et al., 2013; Zhang & Zhang, 2013).

The high glucose recovery recorded was due to the high content of starch in the cassava peel, which could partly be ascribed to the peeling activity which normally leaves some amount of the flesh which contains starch (Chapter one, Table 3.1). The starch was hydrolysed with

amyloglucosidase by catalysing in a stepwise manner the hydrolysis of α (1,4) glycosidic bonds from the non reducing ends to release glucose. The hydrolysis of α (1,6) branch links are relatively slow forming glucose syrups with glucose content of 97% (w/w) (Patil & Patil, 2000). Generally, the reduction in glucose recovery at high substrate water ratio even at high enzyme activity (Figures 4.1a, 4.3a and 4.3b) may be because many enzyme catalysed reactions proceed through a biochemical pathway. The pathways are interconnected such that a product from a reaction serves as a starting material for the next reaction and at the end of the process , a preferred product is synthesised. Product concentration is normally regulated by shutting down the biochemical pathway through feedback inhibition. The product of the final reaction in that pathway reacts with an enzyme somewhere along the pathway at the enzyme's allosteric site, altering the conformation of the enzyme. As a result of conformational changes that enzyme cannot bind to its substrate effectively, thereby closing down that pathway and stopping the final product from synthesising (Berg et al., 2008).

The low level of glucose recovered in α-amylase hydrolysis was because α-amylase is an endoenzyme which breaks down long-chain carbohydrates by cleaving randomly α-1,4 glycosidic linkages (Patil & Patil, 2000) at locations along the starch chain, thereby yielding maltotriose and maltose from amylose, or maltose, glucose and "limit dextrin" from amylopectin.

Positive coefficient for enzyme concentration and time indicates a linear effect of these variables on glucose recovery while negative coefficients reveal the opposite influence. All the interaction terms were not significant except time and substrate water ratio (YZ) in amyloglucosidase and α amylase hydrolysis which were found to have negative effect on glucose recovery (Table 4.4 and 4.10). When an independent variable is insignificant is an indication that the effect of that factor is not dependent on the setting of the other factors.

Although, substrate water ratio is the major factor affecting the glucose recovery, its effect is not significant and have negative interactive effect on glucose recovery with time of hydrolysis (Figure 4.3a and 4.3b). The negative effect means that the shift of the variable from the low level to the high level produced a decrease in recovery (Ferreira et al., 2009). The reason why interactive effect of substrate concentration and time of hydrolysis had negative effect on glucose recovery of enzymatic hydrolysis of cassava peel may be that increasing of substrate concentration caused high end-product concentration thereby inhibiting activities of enzymes. However the effect of time of hydrolysis was found to be very significant on glucose recovery. This can be observed in (Figure 4.3a and 4.3b) which show the effect of enzyme concentration, hydrolysis time and substrate water ratio on mean glucose recovery.

The model fit (R-square) was determined to be 95.6%, 99.2%, 95.1% and 95.5 respectively except that of β-glucosidase which was 49.1% (Table 4.7, 4.9, 4.4, 4.10 and 4.12). Donkoh et al. (2012) reported that an R-squared of (82%) is acceptable. However the high (R-square) of over 90% suggests that the model is adequate in predicting the relationship between the variables and the response. Therefore, the model is appropriate to predict hydrolysis yield.

The significant interactions between β-glucosidase concentration and α-amylase concentration, β-glucosidase concentration and pH, amyloglucosidase concentration and pH, temperature and pH (Table 4.12) showed that the effectiveness of one variable depended on the other. These interactions also showed negative effect on glucose recovery which meant that shifting one of the variables from the low level to the high level produced a decrease in glucose recovery (Ferreira et al., 2009). This phenomenon is clearly shown in Figures (4.6, 4.9 and 4.12) where increasing β-glucosidase concentration from 0.50–2.00 (U/g) decreases mean glucose recovery from 80% to 64% at α-amylase concentration of 30 (U/g). Similarly,

increasing pH from 4-6, reduced glucose recovery from over 90% to less than 40%. This was due to negative interaction between amyloglucosidase and pH.

4.9 Conclusion

Cassava peel which is considered as waste because of its limited use, is essentially a valuable material for producing reducing sugars, because the residual starch content is quite high. A Central Composite Design (CCD) was applied to optimise the enzymatic hydrolysis of cassava peel in order to produce glucose. Two effective approaches were used in the study. The first one was to optimise the enzymatic hydrolysis process using cellulase, β-glucosidase, amyloglucosidase and α-amylase. The second approach was to optimise enzymatic hydrolysis using the mixture of these enzymes. The effects of enzyme loading, hydrolysis time, substrate concentration, pH and temperature on glucose recovery were investigated. The results were subjected to analysis of variance (ANOVA) to produce polynomial regression model. Mean interaction plot and their effect on glucose recovery were drawn to determine the optimal conditions for enzymatic process. Targeted hydrolysis of specific carbohydrate types of cassava peels with single enzymes showed optimised levels of glucose recovery of over 80% for starch hydrolysing enzymes and about 5% for cellulose hydrolysing enzymes at 0.06 g/ml substrate water ratio at 24 hours of hydrolysis. Single step hydrolysis of cassava peel with mixed enzymes of starch and cellulose hydrolysis enzymes at optimised conditions of celulase (30 FPU/g), β- glucosidase (1.25 U/g), amyloglucosidase (30 U/g), α-amylase (30 U/g), pH 4 and 50 ^{o}C at 24 hours produced recovered glucose of about 100%.

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CHAPTER FIVE

Optimisation of conditions for lysine production using mutant *Corynebacterium glutamicum* (AHP3) strain and hydrolysed cassava peel as carbon source

Abstract

Bioprocess optimisation of fermentation conditions for lysine production from mutant *Corynebacterium glutamicum* AHP3 and hydrolysed cassava peel as carbon source was investigated. A Central Composite Design was applied to optimise the amino acid fermentation process. The effects of substrate concentration, microbial load and time of fermentation on growth of *C. glutamicum*, glucose utilisation and lysine production were studied. The results were subjected to analysis of variance to produce polynomial regression model. Mean interaction plot and their effect on microbial growth, glucose consumption and lysine production were drawn to determine the optimal conditions for amino acid fermentation process for lysine production. Glucose utilisation by *C glutamicum* was found to reduce at higher hydrolysate concentration thereby affecting microbial growth. Lysine production was optimum at low initial microbial load of 0.05 nm (OD) and cassava peel hydrolysate concentration not more than 35% at 48 hours.

Keywords: Optimisation, fermentation, amino acid, hydrolysed cassava peel

5.0 Introduction

Microbial production of amino acid requires a carbohydrate source and the use of agricultural by-products as cheap raw materials has gained prominence over the years (Ansari & Montazer, 2007). Amino acids are important macromolecules for human and animal functions and are the basic units of proteins (Ansari & Montazer, 2007). L-lysine, one of the 20 L-amino acids found in most living organisms, is an essential amino acid for human and animal nutrition (Nelofer et al., 2008). In order to enhance industrial amino acid production, optimisation of fermentation media is very important (Coello et al., 2002; Shakoori et al., 2012)

Humans and animals depend largely on cereals, which are deficient in lysine. Hence fortification of cereal based foods/feed is required (Kjeldsen, 2008; Shah, 1998).

L-lysine is mainly produced on industrial scale using *Corynebacterium glutamicum*. *C. glutamicum* is a gram-positive, non-pathogenic soil dwelling bacteria, that is able to utilise a variety of carbohydrates, organic acids and alcohols as sources of carbon for energy and metabolic needs (Kircher & Pfefferle, 2001; Tryfona & Bustard, 2005). Of the 750,000 tons per annum commercial production of lysine, 80% is by fermentation (Malothu, 2012). Fermentations can be done in batch, fed-batch or continuous reactors. Fed-batch reactors are widely used in industrial applications because they combine the advantages from both batch and continuous processes.

The use of agricultural by-products as substrates for fermentation might offer an inexpensive source of substrate for microbial production of products such as amino acids. Reports of lysine production using molasses, pulpy waste, fish silage and silage juice as substrates for *C. glutamicum* fermentation are common in the literature. (Neuner et al., 2013; Ansari & Montazer, 2007; Coelloet al., 2000). However, there are no reports on the utilisation of

cassava peel substrate for lysine production despite its rich carbohydrate content and all year round abundant availability in Ghana (Obueh & Ikenebomeh, 2014; Reis et al., 2011). Cassava ranks among the most essential tubers grown in Ghana. Out of the total amount of cassava tubers produced in 2014, processing of the tuber yielded about 4.4 million metric tonnes of cassava peels (OECD/IEA, 2010; SRID-MoFA, 2014). Due to its high carbohydrate content of about 62% (Adegbola & Asaolu, 1986), it can be used as a cheap carbon source for *C. glutamicum* to produce lysine for animal feed production.

Composition analysis of cassava peels suggest that its high carbohydrate content is mainly composed of (45%) starch and less than 8.4% cellulose. Lignin which is a major limiting carbohydrate in biomass utilisation is about 15% (Aderemi & Nworgu, 2007; Moshi et al., 2014). In order to enhance lysine production research is been focused on strain improvement, process development as well as media optimisation. Physical parameters like pH, agitation and aeration rate, air saturation, temperature, dissolved CO_2 and foaming are very essential in fermentation. But medium composition is equally a vital factor which strongly influence microbial fermentation processes and mostly become an important area for process development and optimisation study (Anastassiadis, 2007).

In the recent past, statistical experimental methods have been applied to media optimisation for industrial purpose (Amdoun et al., 2010). Generally, the experimental method of optimisation is realised by studying a single factor at any one time. This is done by modifying a factor to find the optimal response, whiles keeping other levels constant. This is known as the one-variable-at-a-time-technique. Unfortunately, it has a major disadvantage of not considering the interactions among the factors thereby not reflecting all the potential effects on the process. Additionally, there is the need for large number of

experiments, needing additional time and expense (Lundstedt et al., 1998). More efficient analytical methods based on Response Surface Methodology (RSM) is better option for optimisation study (Bezerra et al., 2008). RSM and central composite designs has been successfully used to produce enzymes, biomass, and metabolites (Udeh & Achremowicz, 1993).

