Characterization of Glucoamylase Produced by *Aspergillus niger* and *Rhizopus sp.*

by

Andrew Nyamful, *Msc. Chemistry*

KNUST

A thesis submitted to the

School of Graduate Studies, Kwame Nkrumah University of Science and Technology

in partial fulfillment of the requirement for the degree of

DOCTOR OF PHILOSOPHY

Department of Chemical Engineering, College of Engineering

August 2013

© 2013, Department of Chemical Engineering

Certification

I hereby declare that this submission is my own work towards the PhD and that, to the best of my knowledge, it contains no material previously published by another person nor material which has been accepted for the award of any other degree of the University, except where due acknowledgement has been made in the text.

KNUST

Andrew Nyamful, **PG2580008** ……. ……..……

Signature Date

Certified by:

Dr. Emmanuel Moses ……..... ….....

Supervisor Signature Date

Certified by:

Dr. Martin Y. Woode ……..... …….....

Supervisor Signature Date

Certified by:

Dr. E. G. Ankudey ...…… …….....

Supervisor Signature Date

Certified by:

Dr. Martin Y. Woode …… ……

Head of Department Signature Date

Dedication

I dedicate this thesis to my father, mother and all my brothers and sisters

KNUST

Acknowledgement

I wish to express my sincere gratitude to the Almighty God who has brought me this far. Words cannot express my gratitude to my research supervisor and mentor, Dr. Emmanuel Moses, of CSIR- Crops Research Instutite, Kumasi; who through his strong dedication, challenging ideas and above all creative and thoughtful insights, has changed my life for good. I learnt a great deal of lessons from him, especially on how to balance life and work.

Apart from providing a furnished laboratory with no bench fee, he further provided some other resources with his own funds as well as keenly followed the progress of this work from beginning to the very end.

I thank Dr. M. Y. Woode, co-supervisor, who originated the idea of this work and through him I joined Chemical Engineering Department as a student. He encouraged me by way of advice and contributed with his own funds and helped by reading and commenting on the thesis.

Many thanks to Dr. E.G. Ankudey, co-supervisor, who generously purchased some equipment from abroad for this work, offered some suggestions and helped by reading and commenting on the thesis.

I thank all workers at CSIR-Crops Research Instutite, Kumasi, for their diverse support they provided for the success of this work, especially, Mrs. Linda Appianimaa Oppong, Mrs. Belinda Akomeah, Miss Lilly N.A. Allotey, Mrs. Ruth Prempeh, Mr. Charles Afiriyie-Debrah, Mrs. Priscilla Francisco-Ribeirrio in the Molecular Laboratory; Mr. Allen Oppong and Mr. David Appiah- Kubi in the Virology Laboratory; Mrs. Zipporah Appiah-Kubi, Mrs. Rose Baafi, Mrs. Rose Osei-Kofi, Mrs

KNUST

Esther Oheneba-Ntim, Miss Abigail Addo and Mr Donkor in the Pathology Laboratory. I also thank Eric Okyere and Anthoniette Nissi, National Service Personals at Dr. Emmanuel Moses' laboratory for their kind support.

I sincerely thank Dr. Harry D. Oduro, MIT (USA), Professor Kwabena Mante Bosompem (Legon, Accra), Mr. Uris S. Mckakpo (Legon, Accra) and Jonas Danquah (Legon, Accra) who assisted me greatly in the determination of molecular weights.

I greatly thank Dr. John Owusu, Mr. Owusu Tawiah, Mr. Theophilus Akurugu, Mr. Joseph Azumah,Mr. Isaac Omari Agyapong, Mr. Kwame Enos Adipa and Mr. Nathaniel O. Boadi who supported me in terms of chemicals, equipment and journals for the work.

I would like to thank all members of Chemical Engineering Department, KNUST, Kumasi, Ghana for providing stimulating environment for my studies. I thank all friends who encouraged me especially Mr. Mohammed Nafiu Zainudeen, Mr Samuel Agyekum, Joseph Adomako and Dr. Marian Nkansah.

Finally my love and appreciation to my family, especially my sister Maame Essumanba, Kofi Nassah, Lady Nassah, Regis Nassah, Patrick Osae Nyamful, Mrs. Evelyn Asare Nyamful and my children Cyril-Devon Nyamful and Cecil-Maris Nyamful for their sacrifice and support.

Lastly I thank my Landlady and her husband, Mr and Mrs Wiafe, who supported me greatly in terms of prayers and all friends in Ghana Education Service, especially, Mrs Victoria Annan (former headmistress of Afua Kobi Girls' Secondary School, Kumasi) for their encouragement.

KNUST

Abstract

Amylase enzymes are important enzymes employed in starch processing industries for hydrolysis of polysaccharides into simple sugars. Microorganisms including a number of fungal species have been used to produce amylases more economically than from other sources. Glucoamylase ($C_{3009}H_{4570}N_{782}O_{1012}S_{13}$) is an exoenzyme that removes glucose units consecutively from the nonreducing ends of starch and oligosaccharides. The enzyme also cleaves α-1, 6- and α-1, 3-bonds but at a slower action. Glucoamylase is used in processed-food industry, fermentation technology, textile and paper industries. In this study, four native fungal isolates, *Aspergillus niger*, *Aspergillus flavus*, *Rhizopus* species and *Fusarium oxysporum* and the potential of five solid substrates, wheat bran, rice bran, groundnut pod, maize bran and cocoa pod for glucoamylase production were investigated using Solid-State Fermentation process. Isolates of *Aspergillus niger* and *Rhizopus* species on wheat bran as substrate at spore concentrations of 1×10^7 per ml produced the highest enzyme activities under optimum growth conditions. Glucoamylase production was found to be affected by temperature, pH, incubation period, nature of substrate and the kind of microorganism used. Glucoamylase production by *Aspergillus niger* was found to be affected by nitrogen. Glucoamylase produced by *Aspergillus niger* yielded maximum enzyme activity of 6.66 U/ml in 18 hours of incubation period at a temperature of 40ºC, nitrogen concentration of 0.2 g/l and at pH 5.0. Similarly, glucoamylase produced by *Rhizopus* species gave maximum enzyme activity of 4.44 U/ml in 18 hours of incubation period at a temperature of 40ºC and pH 4.5. Molecular weights of proteins in culture filtrates were determined by SDS-PAGE. Proteins with molecular weights 61.48, 29.68, 21.06 and 12.33 KDa were identified from culture filtrates of *Aspergillus niger* and proteins with molecular weights 96.40, 65.56, 51.80, 29.05 and

19.75 KDa were found from culture filtrates of *Rhizopus* species. Kinetic studies using Hanes-Woolf's plot and starch as substrate gave Kmax = 0.0009548 g/l and Vmax = 2.387 g/l.min for enzyme produced by *Aspergillus niger* and Kmax = 0.0007443 g/l and Vmax = 2.481 g/l.min for enzyme produced by *Rhizopus* species.

KNUST

Table of Contents

KNUST

KNUST

KNUST

List of Tables

Table **Page**

KNUST

Table of Figures

Figure **Page**

KNUST

Chapter 1

1.0 Introduction

The amylase family of enzymes has been well characterized through the study of various microorganisms (Ellaiah *et al*., 2002). Two major groups, endo- and exo-amylases, have been identified and are among the enzymes most studied (Horváthová *et al*., 2001; Aiyer, 2005; Anto *et al*., 2006). These enzymes represent about 25-33% of the world enzyme market, second after proteases (Cereia *et al*., 2006). Amylase enzymes are important enzymes employed in starch processing industries for hydrolysis of polysaccharides into simple sugars (Pandey *et al*., 1996; Suganthi *et al*., 2011). Microorganisms including a number of fungal species have been used to produce amylases more economically than from other sources.

Glucoamylase is an economically important enzyme because of its capacity to turn starch and related polymers into β-D-glucose as the sole end product (Banks *et al*., 1976). The principal industrial use of glucoamylase is therefore, the production of glucose, which in turn serves as a feedstock for biological fermentations in the production of ethanol or high fructose syrups (Saha and Zeikus, 1989; Pavezzi *et al*., 2008; James and Lee, 1997). Glucoamylase is also used to improve barley mash for beer production (Swift *et al*., 2000). It is a key enzyme in the production of sake and soy sauce (James and Lee, 1997). Glucoamylase also has applications for confectionery, baking and in pharmaceutical industries (Rose, 1980; Pandey *et al*., 2000). The enzyme has also found wide applications in the textile and paper industries (Pandey *et al*., 1996).