The objective of this research was to examine the effect of cassava peel hydrolysate concentration, microbial load and fermentation time, on lysine production. Accordingly, response surface methodology based on Central Composite Design (CCD) was used to determine the significant factors that affect the response and assessing the response in terms of lysine yield, microbial growth and glucose consumption from fermentation and also to determine the optimum conditions for lysine yield.

5.1 Amino acid fermentation process

The objective of this research was to examine the effect of cassava peel hydrolysate concentration, microbial load and fermentation time, on lysine production.

The amino acid fermentation process for lysine production using cassava peel as carbon source was investigated in two stages. The first stage was done in shake flasks to investigate the optimised conditions for the growth of *C. glutamicum* AHP3 in cassava peel hydrolysate, glucose utiliation and lysine production. The optimised conditions established were used in a laboratory fermenter to produce lysine. Separate Hydrolysis and Fermentation (SHF) processes were used. All the experiments were run in batches.

5.2 Preparation of cassava peel hydrolysate for fermentation

The cassava peel sample was prepared according to procedure used in Chapter (3.1.2).

About 100 g of milled cassava peel sample was made into slurry with 500 ml of water in 1000 ml blue cap bottle. Enzymatic hydrolysis of the cassava peel slurry was performed with a mixture of four commercial enzyme obtained from Sigma Aldrich The four enzymes, which were mixed based on previously determined Units (Chapter 3 Fig. 22) [cellulase (30 FPU/g), β-glucosidase (1.25 U/g), amyloglucosidase (30 U/g) and α-amylase (30 U/g)] were added in a one-step hydrolysis at 50 ^{o}C and pH 4 in a water bath (Julabo SW-20C), and was shaken at 160 rpm for 48 hours. Ampicillin concentration of (150 µg/ml) was added to prevent microbial contamination during hydrolysis. The hydrolysed liquor was centrifuged at 10,000 rpm for 25 minutes to precipitate undigested particulate matter and the supernatant filtered through (0.8 µm followed by 0.45 µm and 0.2µm) Whatman Cellulose Nitrate Membrane filters.

Figure 5.1: Cassava peel hydrolysate filtered through 0.8 µm, 0.45 µm and 0.2 µm filter paper

5.3 Preparation of bacteria starter culture for lysine fermentation

A nutrient agar plate (7.4 g of Brain heart Infusion (BHI) and 3 g of agar in 200 ml of distilled water) was inoculated with *C. glutamicum* AHP3 (a genetically modified isolate, kindly provided by the Technical University of Denmark (DTU) and incubated at 30 ^{0}C overnight. A colony of the bacteria was aseptically taken and suspended in 1 ml minimal medium, a growth medium prepared according to Liebl et al. (1998). 150µL of suspended culture was added to 6 culture tubes of which 5 tubes contained (0.2% v/v, 1% v/v, 2% v/v, 10% v/v, 20% v/v) cassava peel hydrolysate with equivalent glucose concentration of (0.02%, 0.08%, 0.2%, 0.8% and 1.60%) respectively. The 6th tube contained 2% laboratory grade glucose as control. All the 6 tubes containing 5 ml of culture medium were incubated overnight at 30^{0}C in a G 25 New Brunswick Scientific CO. (Edison, New Jersey, USA) incubator shaker and the growth of bacteria was monitored by measuring the optical density at 600 nm with UV1800 spectrophotometer (Shimadzu, Japan).

5.4 Determination of glucose utilisation and growth of *C. glutamicum* in cassava peel hydrolysate

The growth of *C. glutamicum* in cassava peel hydrolysate was investigated to find out if it can provide all the necessary nutrients required for the bacteria to grow and synthesis lysine. The experiment was conducted in shake flasks. Duplicate samples of 20% (v/v) cassava peel hydrolysate pre-filtered through 0.2 µm filter paper were prepared in 50 ml aliquots of solution with varying treatments (A) 20 % (v/v) of cassava peel hydrolysate in minimal media at pH 7. (B) 20 % (v/v) of cassava peel hydrolysate at pH 7. A starter culture of 2 ml *C. glutamicum* AHP3 with $(OD)_{600\ nm}$ value of 2.74 was added to all samples making initial starter $(OD)_{600\ nm}$ of the samples as 0.1. The samples were fermented at 30 ^{0}C in a water bath (Julabo SW-20C), shaking at 150 rpm for 48 hrs. Samples were drawn from the flask at 1 hour intervals to determine the growth of the bacteria by measuring the optical density (OD) at 600 nm with UV1800 spectrophotometer (Shimadzu, Japan), and lysine with Ultimate 3000 HPLC system (Dionex, Sunnyvale, USA). Samples were also drawn every 3 hours to analyse for glucose in the fermentum with Ultimate 3000 HPLC system (Dionex, Sunnyvale, USA).

5.5 Optimisation of fermentation condition for lysine production by response surface methodology

Response surface methodology was used to study the effect of fermentation parameters on lysine production. The fermentation experiments were performed using a central composite design (CCD). The effects of three factors, including substrate concentration [5% (v/v)-20% (v/v) & 20% (v/v)-50% (v/v)] cassava peel hydrolysate), *C. glutamicum* AHP3 initial inoculum load (0.05 -0.1 OD) and incubation time (0-48hrs) on lysine production, microbial

growth and glucose concentration were investigated. The optimisation study was carried out in two distinct stages.

In the first stage, effects of two factors, including low substrate concentration [5% (v/v) – 20% (v/v)] and inoculum load (0.05 – 0.1 OD) on microbial growth, lysine production and glucose consumption were studied. The fermentation was run for 24 hours. The experimental design for the fermentation had 13 tests with the centre point at five. The values of the variables were coded to lie ±1 for factorial points, 0 for the centre points in axial, 4 for axial points, 4 for cube points and alpha as 1.

In the second stage, effects of three factors, including high substrate concentration [20% (v/v) – 50% (v/v)], inoculum load (0.05 – 0.1 OD) and fermentation time (0-48 h) on microbial growth, lysine production and glucose consumption were studied. The experimental design for the fermentation has 20 tests with the centre point at six. The values of the variables were coded to lie ±1 for factorial points, 0 for the centre points in axial, 6 for axial points, 8 for cube points and alpha as 1. The experimental factors and corresponding levels as well as the experimental design is presented in (Tables 5.1).

Table 5.1: Process variables in coded and actual units for lysine fermentation in shake flask experiment

	Coded level		
Variables	-1	0	+1
Substrate concentration (%), Y_1	5	12.5	20
Time (h), Z	0	24	48
Substrate concentration (%), Y_2	20	35	50
Microbial load (OD)600nm, X	0.05	0.075	0.1

For all the two stages, the flasks were cleaned and sterilised in autoclave at 121 ^{0}C for 15 minutes. Cassava peel hydrolysate [5% (v/v)-20% (v/v) & 20% (v/v)-50% (v/v)] pre-filtered through 0.2 µm filter paper were prepared in defined minimal medium (MM) in 50 ml aliquots. The media in the flasks were inoculated with an initial *C. glutamicum* load (0.05 - 0.1) OD_{600} as determined by the CCD (Table 5.2 and 5.3) and incubated at 30^{0}C and pH 7 in a (Julabo SW-20C) water bath, shaking at 150 rpm. Lysine production, microbial growth and glucose concentration were measured as response variables.

Table 5.2: Central Composite Design Matrix for Two Independent Variables on Lysine Production, Glucose Consumption and Microbial Growth.

RunOrder	X (OD600nm)	Y_1 (%v/v)	Glucose consumption (%)	Microbial growth (OD 600nm)	Lysine (g/L)
1	0.100	12.5	100	22.8	2.2061
2	0.100	5.0	100	10.6	0.1799
3	0.050	20.0	96	36.2	1.8519
4	0.075	12.5	100	36.2	1.4444
5	0.075	12.5	100	22.2	1.2528
6	0.075	5.0	100	10.6	0.2485
7	0.100	20.0	97	37.0	1.5869
8	0.050	5.0	100	10.2	0.1239
9	0.050	12.5	100	20.2	0.8251
10	0.075	12.5	100	23.0	0.6938
11	0.075	12.5	100	23.2	0.7974
12	0.075	20.0	97	38.2	1.0268
13	0.075	12.5	100	23.0	0.7073

Table 5.3: Central Composite Design Matrix for Three Independent Variables on Lysine Production, Glucose Consumption and Microbial Growth

RunOrder	X (OD600nm)	Y_2 (%v/v)	Z (h)	Glucose consumption (%)	Microbial growth (OD 600nm)	Lysine (g/L)
1	0.075	35	24	50	31	0.8017
2	0.100	50	48	51	33	0.8205
3	0.075	50	24	50	48	0.3441
4	0.050	20	0	0	9	0.0000
5	0.075	35	0	0	12	0.0000
6	0.100	20	48	100	23	0.8205
7	0.100	50	0	0	15	0.0000
8	0.075	35	24	51	46	0.8070
9	0.050	35	24	51	44	0.8695
10	0.100	20	0	0	17	0.0000
11	0.050	20	48	100	25	2.7559
12	0.100	35	24	51	35	0.8947
13	0.075	35	24	51	48	0.7558
14	0.075	35	48	52	40	1.3599
15	0.075	20	24	49	35	0.6439
16	0.050	50	48	52	44	1.3649
17	0.075	35	24	51	46	0.8281
18	0.050	50	0	0	7	0.0000
19	0.075	35	24	51	40	0.7729
20	0.075	35	24	51	37	0.7460

5.6 Production of lysine using optimised fermentation conditions

The fermentation was carried out in 1 L fermenters (600 mL working volume) (B. Braun Biotech International GmbH, Germany). The four 1 L fermenters labelled A-D were filled with 600 ml of 20 % (v/v) hydrolysate (1.6 % glucose) pre-filtered through 0.2 µm and prepared in MM . The fermenters together with connecting hoses were sterilized at 121 ^{0}C for 15 minutes in an autoclave. To each fermenter, 600 µL of vitamin sterile filtered through 0.2 µm filter paper, 600 µL of water based antifoam agent was added to prevent excessive foaming and 8 ml of cultured *C. glutamicum* with $(OD)_{600\ nm}$ value of 7.8 were added. The operating conditions of the fermenter were set at temperature of 30 ^{0}C; pH 7.0, 1 bar air flow and stirring at 500 rpm. An alkali dosing pumps was set at 54.5 ml /min to maintain the pH at 7 during the time course of the fermentation. The initial and final working conditions of the fermenters were recorded. Samples were drawn at every 45 minute intervals for OD analysis for bacteria growth, glucose concentration and lysine production.