In industrialized countries, glucoamylase is produced in economic quantities for industries by using fungal organisms grown on agricultural wastes such as wheat or

rice bran. In a number of developing countries including Ghana, using microorganisms such as fungi to produce glucoamylase for industrial activities have received very little attention. The outcome is the importation of glucoamylase for commercial production of products such as alcohol. A number of small scale alcoholic beverage industries in Ghana for example, have stopped production because of the high cost of imported glucoamylase. A number of surviving alcoholic beverage producing industries in Ghana for example, depend on starch-rich staple foods such as maize, rice, millet and sorghum as sources of amylases for starch hydrolysis (Ocloo and Ayernor, 2010). The use of staple food for enzyme production on large scale has the potential of increasing prices of staple foods and can potentially affect food security. It is therefore, very necessary, that efforts are made to produce amylases using microorganisms grown on cheaper substrates. A number of industries that depend on glucose as raw materials in Ghana will receive a great boost when amylases, particularly, glucoamylase are produced in commercial quantities locally using microorganisms.

This work is an attempt to produce glucoamylase using microbial organisms isolated locally. *Aspergillus niger*, *A. flavus*, *Fusarium* and *Rhizopus* species have been used to produce glucoamylase for industrial activities (Hata *et al*., 1997; Fujio and Morita, 1996; Fogarty and Kelly, 1980; Selvakumar *et al*., 1994; Pandey *et al*., 1993; Pandey and Ashok, 1991; Bhatti *et al.*, 2007). The effectiveness of using *A. niger* and *Rhizopus* species to produce glucoamylase using agricultural wastes or residues as substrates have been investigated in this work. Efforts were also made in investigating the effects of factors such as temperature, pH, nitrogen concentration and incubation period on the production of glucoamylase.

Chapter 2

2.1 Literature Review

2.1.1 Enzymes

Enzymes are proteins that catalyze or increase the rates of chemical reactions (Smith, 1997; Grisham *et al*., 1999). In enzymatic reactions, the molecules at the beginning of the process, called substrates, are converted into different molecules, called products. Like all catalysts, enzymes work by:

(1) lowering the activation energy (E_a) for a reaction. Thus dramatically increasing the rate of the reaction;

(2) lowering the energy of the transition state, but without distorting the substrate, by creating an environment with the opposite charge distribution to that of the transition state;

(3) providing an alternative pathway;

(4) reducing the reaction entropy change by bringing substrates together in the correct orientation to react. (See Figure 2.1).

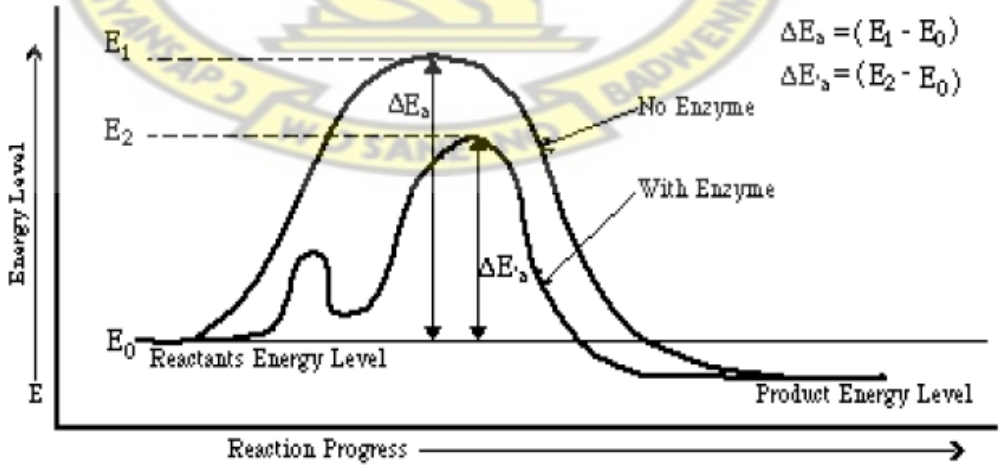


Figure 2.1 Energy Profile for a Catalysed and an Uncatalysed Reaction

An increase in temperature generally makes an enzyme function and develop end products faster. The rate constant, K, for an enzymatic reaction is given by theArrhenius equation as:

$$K = Ae^{-\frac{E_a}{RT}}$$

Where:

K = Enzyme reaction rate constant

R = Gas constant

A = Pre-exponential factor

E_a = Activation energy

T = Temperature in Kelvin

The rates of both the forward and backward reactions increase with increasing temperature (Chang and Raymond, 2005). However, at very high temperatures, the enzyme may be denatured and becomes inactivated. The enzyme unfolds and the three dimensional structure of the protein is disrupted (Fersht and Alan, 1985). Most enzyme reaction rates are millions of times faster than those of comparable un-catalyzed reactions. As with all catalysts, enzymes are not consumed by the reactions they catalyze, nor do they alter the equilibrium of these reactions. However, enzymes do differ from most other catalysts in that they are highly specific for their substrates (Bairoch, 2000).

KNUST

2.1.2 Nature of Enzymes

Like all proteins, enzymes are long, linear chains of amino acids that fold to produce a three-dimensional product. The building blocks of proteins are amino acids, which are small organic molecules that consist of an alpha (central) carbon atom linked to an amino group, a carboxyl group, a hydrogen atom, and a variable component called a side chain. Within a protein, multiple amino acids are linked together by peptide bonds thereby forming a long chain. See Figure 2.2.

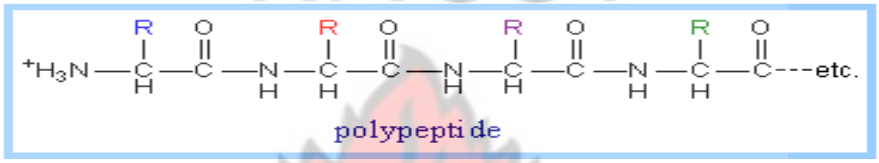


Figure 2.2 Enzyme structure

Peptide bonds are formed by a biochemical reaction that extracts a water molecule as it joins the amino group of one amino acid to the carboxyl group of a neighbouring amino acid. Each unique amino acid sequence produces a specific structure, which has unique properties. Individual protein chains may sometimes group together to form a protein complex.

Enzymes are in general globular proteins and range from 62 amino acid residues in size to over 2,500 residues in the animal fatty acid synthase (Jaeger and Eggert, 2004; Shevelev and Hubscher , 2002). The activities of enzymes are determined by their three-dimensional structure (Tymoczko *et al*., 2002). Most enzymes can be denatured—that is, unfolded and inactivated—by heating or chemical denaturants, which disrupt the three-dimensional structure of the protein. Depending on the enzyme, denaturation may be reversible or irreversible. Enzymes are usually very

specific as to which reactions they catalyze and the substrates that are involved in these reactions. Complementary shape, charge and hydrophilic/hydrophobic characteristics of enzymes and substrates are responsible for this specificity. Indeed, one of the properties of enzymes that makes them so important as diagnostic and research tools is the specificity they exhibit relative to the reactions they catalyze. A few enzymes exhibit absolute specificity; that is, they will catalyze only one particular reaction. Other enzymes will be specific for a particular type of chemical bond or functional group. In general, there are four distinct types of specificity:

KNUST

1. Absolute specificity- the enzyme will catalyze only one reaction.
2. Group specificity- the enzyme will act only on molecules that have specific functional groups, such as amino, phosphate and methyl groups.
3. Linkage specificity- the enzyme will act on a particular type of chemical bond regardless of the rest of the molecular structure.
4. Stereochemical specificity- the enzyme will act on a particular steric or optical isomer.

   (Bennett and Frieden, 1969)

Enzymes can also show impressive levels of stereospecificity, regioselectivity and chemoselectivity (Rodnina and Wintermeyer, 2001). Most enzymes are much larger than the substrates they act on, and only a small portion of the enzyme (around 2–4 amino acids) is directly involved in catalysis (Ibba and Soll, 2000). The region that contains these catalytic residues, known as the active site, binds the substrate, and then carries out the reaction. Enzymes can also contain sites that bind cofactors, which are needed for catalysis. Some enzymes also have binding sites for small molecules, which are often direct or indirect products or substrates of the reaction

catalyzed. This binding can serve to increase or decrease the enzyme's activity, providing a means for feedback regulation (Rodnina and Wintermeyer, 2001).

Some enzymes that produce secondary metabolites are described as promiscuous, as they can act on a relatively broad range of different substrates. It has been suggested that this broad substrate specificity is important for the evolution of new biosynthetic pathways (Savir and Tlusty, 2007). Specificity of enzymes may be attributed to the fact that, both the enzyme and the substrate possess specific complementary geometric shapes that fit exactly into one another (Smith, 1994). This is often referred to as "the lock and key" model. However, while this model explains enzyme specificity, it fails to explain the stabilization of the transition state that enzymes achieve. Hence Koshland (1958) suggested a modification to the lock and key model. Since enzymes are rather flexible structures, the active site is continuously reshaped by interactions with the substrate as the substrate interacts with the enzyme (Chen *et al*., 1992). As a result, the substrate does not simply bind to a rigid active site; the amino acid side-chains that make up the active site are molded into the precise positions that enable the enzyme to perform its catalytic function. In some cases, such as glycosidases, the substrate molecule also changes shape slightly as it enters the active site (Vasella *et al*., 2002). The active site continues to change until the substrate is completely bound, at which point the final shape and charge are determined (Boyer and Rodney, 2002).