5.7 Measurement of glucose concentration, microbial growth and lysine production

Growth of bacteria was monitored before and after fermentation by measuring the optical density (OD) at 600 nm with UV1800 spectrophotometer (Shimadzu, Japan). The concentrations of glucose were determined using an Ultimate 3000 HPLC system (Dionex, Sunnyvale, USA) equipped with an Aminex HPX-87H column (Bio-Rad, Hercules, USA) and a Shodex RI-101 detector (Showa Denko KK, Tokyo, Japan). The column oven temperature was set to 60°C, and the mobile phase consisted of 5 mM H_2SO_4 with a flow rate of 0.5 mL/min. Lysine concentration in supernatant was determined as lysine-HCl with Agilent ZORBAX Eclipse Plus C18 Rapid Resolution HD 2.1 × 50 mm, 1.8-Micron column (Agilent, USA) and Ultimate 3000 HPLC system (Dionex, Sunnyvale, USA) at UV 338 nm

after OPA derivatization (Agilent, USA). The detailed separation conditions were as described by Agilent (High-Speed Amino Acid Analysis: http://www.chem.agilent.com/Library/applications/5990- 4547EN.pdf

5.8 Data analysis

The results of each CCD were analysed using Minitab® Statistical software version 14. Both linear and quadratic effects of the three variables under study were calculated, as well as their possible interactions, on lysine production, glucose consumption and microbial growth. Their significance was evaluated by variance analysis (ANOVA). Interactive and main effect plots were drawn to illustrate the effects of the independent variables (substrate concentration, microbial load and fermentation time) on the dependent variable (lysine yield, microbial growth and glucose concentration), being described by a quadratic polynomial equation, fitted to the experimental data. The fit of the models was evaluated by the determination of R-squared coefficient and adjusted R-squared coefficient. The student's t-test was performed to determine significance (p-values) of the regression coefficients. A p-value of less than 0.05 was used as the model criteria.

5.9 Results

5.9.1 Initial assessment of the growth of *C. glutamicum* in medium containing cassava peel hydrolysate

To determine the adequate amount of cassava peel hydrolysate required to support bacterial growth over the experimental period, the growth of *C. glutamicum* in cassava peel hydrolysate was investigated in overnight cultures prior to shake flask and fermenter fermentation experiments using different concentrations of hydrolysed cassava peel (Figure 24). Optical density measurement of over 12 was recorded for 0.8% and 1.6% glucose in cassava peel hydrolysate. This gave an indication that the cassava peel hydrolysate containing 0.8% glucose and above could provide adequate carbon source for *C. glutamicum* growth (Figure 5.2).

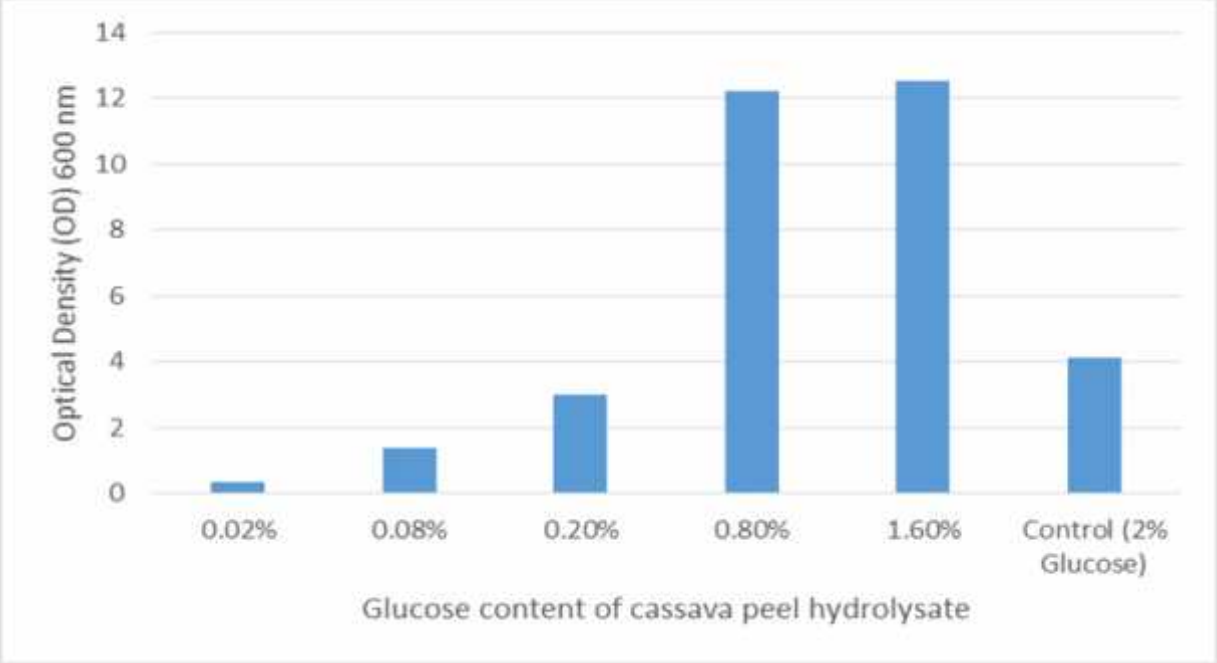

Figure 5.2: Growth of *Corynebacterium glutamicum* AHP3 in overnight culture in single experiment

5.9.2 Determination of glucose utilisation and growth of *C. glutamicum* in cassava peel hydrolysate

The growth of *C. glutamicum* in cassava peel hydrolysate was investigated to find out if it can provide all the necessary nutrients required for the bacteria to grow and synthesis lysine. The experiment was conducted in shake flasks. Shake flask fermentation for 48 hours produced growth of *C. glutamicum* in varying conditions of nutrient medium. The highest growth rate of 0.17 h^{-1} was realised in hydrolysate containing MM, producing 0.14 mmol/L of lysine, (Table 5.4) and about 74% of the glucose in the cassava peel hydrolysate was utilised after 24 hours of fermentation (Figure 5.3). Hydrolysate with no MM added did not produce any lysine and the growth rate of *C. glutamicum* was 0.05 h^{-1}

Table 5.4: Growth and production characteristics of *Corynebacterium glutamicum* AHP3 in 20% (v/v) cassava peel hydrolysate in or without Minimal Medium (MM)

Substrate	**Lysine titer (mmol/L)**	**Maximum OD600nm**	**μ [h-1]**
No addition of MM	0	3.50 ± 0.21	0.05 ± 0.02
Addition of MM	0.14 ± 0.01	14.10 ± 0.28	0.17 ± 0.01

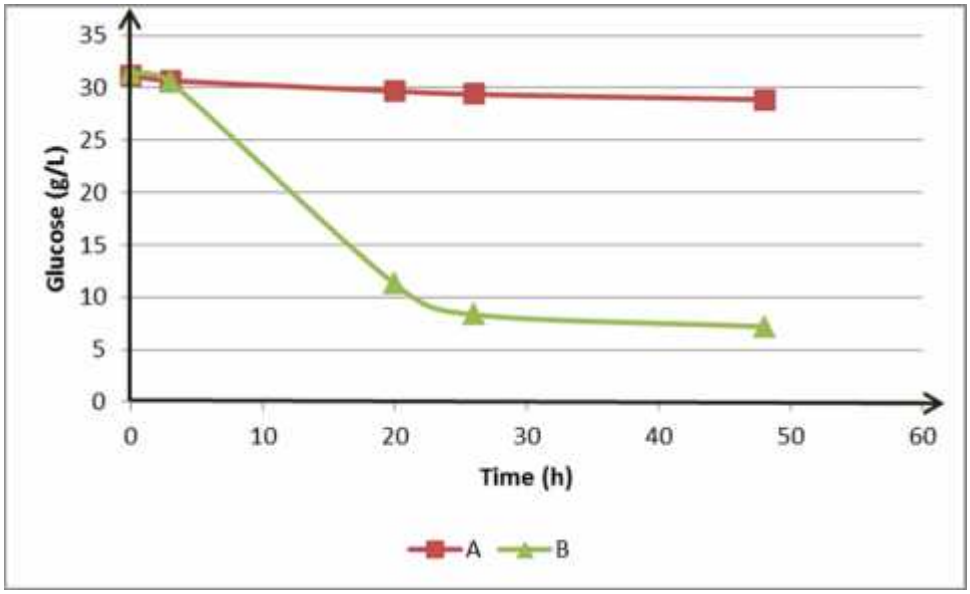

Figure 5.3: Time course of glucose utilisation of *C. glutamicum* on (A) hydrolysed cassava peel with no MM and (B) hydrolysed cassava peel with MM

5.9.3 Optimisation of fermentation condition for lysine production by response surface methodology

The amount of fermentable substrate used in a fermentation process can contribute significantly to the overall cost and economic viability of any industrial process. It is therefore important to determine the optimum level of the substrate required to support microbial life and optimal lysine production per unit time of fermentation. The cassava peel hydrolysate concentration in the shake culture fermentation flasks was varied from [5% (v/v)-20% (v/v) & 20% (v/v)-50% (v/v)], *C. glutamicum* AHP3 initial inoculum load varied from (0.05 -0.1 OD) and incubation time was varied from (0-48hrs).