Some enzymes do not need any additional components to show full activity. However, others require non-protein molecules called cofactors to be bound for activity (Bolster, 1997). Cofactors can be either inorganic (e.g., metal ions and iron-sulfur clusters) or organic compounds (e.g., flavin and heme). Organic cofactors can

be either prosthetic groups which are tightly bound to an enzyme, or coenzymes, which are released from the enzyme's active site during the reaction.

Coenzymes include NADH, NADPH and adenosine triphosphate. These molecules transfer chemical groups between enzymes (Bolster, 1997). An example of an enzyme that contains a cofactor is carbonic anhydrase (Fisher *et al*., 2005). These tightly bound molecules are usually found in the active site and are involved in catalysis. As all catalysts, enzymes do not alter the position of the chemical equilibrium of the reaction. Enzymes catalyze the forward and backward reactions equally. Usually, in the presence of an enzyme, the reaction runs in the same direction as it would without the enzyme, just more quickly. However, in the absence of the enzyme, other possible uncatalyzed reactions might lead to different products, because in those conditions this different product is formed faster.

2.1.3 Production of Enzymes by Microorganisms

A large number of microorganisms including bacteria, yeast and fungi produce different groups of enzymes. The selection of a particular strain remains a tedious task, especially when commercially competent enzyme yields are to be achieved (Pandey *et al*., 1992). *Fusarium* species and *Aspergillus flavus* have been reported as very good sources of glucoamylase especially in Solid State Fermentation processes (Bhatti *et al.*, 2007; Oznur and Kubilay, 2010). Industrially, to achieve high productivity with less production cost, genetically modified strains would hold the key to enzyme production (Pandey *et al*., 1999). Thus, the selection of a suitable strain for the required purpose depends on a number of factors, namely nature of substrate, pre-treatment of the substrate; particle size (inter-particle space and surface area) of the substrate; water content of the substrate; relative humidity; size of the

inoculum; control of temperature of fermenting matter; period of cultivation; maintenance of uniformity in the environment conditions, and the gaseous atmosphere, i.e. oxygen consumption rate and carbon dioxide evolution rate (Pandey *et al*., 1999).

2.1.4 Fermentation Substrates Commonly Used for Enzyme Production

Agro-industrial residues are generally considered the best substrates, especially, in Solid State Fermentation (SSF) processes. SSF therefore offers numerous advantages over Submerged Fermentation (SmF) system, including high volumetric productivity, relatively higher concentration of the products, less effluent generation and requirement for simple fermentation equipment (Hesseltine and Doelle, 1977). Some of the substrates that have been used include wheat bran, rice bran, maize bran, saw dust, starch, arrowroot, arum, maize, potato, pulse, rice, tamarind, palm kernel, cassava, water chestnut, copra waste, etc. (Mitra *et al*., 1994; Tengerdy, 1996). Wheat bran however holds the key, simply because, high enzyme activities are usually produced from it and has most commonly been used in various processes.

2.1.5 Factors that enhance Enzyme Production

Over the years, different types of fermenters (bioreactors) have been employed for various purposes in SSF systems. However, laboratory studies are generally carried out in Erlenmeyer flasks, beakers, petri dishes and glass tubes (as column fermenter). Large scale fermentation has been carried out in tray-, drum- or deep-trough type fermenters. The development of a simple and practical fermenter with automation is yet to be achieved for the SSF processes (Pandey *et al*, 1991). In order to obtain the best fermenter among the rest, a strain of *Aspergillus niger* was used to produce

glucoamylase in solid cultures using different types of bioreactors to evaluate their performances. These included flasks, aluminium trays, and glass columns (vertical and horizontal) (Selvakumar *et al*., 1994; Pandey *et al*., 1993; Pandey *et al*., 1991). The study included screening of a number of agro-industrial residues including wheat bran, rice bran, rice husk, copra waste, etc., individual and in various combinations (Selvakumar *et al*., 1994; Pandey *et al*., 1993; Pandey *et al*., 1991). Apart from the substrate's particle size, which showed profound impact on fungal growth and activity, substrate-moisture content also significantly, influenced the enzyme's yield (Pandey *et al*., 1994; Pandey *et al*., 1991; Pandey *et al*., 1992). Enzyme production in trays occurred optimally in 36 hours in comparison to typically required 96 hours in flasks (Pandey *et al*., 1990).

KNUST

2.2 Amylases

The amylase family of enzymes has been well characterized through the study of various microorganisms. Two major classes of starch-degrading enzymes have been identified in microorganisms, α-amylases and glucoamylases. α-amylase (endo-1, 4-α-D-glucan glucohydrolase, EC-3.2.1.1) randomly cleaves 1, 4-α-D-glucosidic linkages between adjacent glucose units in linear amylase chain. Glucoamylase (exo-1, 4-α-D-glucan glucanohydrolase, EC- 3.2.1.3) hydrolyses single glucose units from nonreducing ends of amylose and amylopectin in a stepwise manner. It is also referred to as glucogeni enzyme, starch glucogenase, amyloglucosidase or gamma amylase. Glucoamylase (GA) is an exoacting enzyme of economic interest because of its capacity to turn starch and related polymers into *β*-D-glucose as the sole end product (Jafari-Aghdam *et al*., 2005). Glucoamylases are mainly used in the production of glucose syrup, high fructose corn syrup, and alcohol (James and Lee, 1997).

Glucoamylases also have applications for dextrose production, confectionery, baking and in pharmaceuticals (Rose, 1980; Pandey *et al*., 2000; Selvakumar *et al*., 1996). This enzyme occurs almost exclusively in fungi and is produced at an industrial level by *Aspergillus* species. Of these species, *Aspergillus niger* was cited as one of the major producers (Selvakumar *et al*., 1998; Wang *et al*., 2006). Unlike α-amylase, glucoamylases are also able to hydrolyse the 1, 6-α-linkages at the branching points of amylopectin, although at a slower rate than 1, 4-linkages (Pandey *et al*., 1999). The ability of glucoamylases to hydrolyse 1, 6-glycosidic bonds is called debranching activity and is of great importance in industrial processes requiring complete degradation of starch to glucose (Fagerstrom *et al*., 1990).

Glucoamylase is mainly made up of two forms (GA1 and GA2) and these two forms can be found extracellularly (Svensson *et al*., 1982; Boel *et al.,* 1984). GA1 possesses the capacity to adsorb onto and to digest raw starch (Svensson *et al*., 1982). This led to the proposal that GAl contains two separate domains: a catalytic domain containing the active site and a granular starchbinding domain that confers on the enzyme the ability to be adsorbed onto the surface of granular starch (Takahashi *et al*., 1985). Partial amino acid sequence determination of GA1 and GA2 has shown that their polypeptide chains are very closely related and it was suggested that GA1 is extended in the COOH-terminal region with a peptide fragment missing in GA2 (Svensson *et al*., 1982). In contrast, multiple forms of glucoamylase from *Rhizopus* species differ in the NH_2-terminal region of the polypeptide chain (Takahashi *et al*., 1982). The smaller forms of the glucoamylases were thought to arise perhaps by limited proteolysis. It is now known that GA2 can be synthesized from GA1 (Boel *et al.,* 1984). However, both enzymes are active towards soluble substrates.

Amylases [α-amylase EC-3.2.1.1 and or β-amylase EC-3.2.1.2] (Selvakumar*et al*., 1998) and glucoamylases are produced by various microorganisms, including bacteria; fungi and yeast, but a single strain can produce these two enzymes. These enzymes have found applications in processed-food industry, fermentation technology, textile and paper industries (Selvakumar *et al*., 1996).

2.3 Isolation and Storage of Enzyme Producing Organisms

KNUST

Aspergillus niger is a filamentous fungus belonging to the Fungi Imperfecti. Its name isderived from the black conidia bearing structures resembling an Aspergillum, an objectused for sprinkling Holy Water (Peter, 2007). It is a cosmopolitan fungus and its spores can be found in air and soil worldwide. Being a saprophyte it is particularly capable of degrading plant cell-wall material using a large variety of enzymes. The sugars that are released are used to sustain growth but can also be metabolised under particular conditions to organic acids such as citric acid, which accumulate extracellularly. *Aspergillus niger* grows at low pH values and is capable of reusing most of these organic acids (Peter, 2007). Because of its ability to produce enzymes and organic acids in large amounts, it is an industrially important fungus. A long history of use in the food industry has provided its products with the GRAS- status, which means that these are generally regarded as safe. For the various production processes, the best producing strains were selected from wild isolates. Subsequently these strains have been further improved, mainly by different rounds of mutation and selection (Peter, 2007).