The results for two independent variables in the optimisation study are presented in (Table 5.2). Over 95 % glucose utilisation was observed in all the 13 experimental runs. The experimental runs with initial glucose concentrations less than 20% (v/v) had 100% glucose utilisation by *C glutamicum* AHP3. Although runs (2, 6 and 8) recorded high glucose utilisation their microbial growth were less than 12 ($OD_{600\ nm}$) which culminated in low lysine production. Lysine production of over 1 g/L was achieved in runs 1, 3, 4, 5, 7 and 12 which also showed high microbial growth of more than 20 ($OD_{600\ nm}$) (Table 5.2). The growth of *C. glutamicum* within 24 hours increased with increasing substrate concentration irrespective of the microbial load used at the beginning of the fermentation. However, the effect of interactions between substrate concentration and microbial load showed slight increase in microbial growth when initial microbial load was 0.075 ($OD_{600\ nm}$) as compared with the others (Figure 5.4a).

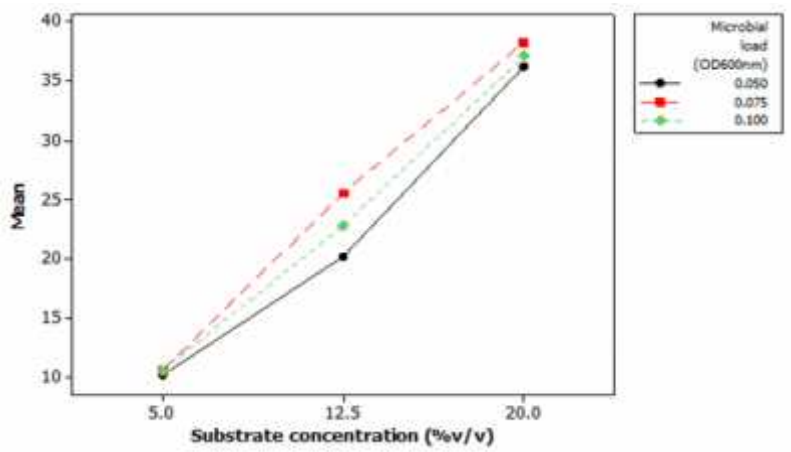

Figure 5.4a: The effect of interaction between microbial load and substrate concentration on microbial growth (OD 600 nm).

The model coefficient of substrate concentration (621.98) was higher than the coefficient of initial microbial load (1.57), which showed the importance of substrate concentration over microbial load in the fermentation process. Positive coefficient was observed for the two independent variables. However, the effect of substrate concentration (p = 0.353) and initial microbial load (p = 0.400) were not significant on microbial growth (Table 5.5). The interactive analysis of the independent variables showed an optimum growth of *C. glutamicum* at 0.075 nm OD and 20% (v/v) hydrolysate after 24 hours of fermentation (Figure 5.4b).

Table 5.5: Estimated Regression Coefficients for Microbial growth (OD 600 nm)

Term	Coef	SE Coef	T	P
Constant	-20.08	26.01	-0.772	0.465
X	621.98	694.52	0.896	0.400
Y_1	1.57	1.58	0.994	0.353
X^2	-4022.07	4484.35	-0.897	0.400
Y_1^2	0.01	0.05	0.138	0.894
XY_1	0.53	12.42	0.043	0.967

S = 4.658 R-Sq = 87.7% R-Sq(adj) = 79.0%

X- Microbial load (OD 600 nm), Y_1- Substrate concentration (%v/v)

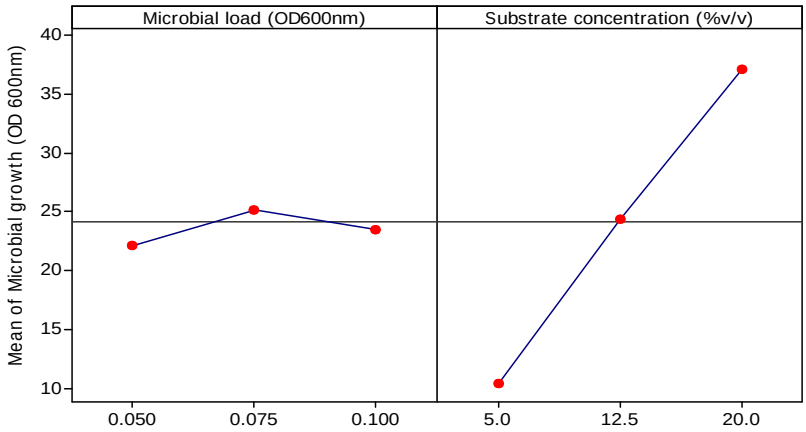

Figure 5.4b: Optimum effect of initial microbial load and substrate concentration interactions on microbial growth (OD 600 nm)

About 100 % glucose consumption was realised in substrate concentrations not more than 12.5 % during the fermentation process (Figure 5.5a). The interaction analysis of the variables indicated that there was 100% glucose utilisation when the substrate concentration was 12.5% and below. However, at substrate concentration higher than 12.5%, the glucose utilisation in the fermentation process started reducing (Figure 5.5a). The microbial load was significant with p-value of 0.000 showing positive effect on glucose utilisation. Substrate concentration was not significant. However, the interaction of substrate concentration and microbial load was significant with p-value of 0.023 (Table 5.6). The optimum effect of independent variables interactions on glucose utilisation of *C. glutamicum* showed that at 0.075 nm (OD) microbial load and glucose concentrations of 12.5% and below provided 100% glucose utilisation (Figure 5.5b).

Table 5.6: Estimated Regression Coefficients for Glucose Consumption (%)

Term	Coef	SE Coef	T	P
Constant	97.974	0.967	101.367	0.000
X	18.966	25.812	0.735	0.486
Y_1	0.398	0.059	6.796	0.000
X^2	-193.103	166.663	-1.159	0.285
Y_1^2	-0.029	0.002	-15.559	0.000
XY_1	1.333	0.462	2.888	0.023

S = 0.1731 R-Sq = 99.2% R-Sq(adj) = 98.6%

X- Microbial load (OD 600 nm), Y_1- Substrate concentration (%v/v)

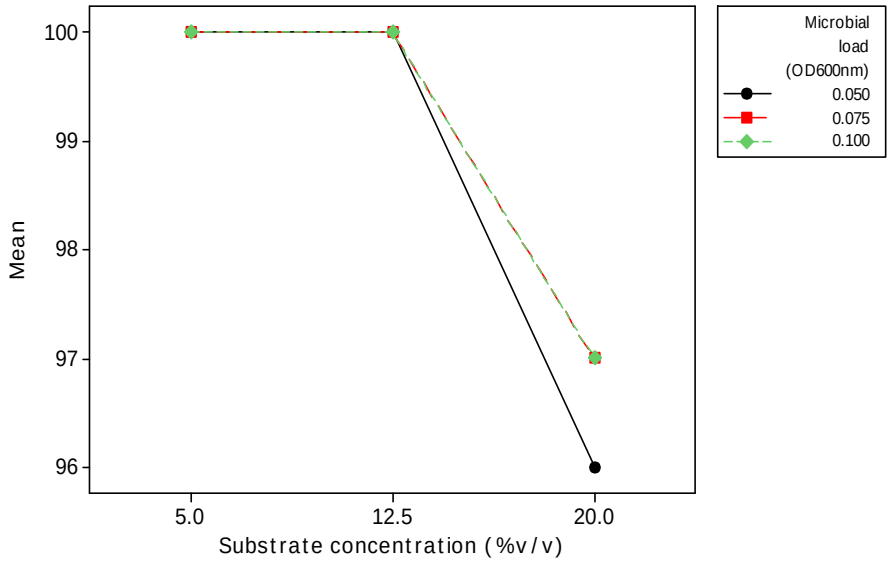

Figure 5.5a: The effect of interaction between microbial load and substrate concentration on glucose consumption (%)

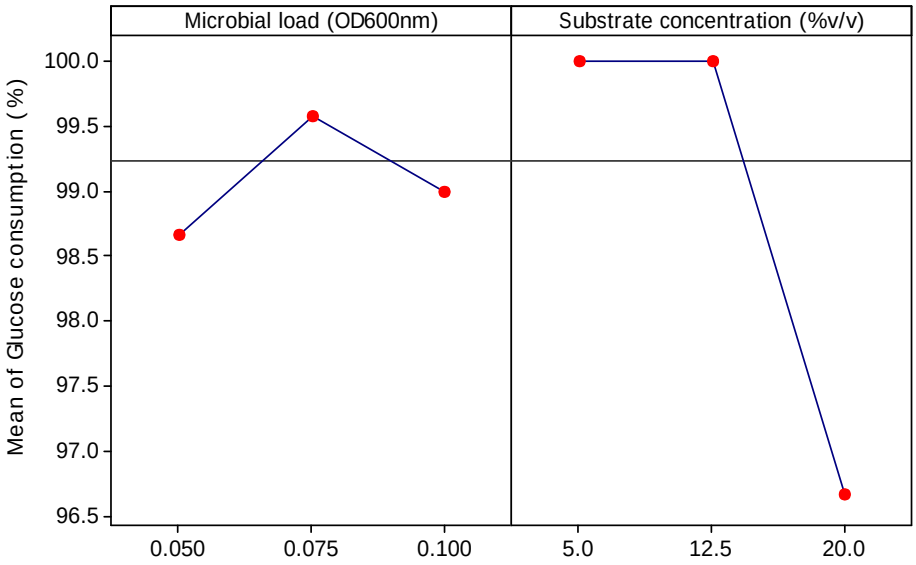

Figure 5.5b: Optimum effect of initial microbial load and substrate concentration interactions on glucose consumption (%)

In lysine production, substrate concentration and microbial load variables were not significant. Substrate concentration had negative coefficient and showed negative effect on lysine production within the period of fermentation (Table 5.7). The interaction results of the variables showed that at low substrate concentration of 5% (v/v) less than 0.5 g/L of lysine was produced by all the microbial loads (Figure 5.6a). Generally lysine production increased with increasing substrate concentration for all the microbial loads except for 0.100 nm (OD) which showed decline in lysine production when substrate concentration was increased above 12.5%. Similarly, 0.075 nm (OD) microbial load did not show any significant increase in lysine production after 12.5% substrate concentration (Figure 5.6a). The main effect of the interactions of the variable showed that lysine production is optimum at higher glucose concentration and high microbial growth for 24 hours of fermentation (Figure 5.6b).

Table 5.7: Estimated Regression Coefficients for Lysine Production (g/L)

Term	Coef	SE Coef	T	P
Constant	1.462	2.552	0.573	0.585
X	-87.961	68.146	-1.291	0.238
Y_1	0.322	0.155	2.082	0.076
X^2	674.163	440.006	1.532	0.169
Y_1^2	-0.008	0.005	-1.660	0.141
XY_1	-0.428	1.219	-0.351	0.736

S = 0.4570 R-Sq = 71.0% R-Sq(adj) = 50.3%

X- Microbial load (OD 600 nm), Y_1- Substrate concentration (%v/v)

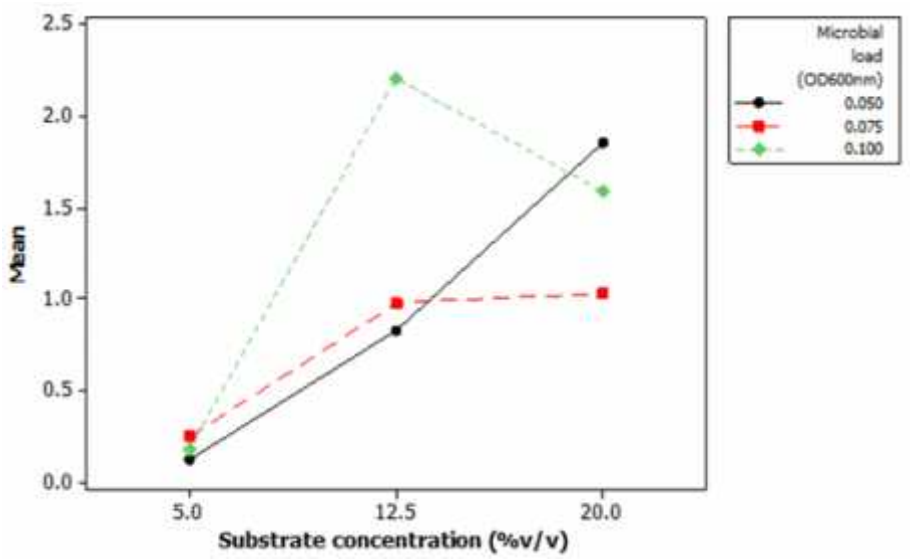

Figure 5.6a: The effect of interaction between microbial load and substrate concentration on lysine production (g/L)

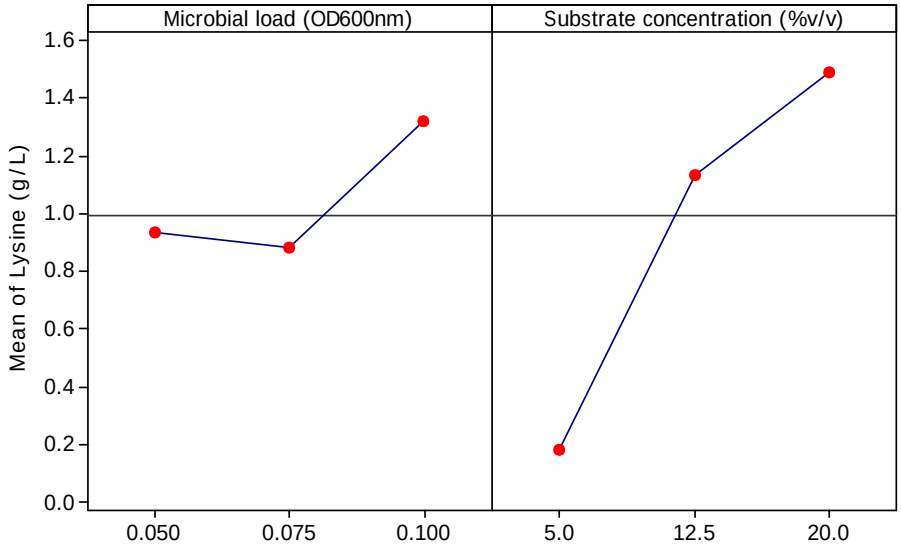

Figure 5.6b: Optimum effect of initial microbial load and substrate concentration interactions on lysine production (g/L)

The results for three independent variables in the optimisation study are presented in (Table 5.3). At substrate concentrations higher than 20% only about 50% of glucose was utilised by *C. glutamicum* during the fermentation period (Table 5.3). Glucose utilisation of the microorganism increased with time. However, for substrate concentration higher than 20%, glucose utilisation remained constant till 48 hours (Figure 5.7a). The interaction of microbial load with substrate concentration showed no difference in glucose utilisation up to 35% substrate concentration. However glucose utilisation dropped slightly below 50% when 0.100 nm and 0.050 nm (OD) microbial load was used (Figure 5.7a). Regression analysis also showed that the interaction between substrate concentration and microbial load was not significant and showed negative interaction. The interaction between substrate concentration and time was significant with p-value of 0.001 and showed negative interaction. All the independent variables were not significant except time which had p-value of 0.000 for glucose consumption (Table 5.8). Generally, glucose consumption reduced at higher hydrolysate concentration but increased with time (Figure 5.7b).

Table 5.8: Estimated Regression Coefficients for Glucose Consumption (%)

Term	Coef	SE Coef	T	P
Constant	49.25	41.83	1.177	0.266
X	-1165.52	1115.70	-1.045	0.321
Y_2	-0.87	1.51	-0.573	0.579
Z	4.35	0.57	7.635	0.000
X^2	7854.55	7193.39	1.092	0.300
Y_2^2	0.02	0.02	0.758	0.466
Z^2	-0.03	0.01	-4.469	0.001
XY_2	-0.33	7.03	-0.047	0.963
XZ	-0.21	4.39	-0.047	0.963
Y_2Z	-0.03	0.01	-4.600	0.001

S = 7.456 R-Sq = 96.6% R-Sq(adj) = 93.6%

X- Microbial Load (OD 600 nm), Y_2- Substrate Concentration (%), Z- Time (h)

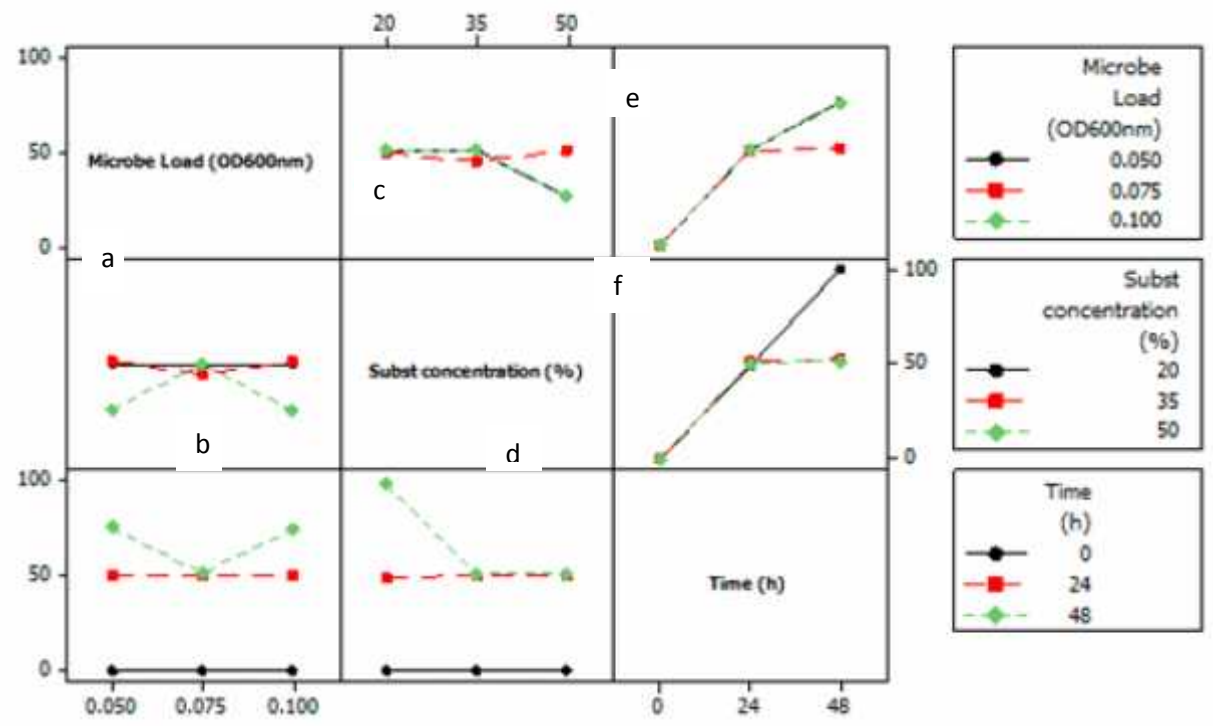

Figure 5.7a: The effect of interaction between microbial load, substrate concentration and fermentation time on glucose consumption (%).Data plotted are the means of glucose consumption (%) resulting from; (a and c) Substrate concentration and microbial load; (b and e) Microbial load and fermentation time; (d and f) Substrate concentration and fermentation time.