Aspergillus niger is a morphologically complex organism. It shows different morphologies at different times of its life cycle. It shows different forms between surface and submerged growths. Again, it shows different forms with respect to the

nature of the growth medium and physical environment (Papagianni, 2004).When grown in submerged culture, *Aspergillus niger* can exhibit different morphological forms, ranging from dispersed mycelial filaments to densely packed mycelial spheres better known as pellets. The different morphological states have a significant effect on the rheology of the fermentation medium. Filamentous growth results in very viscous media, leading to a significant change in the mass transfer properties of the medium, especially the gas-liquid mass transfer. Pelleted growth exhibits low viscosity and therefore achieves better agitation and oxygen transfer compared to filamentous growth. However, the central region of larger pellets is prone to autolysis as a result of nutrient and oxygen deficiency. This autolysis can have a significant effect on the cellular behaviour and product synthesis (Meijer, 2008). The morphological form is very important to achieve the maximal performance. For example, filamentous growth of *Aspergillus* species (*niger and flavus*) is preferred for pectic enzyme production, whereas the pelleted form is preferred for citric acid production (Liao, 2007; Meijer, 2008). The particular form exhibited is not only stipulated by the genetic material in the cell, but also by other factors. Factors affecting the fungal morphology include the nature of the inoculum, the type and concentration of the carbon source, the levels of nitrogen and phosphate, the levels of trace minerals, and the concentrations of dissolved oxygen and carbon dioxide. At least 13 elements are essential for growth of *Aspergillus niger*, namely oxygen, carbon, hydrogen, nitrogen, phosphorus, potassium, sulfur, magnesium, manganese, iron, zinc, copper and molybdenum (Papagianni, 2004). The first eight are macronutrients, needed in relatively large quantities (in the order of g/l). The latter five are micronutrients, which are required in small amounts (in the order of mg/l). Some other physical factors that affect the

KNUST

morphology of *Aspergillus niger* are pH, temperature, and culture mode (Meijer, 2008).

Oxygen availability is a critical parameter for *Aspergillus niger*, since it is unable to grow without it. The oxygen availability in the reactor is measured by the dissolved oxygen tension (DOT). There are critical DOT levels for growth, but also for product formation. This indicates a general role of oxygen in metabolic regulation (Meijer, 2008). The fixation of carbon dioxide also plays a major role in the production of commercially important organic acids (Meijer, 2008). It is well known that for fungal growth certain essential metals are required. When these metals are absorbed, some traces of it like iron and zinc are incorporated into the enzymes that catalyse biochemical transformations occurring at key points in the carbon cycle. The effect of trace metals on growth in *Aspergillus niger* has been extensively investigated. Zinc, manganese, copper and iron have been shown to affect fungal morphology (Meijer, 2008).

The pH is an important parameter in the cultivation of micro-organisms. It is a measure of the concentration of hydrogen ions present in the medium. *Aspergillus niger* can grow over a wide range of pH ranging from 1.5 to 8. However, its metabolite production profile changes completely at different pH. At pH less than 3, the major acid produced is citrate (Papagianni *et al.*, 1994). At pH between 3 and 6, mainly oxalate is produced (Kubicek and Rohr, 1986; Ruijter *et al*., 1999). At pH 5.5 or higher, gluconate production may occur (Meijer, 2008; Mattey, 1992). This indicates that the pH has a major impact on the flux distributions inside the cell as well as on the enzyme activities. The pH also influences the solubility of compounds, for example, CO_2 dissolves better at higher pH in the form of bicarbonate.

The genus *Rhizopus* can grow in submerged culture in different forms ranging from dispersed filaments to pellets. Some factors that affect its growth morphology include type of inoculum, medium shear, medium constituents, and pH (Byrne and Ward, 1989). Their fermentations require different growth morphologies for optimum product yield (Konig *et al.*, 1982; Liao, 2007). Growth morphology affects nutrient and oxygen uptake rate in submerged culture. Filamentous growth increases the viscosity of the medium, thereby requiring a higher power input to maintain adequate mixing and aeration. Viscosity of media containing pellets is substantially lower and less power is required for aeration and agitation. Higher specific growth rates are achieved with filamentous growth (Ward, 1988; Mishra *et al*., 2004). Pellet interiors often become anaerobic and the growth is confined to the external region of the pellet (Huang and Bungay, 2004; Meijer, 2008). Numbers and sizes of pellets produced in submerged culture can also vary. Citric acid production requires pellets of a particular size for optimum production. Problems relating to nutrient limitation and product toxicity will be proportionally more acute in larger pellets than smaller pellets. The pellets also have potential applications as immobilized cell systems which, because of their shape, may not require cross linking or entrapment. The benefits of using smaller particle sizes in immobilized cell systems has been noted previously (Meijer, 2008). It hasalso been suggested that the pellets could effectively be used as supporting matrices for immobilization of cells of other species. Controlling the physical properties of *Rhizopus* pellets should increase the flexibility and potential of fungal fermentation processes.

KNUST

2.4 Production of Glucoamylase on Different Media

Glucoamylase ($C_{3009}H_{4570}N_{782}O_{1012}S_{13}$) is a widely utilized exoenzyme that removes glucose units consecutively from the nonreducing ends of starch and oligosaccharides (Robert *et al.*, 1987). The enzyme is produced by a variety of microorganisms, with the ones extracted from *Aspergillus niger*, *Aspergillus awamori* and *Rhizopus oryzae* being considered as most important (Coutinho and Reilly, 1997).

Out of eight agro residues screened, (wheat bran, rice husk, maize bran, bagasse powder, groundnut oil cake, coconut oil cake, corn steep solid, cassava and copra waste), wheat bran gave the highest enzyme production followed by rice husk. Wheat bran as the most promising substrate for glucoamylase production has been reported by several researchers (Kaur *et al.* 2003; Anto *et al.*, 2006; Pandey *et al*., 1999). Production of very high levels of a hard starch-gel digesting amyloglucosidase under SSF using wheat bran, rice bran, other rice components and combination of these has been reported (Singh and Soni, 2001). Rice husk and cotton seed powder yielded almost the same enzyme activity. Enzyme production was lower with bagasse powder, groundnut oil cake, coconut oil cake and corn steep solid (Kaur *et al.* 2003; Anto *et al.*, 2006; Pandey *et al*., 1999). Cereal bran and flours, potato residue and other starchy waste materials have been utilized as fermentation substrate for glucoamylase production by filamentous fungi (Joshi *et al.* 1999; Biesebeke *et al.* 2005). Shivaramkrishnan *et al.*, (2007) also studied agro residues for glucoamylase production from *Aspergillus oryzae var brunneus* and obtained maximum production with wheat bran and significantly good production with oil cakes. Glucoamylase production increases with an increase in initial moisture content with optimum at

initial moisture content 100% (v/w). Generally 40-70% of initial moisture content has been reported for fungal growth and substrate utilization (Zambare, 2010).

Except urea and ammonium sulphate, all other organic and inorganic nitrogen sources used had diminishing effect on glucoamylase production (Zambare, 2010). Urea (0.25%) was found to enhance enzyme activity by 10% compared to basal media. The beneficial effects of the addition of urea nitrogen have been reported in submerged fermentations for glucoamylase production by *Aspergillus awamori* (Bertolin *et al.* 2003). According to (Cereia *et al*., 2006) glucoamylase activity is enhanced by 1 mM each of: Mg^{2+} (8.9%), Cu^{2+} (8.2%), Mn^{2+} (4.4%), Na^{+} (3.6%) and EDTA (2%). On the other hand, glucoamylase activity is inhibited by 1 mM each of: Ca^{2+} (21%) and Hg^{2+} (20%) when starch is used as a substrate. However, when the effect of metal ions on activity of glucoamylase (G2) was investigated using maltose as substrate, no increament of activity was observed with 1 mM ions and the activity levels decreased by 15-46% (Cereia *et al*., 2006). 10 mM each of Ba^{2+} and NH_4^{+} enhanced glucoamylase activity by 6%; and 10 mM of Hg^{2+} inhibited glucoamylase activity by 90%. β-Mercaptoethanol a reducing agent that disrupts disulfide bonds, inhibited glucoamylase activity completely, thus indicating the presence of such bonds in the enzyme molecule. EDTA, an ion chelater, was not effective on glucoamylase (G2) activity suggesting that this enzyme does not require metallic ions in its active site for its activity (Cereia *et al*., 2006).