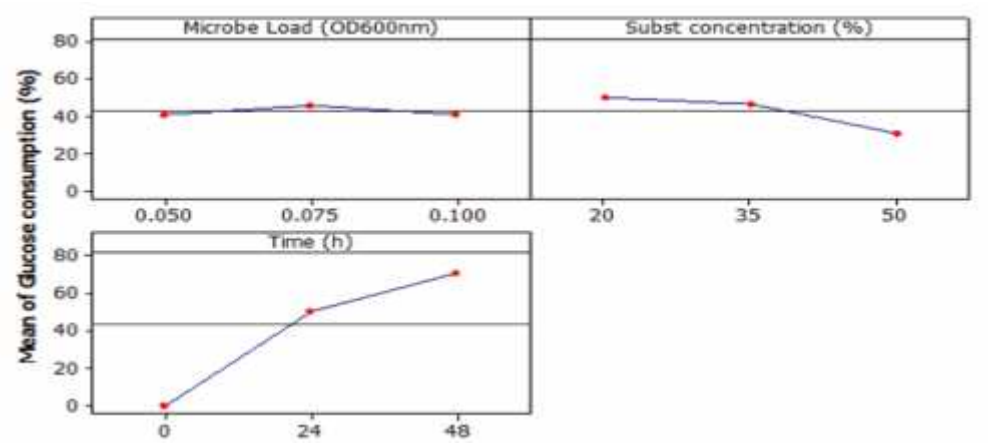

Figure 5.7b: Optimum effect of initial microbial load, substrate concentration and fermentation time interactions on glucose consumption (%)

Microbial growth of over 40 nm (OD) was recorded by experimental run 3, 8, 9, 13, 16 and 17 (Table 5.3). The regression coefficient of microbial load (915.64) was bigger than that of substrate concentration (0.45) and time (1.87) (Table 5.9). This showed the importance of microbial load on the overall growth of the microorganism in the fermentation process. All the independent variables were not significant for microbial growth except time. Although all the interactions were not significant, XY_2 and XZ in addition had negative impact on microbial growth (Table 5.9). This phenomenon was shown in (Figure 5.8a and 5.8b) where increasing microbial load did not result in increased growth of *C. glutamicum* even at high substrate concentration. Generally, microbial growth decreased at a very high glucose concentration above 35% and optimum growth was attained at 24 hours (Figure 5.8b)

Table 5.9: Estimated Regression Coefficients for Microbial Growth (OD 600 nm)

Term	Coef	SE Coef	T	P
Constant	-31.44	31.61	-0.995	0.343
X	915.64	843.09	1.086	0.303
Y_2	0.45	1.14	0.390	0.705
Z	1.87	0.43	4.354	0.001
X^2	-4610.91	5435.74	-0.848	0.416
Y_2^2	-0.00	0.02	-0.230	0.823
Z^2	-0.03	0.01	-4.881	0.001
XY_2	-3.00	5.31	-0.565	0.585
XZ	-5.96	3.32	-1.795	0.103
Y_2Z	0.01	0.01	2.046	0.068

S = 5.634 R-Sq = 91.2% R-Sq(adj) = 83.4%

X- Microbial Load (OD 600 nm), Y_2- Substrate Concentration (%), Z- Time (h)

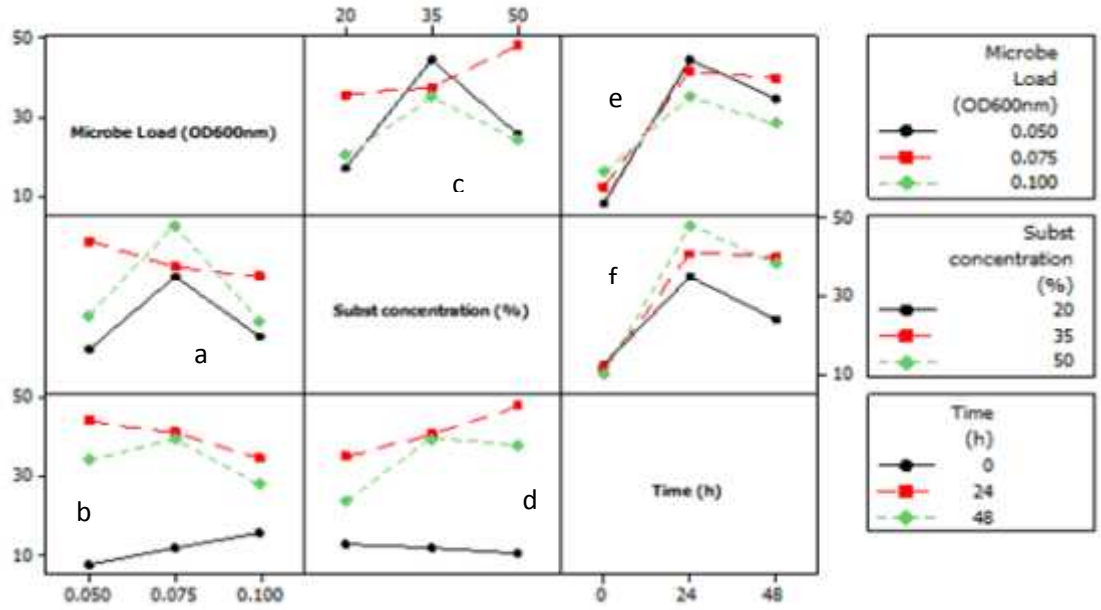

Figure 5.8a: The effect of interaction between microbial load, substrate concentration and fermentation time on microbial growth (OD 600 nm). Data plotted are the means of microbial growth (OD) resulting from; (a and c) Substrate concentration and microbial load; (b and e) Microbial load and fermentation time; (d and f) Substrate concentration and fermentation time.

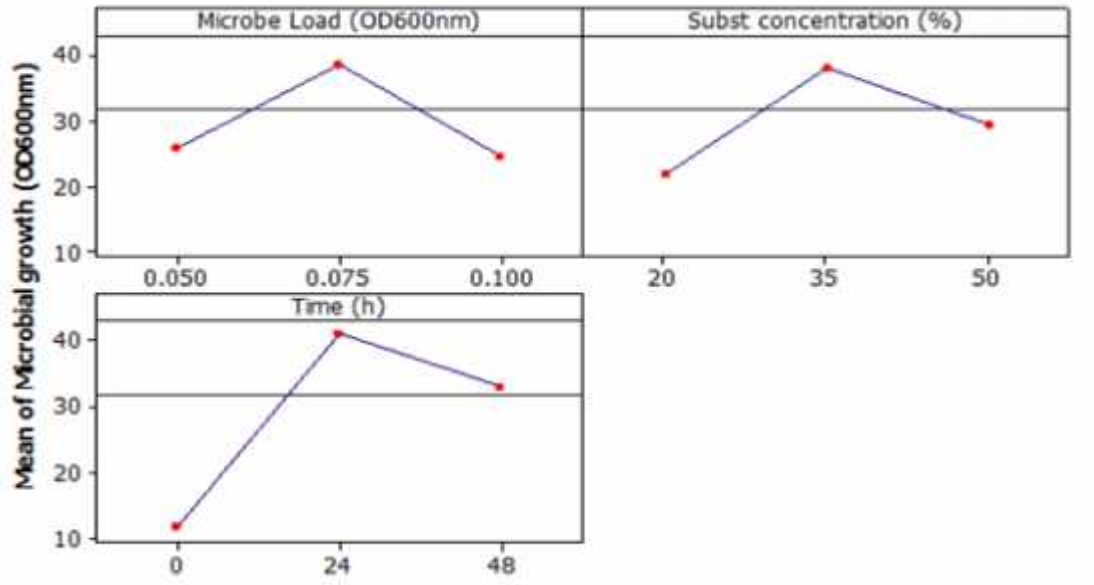

Figure 5.8b: Optimum effect of initial microbial load, substrate concentration and fermentation time interactions on microbial growth (OD 600 nm)

The highest lysine of 2.7559 g/L was produced by the experimental run 11, whiles run 3 had the lowest of 0.3441 g/L (Table 5.3). Lysine was not produced by experimental run 4, 5, 7, 10 and 18 because time was a very important factor in fermentation process for microorganisms to utilise the substrate for cell growth and lysine synthesis (Table 5.3 and 5.10). Regression analysis showed that time was significant for lysine synthesis. Substrate concentration and microbial load were not significant but substrate concentration had negative influence on lysine synthesis. All the variable interactions were significant; nonetheless XZ and Y_2Z have negative impact on lysine production (Table 5.10). This phenomenon is clearly demonstrated in (Figure 5.9a and 5.9b) where increasing substrate concentration did not increase lysine production, likewise increasing microbial load did not result in increased production of lysine during 48 hours of fermentation. Lysine production was optimum at low initial microbial load of 0.05 nm (OD) and cassava peel hydrolysate concentration not more than 35% at 48 hours at 48 hours although microbial growth was highest at 24 hours (Figure 5.8b and 5.9b).

Table 5.10: Estimated Regression Coefficients for Lysine Production (g/L)

Term	Coef	SE Coef	T	P
Constant	1.550	1.178	1.315	0.218
X	-57.369	31.427	-1.825	0.098
Y_2	0.030	0.043	0.695	0.503
Z	0.087	0.016	5.423	0.000
X^2	291.478	202.622	1.439	0.181
Y_2^2	-0.001	0.001	-1.626	0.135
Z^2	-0.000	0.000	-0.158	0.878
XY_2	0.464	0.198	2.342	0.041
XZ	-0.517	0.124	-4.175	0.002
Y_2Z	-0.000	0.000	-2.342	0.041

S = 0.2100 R-Sq = 94.4% R-Sq(adj) = 89.3%

X- Microbial Load (OD 600 nm), Y_2- Substrate Concentration (%), Z- Time (h)