In the SSF process, the solid substrate not only supplies the nutrients to the culture, but also serves as an anchorage for the microbial cells. Agro-industrial residues used as carbon sources are generally considered the best substrates for the SSF processes (Ellaiah *et al.* 2002). During fermentation, the moisture content of the medium changes as a result of evaporation and metabolic activities and thus optimum moisture

level of the substrate is the most important factor in enzyme production (Baysal *et al.* 2003). Zambare (2010) reported that the optimization of the SSF media and parameters resulted in a 24% increase in the glucoamylase activity. The optimum glucoamylase production observed was 1,986 µmoles of glucose per minute per gram of dry fermented substrate (i.e. wheat bran) at pH 6. The activity of glucoamylase increases with longer chain length of the substrate molecule (Suntornsuk and Hang, 1997). Among the several factors that are important for microbial growth and enzyme production using a particular substrate, particle size, moisture level and water activity are the most critical. Generally, smaller substrate particles provide larger surface area for microbial attack and, thus, are a desirable factor. However, too small a substrate particle may result in substrate agglutination, which may interfere with microbial respiration and aeration, and therefore result in poor growth. In contrast, larger particles provide better respiration and aeration efficiency (due to increased inter-particle space), but provide limited surface for microbial attack. This necessitates a compromised particle size for a particular process (Pandey *et al*., 1994; Couto and Sanroman, 2006). Increase in water activity of substrate increases specific growth rate and spore germination time of fungus (Oriol *et al.*, 1988). Grajek and Grevais (1987) reported that decrease of water activity by 0.01 (equivalent to 1 % of relative air humidity) causes reduction in biomass production and protein content of culture medium. Narahara (1977) found that optimum water activity for *Aspergillus* species was between 0.970 and 0.990. Fungus was unable to grow below water activity of 0.97. These data prove that fungal growth and their secondary metabolite production during SSF are strongly affected by water activity of the substrate (Grajek and Grevais, 1987).

KNUST

In SSF the fungal hyphae forms a mat on the substrate surface and penetrates by secreting secondary metabolites and enzymes (Ge´linas and Barrette, 2006; Mudgett *et al*., 1986). Inter particle concentration gradients due to nutrient consumption in combination with mass transfer limitations can have a strong effect on the rate and efficiency of the process (Bhargav *et al*., 2008; Moo-Young *et al*., 1983; Prosser *et al*., 1994). Mass transfer in SSF involves micro-scale and macro-scale phenomenon. Micro-scale mass transfer depends on the growth of microorganisms which depends on inter and intra particle O_2 and CO_2 diffusion, enzyme, nutrient absorption and metabolites formation. Macro-scale mass transfer includes airflow into and out of the SSF system, types of substrate, mixing of substrate, bioreactor design, space between particles, variation in particle size and microorganisms within the SSF system (Raghavarao *et al*., 2003; Bhargav *et al*., 2008; Ramesh *et al*., 1990).

KNUST

2.5 Comparison of Structures of Glucoamylases

Some properties of glucoamylase from *Rhizopus niveus* have been determined and compared with properties of glucoamaylase from *Aspergillus niger*. The enzymes from these organisms possess the following common properties: quantitative conversion of starch to glucose, molecular weights in the range 95,500 to 97,500, and glycoprotein structures with many oligosaccharide side chains attached to the protein moieties of the enzymes. Differences in the glucoamylases exist in electrophoretic mobility, amino acid composition, nature of carbohydrate units, and types of glycosidic linkages. Lysine, threonine, serine, glutamic acid, tyrosine, and phenylalanine differ in the two glucoamylases by 25 to 50%. Whereas the enzyme from *Rhizopus niveus* contains mannose and glucosamine, in the N-acetyl form as the carbohydrate constituents, the enzyme from *Aspergillus niger* contains mannose,

glucose, and galactose. The carbohydrate chains of the *Rhizopus niveus* enzyme are linked by O-glycosidic and N-glycosidic linkages to the protein, while those of the *Aspergillus niger* enzyme are linked by O-glycosidic linkages only. Antibodies directed against the two glucoamylases have been isolated by affinity chromatography and found to be specific for the carbohydrate units of the glucoamylases. Cross reactions did not occur between the glucoamylases and the purified antibodies (Pazur *et al*., 2010).

KNUST

2.6 Effects of Temperature, pH, Nitrogen and Incubation Period on GlucoamylaseProduction

2.6.1 Effect of Temperature

Aspergillus niger and *Rhizopus* are fungal species that produce glucoamylase (Bhargav *et al.*, 2008; Sun *et al*., 2009). These fungal species can grow over a wide range of temperatures from a range of 20 °C to 55 °C. However, the optimal temperatures for these fungal growths could be different from their required temperatures for their product formation (Bhargav *et al*., 2008). High temperature affects fungal germination, metabolites formation and sporulation. Fungal growth and secondary metabolite production in Solid State Fermentation (SSF) are also greatly influenced by temperature and heat transfer processes in the substrate bed. During SSF a large amount of heat is generated, which is proportional to the metabolic activities of the microorganism (Raghavarao *et al.*, 2003).

A report by (Madihah *et al*., 2000) revealed that the activity of glucoamylase varies with temperature. In the report, the enzyme was stable at high temperatures where 80% of its activity was retained after 1 hour incubation at 60 °C. However, the highest glucoamylase activity was observed at 40°C on starch and was still stable at

temperature up to 70°C. The activity was greatly reduced to relative activity of about 15% at temperature above 80°C. The activity was completely lost after heating above 80°C for one hour. This investigation about the activity of Glucoamylase with respect to temperature was extended to the types G1 and G2 (Boel *et al.*, 1984). The results showed that, the two forms have the same thermal properties (Didier *et al.*, 1982). (Jafari-Aghdam *et al.*, 2005) carried out an experiment on the stability of glucoamylase at a temperature of 70°C. It was disclosed that the stability was because of its native structure or form and not the deglycosylated form. A reduction of thermal stability of glucoamylase upon removal of the carbohydrate moieties was evident at temperatures between 60 - 65 - 75 and 80°C.

KNUST

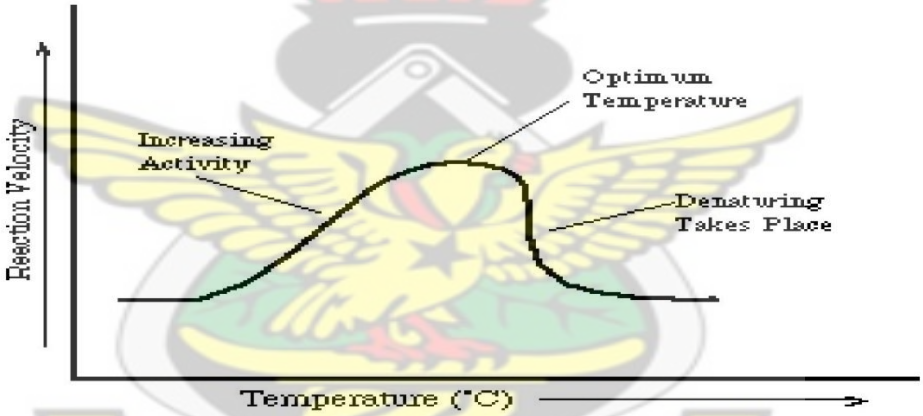


Figure 2.3 General temperature profile of enzymatic reactions

The ascending part of Figure 2.3 is known as temperature activation. The rate, v, varies according to the Arrhenius equation in this region.

$$v = K_2[E] \quad \text{..... 1.0}$$

$$\text{and } K_2 = Ae^{-E_a/RT} \text{..... 2.0}$$

$[E]$ is the active enzyme concentration and K_2 the reaction constant. A plot of In v versus $1/T$ results in a graph of slope $-E_a$/R. The descending part of Figure 2.3 is

known as temperature inactivation or thermal denaturation. The kinetics of thermal denaturation can be expressed as:

$$-\frac{d[E]}{dt} = K_d[E] \text{.....3.0}$$

$$\text{Or} \quad [E] = [E_o]e^{-k_d t} \text{.....4.0}$$

Where $[E_o]$ is the initial enzyme concentration and K_d is the denaturation constant. K_d also varies with temperature according to the Arrhenius equation:

$$K_d = A_d e^{-E_d/RT} \text{.....5.0}$$

Where E_d is the deactivation energy (kcal/g mol). Consequently,

$$v = Ae^{-E_a/RT} E_o e^{-k_d t} \text{.....6.0}$$

The activation energies of enzyme-catalyzed reactions are within 4 to 20 kcal/g mol range (mostly about 11 kcal/g mol). Variations in temperature may affect both v_m, the maximum forward velocity of the reaction and k_m , the Michaelis-Menten constant. Deactivation energies E_d vary between 40 and 130 kcal/g mol (mostly about 70 kcal/g mol). That is, enzyme denaturation by temperature is much faster than enzyme activation. A rise in temperature from 30°C to 40°C results in a 1.8-fold increase in enzyme activity, but a 41-fold increase in enzyme denaturation (Shuder and Kargi, 2002).

2.6.2 Effect of pH

Madihah *et al*., (2000) studied the effect of pH on glucoamylase activity and its stability at pH 3−11. The study disclosed that at pH between 4 and 5, glucoamylase was very stable and retained its original activity above 90 % even after 24 hours of

incubation. However, between 10 and 11, the enzyme retained less than 50 % of its relative activity. The study further revealed that at pH above 11, glucoamylase activity was greatly reduced and less than 30 % of its original activity was retained. The optimal pH for glucoamylase was observed at 4.4 on starch. A recent report by (Bhargav *et al*., 2008) also puts the best pH for glucoamylase activity at 5. Interestingly, (Suntornsuk and Hang, 1997) have also reported that, glucoamylases from *Rhizopus* species are usually stable at pH 3 – 8 and show optimal activities at pH 4 – 5. Further work on types G1 and G2 showed that the two forms have the same pH properties (Didier *et al*., 1982). A study of the (thermal) unfolding temperature, *T*m, of glycosylated and deglycosylated proteins as a function of pH was carried out in the pH range 4.5-8.8. When *T*m was plotted against pH, an apparently linear function was obtained (Sun *et al*., 2009). The pH of a medium may affect the maximum reaction rate,K_m, and the stability of the enzyme. In some cases, the substrate may contain ionic groups, and the pH of the medium would affect the affinity of the substrate to the enzyme (Shuder and Kargi, 2002).

The rate of an enzyme catalysed reaction varies with pH of the system. The rate passes through a maximum of a particular pH, known as the optimum pH (Bahl *et al*, 1997). The enzyme activity is lower at other values of pH. See Figure 2.4.

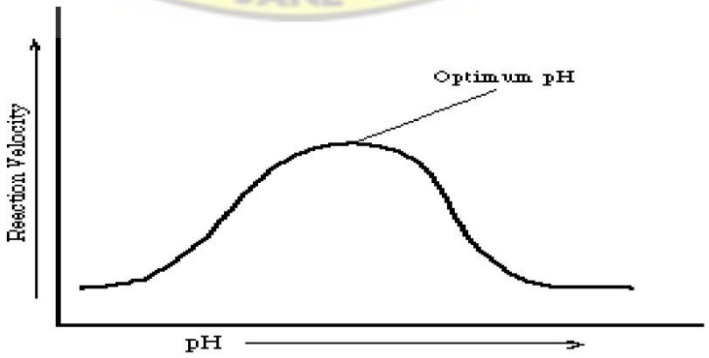


Figure 2.4 Distribution of molecular kinetic energies at different pH

Glucoamylase has ionic groups on its active sites, and these ionic groups must be in a suitable form (acid or base) to function. Variations in the pH of the medium result in changes in the ionic form of the active site. Hence correspondingly, it results in changes in the activity of glucoamylase and hence its reaction rates. Changes in pH may also alter the three-dimensional shape of glucoamylase (Sun *et al*., 2009). Hence, glucoamylase is only active over a certain pH range.

For *Aspergillus niger*, growth on glucose is normally associated with organic acid production and an associated drop in pH, called phase I. When glucose becomes limited, the organic acids are used as a secondary carbon source and the pH rises again (phase II). After reaching a maximum, the pH stabilizes (phase III). Phase I includes logarithmic growth. Phase II starts with a declining growth rate followed by stationary growth. The end of phase II and phase III are characterized by lyses and breakdown of mycelium (Monique *et al*., 2004).

2.6.3 Effect of Nitrogen

Two molecular forms of glucoamylase namely, G1 and G2 are produced by *Aspergillus niger* and *Rhizopus* species. These two forms liberate glucose from starch by hydrolyzing α-D-(1-4) and α-D-(1-6) glucosidic linkages (Boel *et al.*, 1984; Sun *et al*., 2009). Production of these two different forms arises when growth results from various organic nitrogen sources (Barton *et al*., 1972). Fogarty and Kelly (1980) and Anto *et al.*,(2006) also found that organic sources are better supplements than inorganic nitrogen sources. For the inorganic source, a report by Koukab Rana *et al.*, (2005) shows that the best nitrogen source is ammonium sulphate.

Barton *et al*., (1972) showed by different experiments that when ammonium salt is the nitrogen source, the G1 type appears to be the only glucoamylase produced. However,

if the nitrogen source is yeast extract, then the two forms i.e. G1 and G2 which are both glycoproteins containing D-glucose, D-mannose, and D-galactose are produced. It was observed that in addition to G2 formation, glucosyltransferase and α-amylase were also produced. The G1contains 18% carbohydrate and G2 contains 10% carbohydrate. Although the two forms have the same K_m and V_{max} values, the G1 hydrolyzes starch about three times faster than the G2. Barton *et al*., (1972) also observed that glucoamylase activity decreased after day 5 or 6 when ammonium salts or yeast extract was the nitrogen source.The role of the non-nitrogenous compounds in the yeast extract on the formation of α-amylase, glucosyltransferase and glucoamylase forms is still under investigation. A mixture of ammonia and complex nitrogen sources, such as yeast extract or casein hydrolysate, produced glucoamylase with higher enzyme activity than ammonia as the sole nitrogen source. Even a low concentration of casein hydrolysate ($0.05\ g\ l^{-1}$) resulted in a 35% increase in the α-amylase productivity. The higher α-amylase productivity during growth on casein hydrolysate was not caused by increased transcription of the α-amylase genes but was caused by a faster secretion of α-amylase or by a lower binding of α-amylase to the biomass (Pedersen and Nielsen, 1999).

2.6.4 Effect of Incubation Period

There are different incubation methods for substrates depending largely upon the nature and nutritional composition of the substrates. These include tray drying, tunnel drying, conventional sun drying, solar drying, microwave drying, vacuum drying or a combination of some of these methods. These are employed depending upon the desired colour and flavour of the dried product, initial moisture content of the substrate in question and the composition of the substrate with respect to its fibre,

protein and vitamin content. One of the important aspects considered in incubation of substrates is incubation time, because the lower the incubation period, the better it is in terms of time, money and energy savings. However, one must be careful in order not to lose some important properties like flavour, colour and other desired characteristics through rapid incubation methods. A study on incubation period indicates that, incubation at high temperatures or incubation at low temperatures results in a product of poorer quality (Oberoi *et al*., 2007). Different incubation methods have direct impact on the nutrient availability for the growth of fungus and subsequent enzyme production (Wadhwa *et al.,* 2005). Incubation period is largely controlled by a number of factors. These include (1) temperature, (2) substrate's particle size, (3) substrate-moisture content and (4) surface area of substrate. Studies indicate that incubation periods for experiments conducted in trays with bigger surface areas are usually shorter than those conducted in narrow tubes with smaller surface areas (Pandey *et al*., 1994; Pandey *et al*., 1991; Pandey *et al*., 1992; Pandey *et al*, 1990).

2.7 Partial Purification of Glucoamylase

Glucoamylase can partially be purified by: (1) Ammonium sulphate precipitation, (2) Acetone precipitation, (3) Ethanol precipitation and (4) Activated charcoal.

2.7.1 Ammonium Sulphate Precipitation

The culture filtrate is first subjected to ammonium sulphate fractionation (30 - 100% saturation). The precipitated protein is then dissolved in acetone buffer (0.1 M, pH 4.4) and dialysed overnight against distilled water. A volume of chilled acetone (-16°C) is added slowly to the re-dissolved protein precipitate fraction with a constant

stirring. After standing overnight at 4°C, the solution is centrifuged at 10 000 g for 30 minutes. The resultant precipitate is then dissolved in a small amount of acetone buffer (0.1M, pH 4.4) and dialysed overnight against distilled water (Madihah *et al*., 2000).

2.7.2 Acetone Precipitation

To one volume of the protein solution, four volumes of cold acetone solution are added. The mixture is well mixed and placed in a refrigerator at either -70°C for 10 minutes or 90 minutes at -20°C for the protein in the mixture to precipitate. If the protein concentration is however low, the mixture could be left to stay in the fridge overnight. The precipitated protein is then centrifuged at 4°C for 15 minutes at a maximum speed of 15000g. The supernatant is carefully discharged and the protein pellet is retained. The pellet may be dried under vacuum or in dry air. (Wessel and Flugge, 1984).

2.7.3 Ethanol Precipitation

To one volume of the protein solution, nine volumes of cold ethanol solution (100% concentration) are added and mixed thoroughly. The mixture is then kept in a refrigerator at -20°C for at least 60 minutes. For low protein concentration, it may be left to stay overnight. The cold mixture is then centrifuged at a maximum speed of 15000g at 4°C. The supernatant is carefully discharged and the protein pellet retained. The pellet is then washed with ethanol solution (90% concentration). This is done by placing the pellet in a small amount of cold ethanol solution (90% concentration) and kept in the refrigerator at -20°C. The protein pellet and ethanol mixture is vortexed and centrifuged at 15000 g maximum speed for 5 minutes at 4°C. The pellet is

separated from the supernatant by filtration. The pellet is dried under vacuum or dried in dry air to eliminate any ethanol residue. For PAGE-SDS, the pellet is re-suspended in the sample buffer.