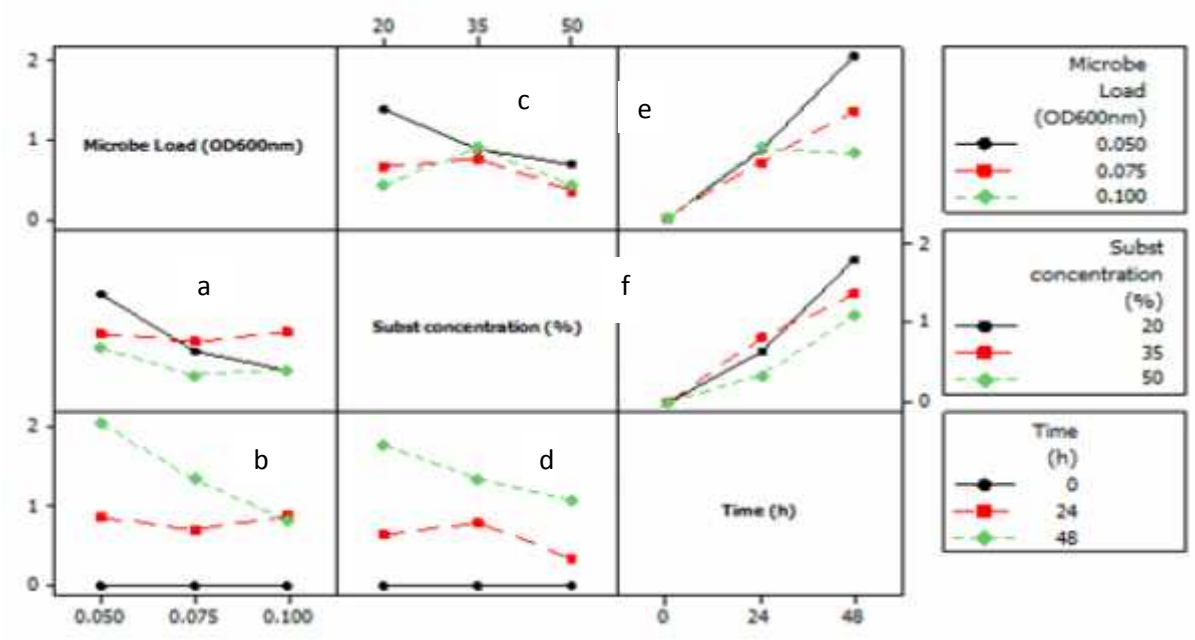

Figure 5.9a: The effect of interaction between microbial load, substrate concentration and fermentation time on lysine production (g/L). Data plotted are the means of lysine production (g/L) resulting from; (a and c) Substrate concentration and microbial load; (b and e) Microbial load and fermentation time; (d and f) Substrate concentration and fermentation time.

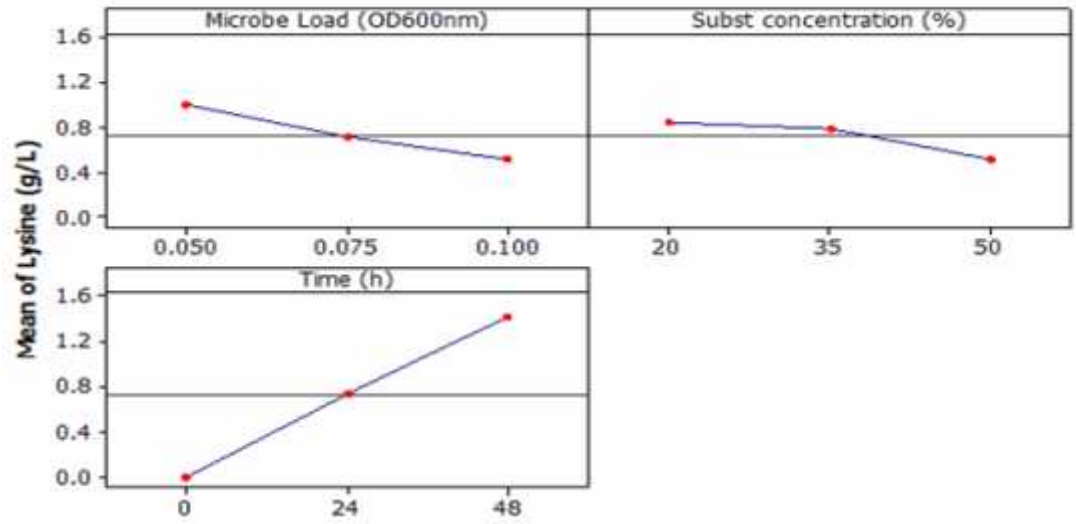

Figure 5.9b: Optimum effect of initial microbial load, substrate concentration and fermentation time interactions on lysine production (g/L)

In batch fermentation using laboratory fermenter the growth rates of the bacteria vary from 0.38 h^{-1} to 0.41 h^{-1} with an average of 0.40 ± 0.02 h^{-1}. Production of lysine also varies from 5.96 mmol/L to 7.60 mmol/L with average production of 6.73 ± 0.7 mmol/L. The average lysine production yield was 76 ± 8 mmol/mol glucose (Table 5.11). This result was comparable to work done by Wang et al., (2014) using 1% glucose as a source of carbon to produce lysine.

Table 5.11: Growth and production characteristics of *Corynebacterium glutamicum* in 20% (v/v) cassava peel hydrolysate (1.6% glucose) in Minimal Medium. Specific growth rate μ [h^{-1}], $Y_{p/s:}$ lysine production yield (mmol lysine per mol glucose)

Fermenter	Initial pH	Maximum OD_{600nm}	Initial glucose (g/L)	μ [h^{-1}]	Lysine titer (mmol/L)	$Y_{p/s}$ (mmol/mol
A	6.85	19.2	16.2	0.41	6.54	73
B	6.84	21.8	16.1	0.38	7.60	85
C	7.04	22.4	15.8	0.41	6.82	78
D	6.88	22.6	16.3	0.38	5.96	66
Average	6.90	21.5	16.1	0.40	6.73	76
STDEV	0.09	1.6	0.2	0.02	0.68	8

5.10 Discussions

The use of agricultural waste as biomass for bioprocess is becoming very prominent because it can contribute significantly towards cost reduction in industrial manufacturing. Bawjiase in Ghana is a major cassava producing and processing community which is estimated to process 3 million metric tonnes of cassava per annum. Various researchers have demonstrated that cassava peel (without trimmings) is composed of 45-56.5% starch and 5.4-8.4% cellulose (Gerberet al., 2013; Moshiet al., 2014), making it a good source of biomass for bioprocessing at Bawjiase however, the traditional method of preparing cassava for processing which is peeling involves the trimming of part of the starch storage tissue, thus potentially making the waste generated in this location more valuable as a rich fermentable carbohydrate enclave for bioprocessing industries. The goal of this work was to determine the suitability of cassava peel hydrolysate prepared from waste collected from the Bawjiase processing community to as a source of biomass for lysine fermentation.

The cassava peel hydrolysate from Bawjiase is suitable for the growth of *C. glutamicum*, because it contains adequate glucose to provide the carbon requirement of the microorgainism for lysine synthesise. The nitrogen content of cassava peel hydrolysate is low hence additional nutrient supplement is needed for the growth of *C. glutamicum* to synthesis lysine. In lysine fermentation, nutrient supplementation is very important and mostly achieved by adding minimal medium (Lieblet al., 1998). Cassava peel hydrolysate concentration of about 20% (v/v) containing 1.6 % glucose content is adequate for *C. glutamicum* growth and lysine production. Lysine production is optimum at low initial microbial load of 0.05 nm (OD) and cassava peel hydrolysate concentration not more than 35% at 48 hours. The main effect, of the interactions of the variable shows that, lysine

production increases with increasing glucose concentration, up to a threshold and at high microbial growth over 48 hours of fermentation.

Availability of cheap and sustainable feedstock is key to lysine production. Cassava peel is abundant, sustainable and available at Bawjiase to feed lysine bioprocessing plant. The technology for lysine production is available but the optimised use of local raw material which this project addressed is paramount for lucrative business venture. Therefore, Ghana Government can support private sector to venture into lysine production through the one district one factory policy being rolled up to provide jobs and also to reduce lysine concentrate importation for poultry feed formulation.

Understanding the composition of cassava peel played an important role in its usage as substrate for fermentation. Cassava peel composition of crude protein 4.1–6.5%; hemicellulose and cellulose 34.4%; starch 56.5%; carbohydrate 62.0% and lignin 8.4% has been cited in literature (Adegbola & Asaolu, 1986; Gerber et al., 2013; Kongkiattikajorn & Sornvoraweat, 2011). Hydrolysis of the cassava peel with enzymes converted the complex polysaccharide to monosaccharide especially glucose, making it possible for *C. glutamicum* to utilise as a source of carbon for lysine fermentation. The culture medium must provide the necessary environment suitable for microbial growth and production (Anastassiadis, 2007).

It is observed that as fermentation time increases the concentration of the residual glucose decreases (Figure 5.7a). Similar trend was reported by Amin & Al-Talhi (2007); Razak & Viswanath (2015) in production of L-Glutamic acid using immobilized cells of *C. glutamicum*. Lysine concentration was relatively lower for the first 24 h when the microbial cells multiplied with rapid growth of biomass (Figure 5.6b, 5.8b and 5.9b). As the fermentation proceeds the glucose consumption and L-lysine production increased at 48 hours with high glucose utilisation and maximum L-lysine concentration.

In fermentation microbial cell concentration and glucose concentration play important roles in the performance of a bio-process for lysine production. The efficient utilisation of the available glucose by microorganisms depends on suitable process conditions. The rate of the microbial growth in fermentation is largely influenced by the amount of glucose present in the fermentum. The ability of *C. glutamicum* to utilise a variety of carbon sources, such as glucose, fructose, sucrose, and maltose for lysine production makes it more relevant in amino acid fermentation. The effect of glucose concentration on the L-lysine yield was expressed on the basis of lysine produced per unit substrate utilised (Table 5.11). From running different batches, it was clearly seen that lysine production is cell-growth associated and the growth of the cells is influenced by glucose concentration (Table 5.8, Figures 5.7b and 5.8b) and Lysine production was enhanced because the microbes use their energy to metabolite lysine than cell growth. Microbial growth decreased at a very high glucose concentration above 35% resulting in low lysine production (Figure 5.8b and 5.9b). One of the reasons might be due to oxygen limitation resulting from high oxygen demand during rapid growth at high substrate concentrations. This finding is supported by work done by Hirose & Shibai (1985) on lysine production in which it was reported that higher concentration of glucose inhibited bacterial growth along with low yield of lysine. Costa-Ferreira & Duarte (1991) also reported that 10% sugar concentration is optimum for cell growth and lysine production. They attributed this to bacteria sensitivity to osmotic pressure of the medium.