2.7.4 Activated Charcoal

Purification of crude glucoamylase can be carried out using activated charcoal (Kareem *et al*., 2011). 3 g of the activated charcoal are added to 100 cm^3 of the crude enzyme solution at a pH of 5.0. The homogenized mixture is then incubated for 20 minutes at 50°C with occasional stirring. The homogenized mixture is then centrifuged at 7000 rpm for 15 minutes to obtain the clear supernatant.

2.8 Determination of Michaelis Constants of Glucoamylases

2.8.1 Enzymatic Reactions

Applying enzymatic reactions:

$$E + S \underset{K_{-1}}{\overset{K_{+1}}{\rightleftarrows}} ES \underset{K_{-2}}{\overset{K_{+2}}{\rightleftarrows}} P + E \quad \ldots\ldots (1)$$

Where E denotes enzyme, S substrate and P the product formed.K_{+1}, K_{+2} are the forward reaction rate constants and K_{-1}, K_{-2} rate constants for the reverse reactions. Equilibrium or a steady state condition is reached when the forward reaction rates equal the backward reaction rates. This is the basic equation upon which most enzyme activity studies are based. Several factors affect the rate at which enzymatic reactions proceed i.e. temperature, pH, enzyme concentration, substrate concentration, and the presence of any inhibitors or activators (Roskoski, 2007).

2.8.2 Michaelis Constants

It is theorized that when the maximum velocity had been reached, the entire available enzyme has been converted to ES, the enzyme substrate complex. This point is designated Vmax in Figure 2.5. Using this maximum velocity and equation (1),

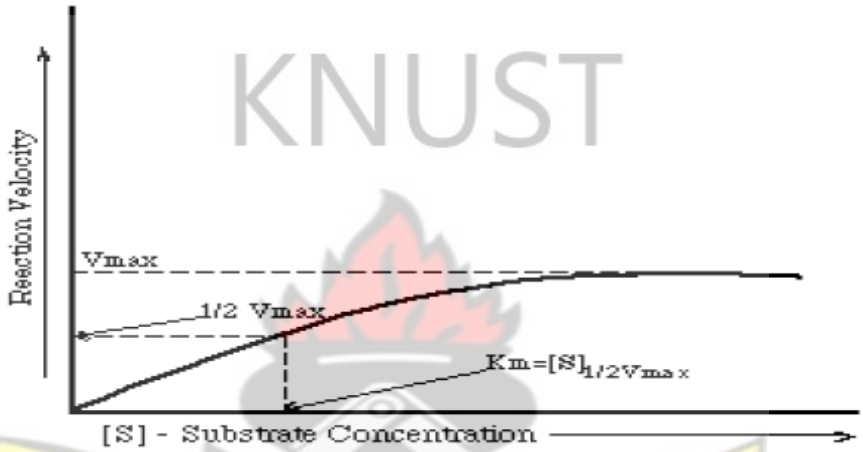


Figure 2.5 Effect of substrate concentration

Michaelis (Roskoski, 2007) developed a set of mathematical expressions to calculate enzyme activity in terms of reaction speed from measurable laboratory data. The Michaelis constant Km is defined as the substrate concentration at 1/2 the maximum velocity. See Figure 2.5. Using this constant and the fact that Km can also be defined as:

$$\mathrm{Km} = \frac{K_{+1}+K_{+2}}{K_{-1}} = [S]_{\frac{1}{2}V_{max}} \ldots\ldots(2)$$

Where K_{+1}, K_{-1} and $K_{+2}$are the rate constants from equation (1).Michaelis developed the following expression for the reaction velocity in terms of this constant and the substrate concentration.

$$V_t = \frac{V_{max}[S]}{Km+[S]} \ldots\ldots\ldots\ldots\ldots\ldots\ldots (3)$$

V_t = the velocity at anytime,

[S] = the substrate concentration at this time,

V_{max} = the highest forward velocity under this set of experimental conditions (pH, temperature etc.),

Km = the Michaelis constant for the particular enzyme being investigated. Derivation of equation (3) is shown in Appendix 25.

Michaelis constants have been determined for many of the commonly used enzymes. The size of Km tells several things about a particular enzyme:

1. A small Km indicates that the enzyme requires only a small amount of substrate to become saturated. Hence, the maximum velocity is reached at relatively low substrate concentrations.
2. A large Km indicates the need for high substrate concentrations to achieve maximum reaction velocity.
3. The substrate with the lowest Km upon which the enzyme acts as a catalyst is frequently assumed to be enzyme's natural substrate, though this is not true for all enzymes.

According to (Cereia *et al*., 2006), glucoamylase shows Michaelian behaviour with major affinity for starch. Km values measured for some G2 and G1 are 0.14 mg/ml and 0.28 mg/ml respectively (Campos and Félix, 1995; Cereia *et al*., 2006).

2.8.3 Effect of Substrate on Michaelis Constants

Substrate inhibition will sometimes occur when excessive amounts of substrate are present. Figure 2.6 shows the reaction velocity decreasing after the maximum velocity has been reached (Roskoski, 2007).

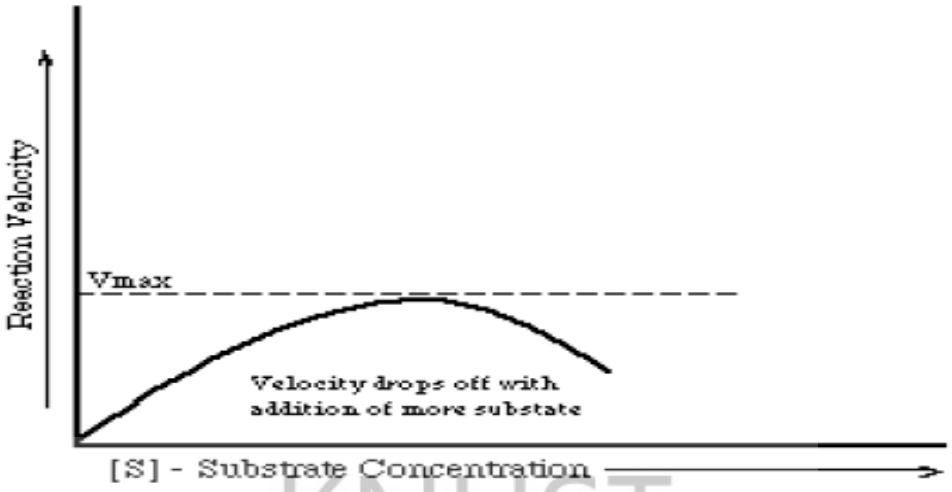


Figure 2.6 Substrate becomes rate-inhibiting

Additional amounts of substrate added to the reaction mixture after this point actually decreases the reaction rate. This is thought to be due to the fact that there are so many substrate molecules competing for the active sites on the enzyme surfaces that they block the sites and prevent any other substrate molecules from occupying them. In the study and characterization of an enzyme, it is important to know the optimal concentration of substrates. These concentrations are related to the substrate concentration, or Km values, that result in a reaction velocity that is one-half of the maximal velocity (Vmax). Also from the Vmax, the turnover number of the enzyme or thecatalytic rate constant(kcat) which is the maximal number of molecules of substrate converted to product per active site per unit time when the enzyme is saturated with substrate can be calculated (Garrett and Grisham, 1999). Determination of these values is important in developing and using enzyme assays for studying normal physiological processes (Roskoski, 2007). Measured Vmax values for G2 and G1 are 0.21 µmol glucose/min/mg and 67.2 µmol/min/mg respectively (Campos and Félix, 1995; Cereia *et al*., 2006).

Chapter 3

3.1 Methodology

3.1.1 Sources of Fungal Isolates for the Studies

Isolates of *Aspergillus niger*, *Aspergillus flavus*, *Fusarium* and *Rhizopus* species were obtained from the Plant Pathology Laboratory of Crops Research Institute, Kumasi for this study.

KNUST

3.1.2 Preservation of Fungal Isolates for the Production of Glucoamylase

The fungal isolates were sub-cultured on PDA (Potato Dextrose Agar) in Petri Dishes for use. Slants of these fungal organisms on PDA were maintained at 4°C in a refrigerator and regenerated for use whenever needed.