Generally, it is important to optimise inoculum density because too low a density may give insufficient biomass and too high density may produce too much biomass and deplete the substrate of nutrients necessary for lysine fermentation. Initial microbial load higher than 0.075 nm (OD) did not have any significant effect on glucose utilisation, microbial growth and lysine production (Figures 5.7b, 5,8b and 5.9b). Inoculum size of 2-14% was studied on

shake flask experiment in lysine fermentation by Shah et al. (2002) and the optimum result was 10% inoculum size. They further stated that it might not be fair to say that 10% inoculum is required in all cases of fermentation since it depends on cell mass and the composition of the seed medium to be transferred.

Positive coefficient for substrate concentration and time indicates a linear effect of these variables on lysine production while negative coefficients reveal the opposite influence (Table 5.10). Microbial load and substrate concentration were not significant but the interaction between microbial load and time; substrate concentration and time were significant and have negative impact on lysine production (Table 5.10). When an independent variable is insignificant is an indication that the effects of that factor are not dependent on the setting of the other factors. Although, microbial load and time; substrate concentration and time were significant for the fermentation process, which showed that the effectiveness of one variable depended on the other, their interactions have negative effect on lysine production (Figure 5.9a and 5.9b). The negative effect means that the shift of the variable from the low level to the high level produced a decrease effect on lysine production (Amdoun et al., 2010; Ferreira et al., 2009).

The model fit (R-square) was determined to be 87.7%, 99.2%, 96.6% 91.2% and 94.4% respectively except that of lysine fermentation for 24 hours which was 71% (Table 5.5, 5.6, 5.7, 5.8, 5.9 and 5.10). Donkoh et al. (2012) reported that an R-squared of (82%) is acceptable. However the high (R-square) of over 90% suggests that the model is adequate in predicting the relationship between the variables and the responses. Therefore, the model is appropriate to predict glucose uitisation of *C glutamicum*, growth of *C glutamicum* and lysine production.

5.12 Conclusion

The conclusion drew from the present studies showed that the production of lysine from *C. glutamicum* can be significantly improved by optimising the fermentation process. The role of various ingredients in the metabolic pathway of the organism for lysine production is very vital. Carbon and nitrogen sources from cassava peel hydrolysate have also been found to have influential role in the amino acid production.

A Central Composite Design (CCD) was applied to optimise the amino acid fermentation process in order to produce lysine. The effects of substrate concentration, microbial load and time of fermentation on growth of *C. glutamicum*, glucose utilisation and lysine production were investigated. The results were subjected to analysis of variance (ANOVA) to produce polynomial regression model. Mean interaction plot and their effect on microbial growth, glucose consumption and lysine production were drawn to determine the optimal conditions for amino acid fermentation process for lysine production. Glucose utilisation reduced at higher hydrolysate concentration thereby affecting microbial growth. Lysine production was optimum at low initial microbial load of 0.05 nm (OD) and cassava peel hydrolysate concentration not more than 35% at 48 hours.

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CHAPTER SIX

6.0 Conclusion and discussion

To achieve the overall goal of the present PhD project various strategies have been applied in optimisation of the parameters for bio-processing of cassava peel to increase fermentable sugars and lysine production. The experimental part of the project was divided into three separate parts all of which are described below.

6.1 Characterisation of cassava peel for carbohydrate types

Cassava peel is a biomass generated as a result of processing cassava tuber by peeling operations. It is a natural resource that shows heterogeneity in structure and chemical composition. Physical and chemical composition analysis was done using standard methods.

The results from the analysis showed that nearly 83 % dry matter (DM) composition of the cassava peel was glucose whiles arabinose and xylose have made up only small amount of 2.35 and 2.31 % respectively. The cellulose and hemicellulose were 6.0 % DM and 2.23 % DM respectively and the residual starch content was 47.16 %. The protein was 2.40 % and the cyanide level was 9.3 mg/kg. The lignin and the ash contents were 1.92% and 6.30% respectively.

The high level of residual starch and low amount of lignin make the cassava peel more susceptible to enzymatic hydrolysis without laborious pretreatment regimes. Importantly, this study provides a useful base line data for agro-economic evaluation of cassava peel as a feedstock for an integrated biorefinery, because the valorisation of cassava peel is still overlooked and not fully exploited. Additionally, a deep understanding of the biomass chemical and physical properties, are required for the design and safe operation of processing facilities.

6.2 Optimisation of enzymatic hydrolysis of cassava peels to produce fermentable sugars using response surface methodology

Cassava peel which is considered as waste, because of its limited use is essentially a valuable material for producing reducing sugars, because the residual starch content is quite high. A Central Composite Design (CCD) was applied to optimise the enzymatic hydrolysis of cassava peel in order to produce glucose. Two effective approaches were used in the study. The first one was to optimise the enzymatic hydrolysis process using cellulase, β-glucosidase, amyloglucosidase and α-amylase. The second approach was to optimise enzymatic hydrolysis using the mixture of these enzymes. The effects of enzyme loading, hydrolysis time, substrate concentration, pH and temperature on glucose recovery were investigated. The results were subjected to analysis of variance (ANOVA) to produce polynomial regression model. Mean interaction plot and their effect on glucose recovery were drawn to determine the optimal conditions for enzymatic process. Targeted hydrolysis of specific carbohydrate types of cassava peels with single enzymes showed optimised levels of glucose recovery of over 80% for starch hydrolysing enzymes and about 5% for cellulose hydrolysing enzymes at 0.06 g/ml substrate water ratio at 24 hours of hydrolysis. Single step hydrolysis of cassava peel with mixed enzymes of starch and cellulose hydrolysis enzymes at optimised conditions of celulase (30 FPU/g), β- glucosidase (1.25 U/g), amyloglucosidase (30 U/g), α-amylase (30 U/g), pH 4 and 50 ^{o}C at 24 hours produced recovered glucose of about 100%.

6.3 Optimisation of conditions for lysine production using mutant *Corynebacterium glutamicum* (AHP3) strain and hydrolysed cassava peel as carbon source

The present study has concluded that production of lysine from *Corynebacterium glutamicum* can be substantially enhanced by optimising the fermentation process. Different ingredients have an important role in the metabolic pathway of the organism for lysine production.

Carbon and nitrogen sources from cassava peel hydrolysate have also been found to have influential role in the amino acid production.

A Central Composite Design (CCD) was applied to optimise the amino acid fermentation process in order to produce lysine. The effects of substrate concentration, microbial load and time of fermentation on growth of *C glutamicum*, glucose utilisation and lysine production were investigated. The results were subjected to analysis of variance (ANOVA) to produce polynomial regression model. Mean interaction plot and their effect on microbial growth, glucose consumption and lysine production were drawn to determine the optimal conditions for amino acid fermentation process for lysine production. Glucose utilisation reduced at higher hydrolysate concentration thereby affecting microbial growth. Lysine production was optimum at low initial microbial load of 0.05 nm (OD) and cassava peel hydrolysate concentration not more than 35% (v/v) at 48 hours.

6.4 Significance of project

Processing of agricultural waste into value-added products is an important facet of any country's changing agro economy. Large volumes of agricultural residues are generated in Ghana annually which can be converted into food, animal feed, energy or industrial raw materials. The over 2 billion MT of maize stalks, 0.5 billion of maize cob and 3.8 million MT of cassava peel produced per annum in Ghana are classical examples of conventional feed stocks. Other opportunities for value-added processing are in the large amounts of plant residues associated with rice, sorghum and millet that we produce. Increasing value-added processing of agricultural wastes in Ghana will improve the demand for our agricultural waste, increase farm profitability, provide jobs, and help minimise rural urban drift.

The findings from the research showed that cassava peel can be used as a substrate to provide cheap source of lysine for animal feed production. The strategy used in this project can be

adopted and used for other agricultural wastes generated in Ghana. This can help reduce cost of feed for poultry and pig husbandry thereby promoting local meat production at affordable cost. Protein energy malnutrition in children in Ghana can also be reduced through adequate consumption of meat protein.

6.5 Recommendations

Over the years, research focus has been on cassava peels because of its availability as process residue at small and medium scale cassava processing units as well as its potential as a renewable feedstock for biorefinery. One of the most important considerations for cassava peel utilisation is to look for economic and viable ways of exploiting this inevitable process residue. Residues may have several substances of high value which can be transformed into commercial products or raw materials for secondary processes by means of appropriate technology.

The findings from composition analysis of cassava peel showed high level of residual starch (46%) and low amount of lignin (1.92%) making cassava peel very susceptible to enzymatic hydrolysis without laborious pretreatment regimes. Importantly, this research provides a useful base line data for agro-economic evaluation of cassava peel as a feedstock for an integrated biorefinery. Based on this finding, further work can be done employing the same analytical procedures to generate national data on composition of agro-processing waste in Ghana. This will provide basic data on agro-processing wastes as feedstock, their availability, quality and location for establishment of a biorefinery.

However, availability of cassava peels as feedstock for biorefinery in Ghana is assured because of the increasing demand for High Quality Cassava Flour (HQCF) in the alcoholic beverage industries. The hand peeling of cassava generates huge volumes of cassava peels at

these facilities making this feedstock readily available for valorisations. The high amount of hexose sugar, glucose (83%) found in cassava peel makes it a good substrate for fermentation after hydrolysis with mixed enzymes of cellulase, glucosidase, amyloglucosidase and α-amylase. The cassava peel hydrolysate can be used to produce lactic acid, lysine and ethanol through various fermentation processes.

In order to reduce the high importation of ethanol and promote its local manufacturing for use in Senior High Schools in Ghana, a small scale ethanol manufacturing plant can be established at Bawjiase in the Central Region. Additionally, low grade lysine can also be produced from cassava peel hydrolysate after microbial fermentation with *C. glutamicum* without complex purification process. The recovered lysine can be used to prepare animal feed for the poultry and pig husbandry. Similar processing plants can be replicated in the country at cassava processing enclaves as one of the Government's industrialisation programmes of providing manufacturing industries in each district in Ghana. Before commencing a full scale production, hydrolysis and fermentation processes optimisation would have to be carried out in a pilot scale processing unit to establish optimised conditions for upscaling production.