3.2 Preparation of PDA and Maintenance of Microorganisms

200g of Irish potatoes were washed and slashed into pieces and boiled in 500 ml of distilled water till the potato pieces softened. The softened pieces were mashed and 20g of glucose and 20g of agar added. Distilled water was added to produce a one litre (1 L) mixture. The mixture was autoclaved at a temperature of 121°C under 1.1 kg/cm^2 of pressure for 25 minutes. The prepared PDA was allowed to cool and poured into sterilized petri dishes inside a laminar flow chamber. This prepared media was used to grow the fungal isolates for the various experiments.

3.3 Preparation of Fungal Isolates for Inoculation of Substrates

Spores from six day old cultures of *Aspergillus niger, A. flavus, Fusarium oxysporum* and four day old of *Rhizopus* species were taken up in 12 ml of sterile distilled water.

The spore suspensions were filtered through sterilized cheese clothes and their concentrations adjusted to 1 x 10^7 spores/ml following the method used by (Bhatti *et al*., 2007) with some modifications. Prepared spore suspension of each organism was used to inoculate different substrates as described in section 3.4.

3.4 Identification of Suitable Substrate for Glucoamylase Production

Cleaned healthy cocoa pods that have been chopped into pieces, maize bran, rice bran, wheat bran and groundnut pod pieces were dried in an oven at 70°C to a constant mass. Each substrate was ground in a Wiley mill to 0.5 mm particle size (Paulchamy, 2008). Ten grams (10 g) of each substrate was placed in a 250 ml Erlenmeyer flask and mixed with ten millilitres (10 ml) of appropriate mineral salts solution. Substrates for growing *Aspergillus niger* and *A. flavus* were mixed with liquid Aspergillus Complete Medium (ACM) (Zaldivar-Aguero *et al*., 1997). ACM was prepared according to the protocol in Appendix 1. Substrates for growing *Rhizopus* species and *Fusarium oxysporum* were mixed with mineral salts solutions described in Appendices 2 and 3 respectively (Pandey, *et al*., 1994). The contents in each flask were autoclaved at 121°C under 1.1 kg/cm^2 of pressure for 25 minutes. Each flask was next inoculated with three (3) millilitres of spore suspension of the appropriate organism at spore concentrations of 1×10^7 per ml and incubated at 30°C. Each investigation was set up in triplicates. Inoculated flasks were harvested at 24 hours, 48 hours, 72 hours, 96 hours and 120 hours. At harvest, ten millilitres (10 ml) citric acid buffer described in Appendix 4 was added to the contents of each flask. The buffer was mixed up properly with the contents of flask and the mixture squeezed through cheese cloth to obtain a filtrate. The filtrates obtained were centrifuged at 7000 rpm

for 15 minutes. The solid free supernatants were tested for glucoamylase activities as described in section 3.4.1 (Madihah *et al*., 2000).

3.4.1 Testing for Glucoamylase Activity

A mixture of one millilitre (1 ml) citric acid buffer (pH 4.6) and one millilitre (1 ml) 1% (w/v) soluble starch (Harris Chemical, England) solution was prepared in a test tube. One millilitre (1 ml) culture filtrate was added to the mixture to convert the starch to glucose (at room temperature of 28°C). The reaction was terminated by the addition of two millitres (2ml) of 3, 5-Dinitrosalicylic acid (DNS) reagent after 10 minutes (Miller, 1959). The DNS reagent was prepared as described in Appendix 5. After adding the DNS reagent, the test tube was quickly placed in boiling water for 5 minutes to develop the colour complex if DNS was reduced by produced glucose. When DNS comes into contact with glucose, it is reduced to 3-amino-5-dinitrosalicylic acid which has a red complex colour. The red complex colour is formed when the reduced DNS is heated in boiling water. The heat denatures the enzyme and ensures that the enzymatic reaction is completely stopped. The contents (mixture of citric acid, starch, culture filtrate and DNS) in the test tube were next cooled by placing the test tube in ice-cold water. Corresponding mixture serving as blank (mixture of citric acid, distilled water, culture filtrate and DNS) was prepared and treated just like the mixture containing the starch solution.The two mixtures were set in triplicates. Absorbance of the mixture comprising citric acid, starch, enzyme and DNS was taken at 540 nm and that of the blank (control) also taken at 540 nm. In the blank, there was no glucose. Hence the difference in absorbances between the mixture (citric acid, starch, enzyme and DNS) and its blank is proportional to concentration of glucose produced by the enzymatic reaction (which occurred in the

mixture comprising citric acid, starch, enzyme and DNS) as described by Miller, 1959 in accordance with Beer Lambert's law. Using a standard glucose curve shown in Appendix 11, concentration of glucose due to the enzymatic reaction was obtained. Glucoamylase activity was calculated as shown in Appendix 24.

3.4.2 Effect of Temperature on Glucoamylase Production on Wheat Bran

To investigate the effect of temperature on glucoamylase production, crude culture filtrates of *Aspergillus niger* and *Rhizopus* species produced from inoculated substrates mixed with the appropriate mineral salts solution; and had been incubated at 30ºC, 40ºC and 50ºC for 24 hours, 48 hours, 72 hours, 96 hours and 120 hours were obtained as described in section 3.4 and glucoamylase activities determined as described in section 3.4.1.

3.4.3 Effect of pH on Glucoamylase Production on Wheat Bran

To investigate the effect of pH on glucoamylase production, the pHs of ACM and mineral salts solution for growing *Rhizopus* species were adjusted with 2M HCl and 2M NaOH. ACM and the mineral salts solution with pH range of 4 - 8 and 4 - 6 respectively were investigated at intervals of 1.0 and 0.5 units respectively. Crude culture filtrates of *Aspergillus niger* and *Rhizopus* species produced from inoculated substrates mixed with the appropriate mineral solution that had been incubated at 40°C for 24 hours, 48 hours, 72 hours, 96 hours and 120 hours were obtained as described in section 3.4 and glucoamylase activities determined as described in section 3.4.1.

3.4.4 Effect of Nitrogen on Glucoamylase Production on Wheat Bran

The effect of nitrogen on glucoamylase production was investigated by preparing mineral solutions (ACM) containing 0.1, 0.2, 0.3 and 0.4 g/L of yeast extract as the nitrogen source. Crude culture filtrates of *Aspergillus niger* produced from inoculated substrates mixed with the appropriate mineral solution (ACM) which had been incubated at 40°C for 24 hours, 48 hours, 72 hours, 96 hours and 120 hours were obtained as described in section 3.4 and glucoamylase activities determined as described in section 3.4.1. *Rhizopus* species depend on very little amount of nitrogen for their growth (Irfan *et al*., 2012) and that effect of nitrogen for glucoamylase production was only investigated using *Aspergillus niger*.

3.4.5 Effect of Incubation Period on Glucoamylase Production on Wheat Bran at the Optimum Regimes

Effect of incubation period for glucoamylase production was investigated by obtaining crude culture filtrates ohnf *Aspergillus niger* and *Rhizopus* species harvested at six (6) hours interval. The culture filtrates of *Aspergillus niger* and *Rhizopus* species were produced from inoculated wheat bran mixed with the appropriate mineral solution and had been incubated at 40°C as described in section 3.4. Glucoamylase activities of the crude culture filtrates obtained were determined as described in section 3.4.1. ACM for growing *Aspergillus niger* was prepared at pH 5 and nitrogen concentration of 0.2 g/l.

3.5 Determination of Molecular Weights of Glucoamylase Produced by *Aspergillus niger* and *Rhizopus* species

3.5.1 Purification of Crude Culture Filtrates for Molecular Weights Determination

Crude culture filtrates for the determination of molecular weights of glucoamylase produced by *Aspergillus niger* and *Rhizopus* species were produced following the procedure in section 3.4 using wheat bran as substrate and incubated at 40°C for 18 hours. Hundred millilitres (100 ml) each of the crude culture filtrate were adjusted to pH 5.5 with 2M HCl and 2M NaOH. Each crude culture filtrate was mixed properly with three (3) grams of activated charcoal and placed in an incubator and intermittently shaken at a temperature of 50°C for 20 minutes. Crude culture filtrates were centrifuged at 7000 rpm for 15 minutes with activated charcoal (Kareem *et al.*, 2011). The clear purified supernatant was assayed for glucoamylase activity following procedure described in section 3.4.1.

3.5.2 Determination of Protein Concentrations of the Purified Culture filtrates

The protein concentrations in the purified culture filtrates obtained as described in section 3.5.1 were determined using Bio-Rad Protein Assay. Eight different concentrations of protein (in decreasing order) were first prepared from a 2.0 mg/ml Bovine Serum Albumin (BSA) by dilution shown in Appendix 6. Twenty microlitres (20µl) each of these concentrations were placed in the first set of wells in a microtitre plate alongside with twenty microlitres (20µl) each of the purified culture filtrates (set in triplicates). This arrangement was repeated in the second row. Twenty microlitres (20µl) of phosphate buffered saline solution (PBS) at pH 7.2 were added to each well in the second row (without adding PBS to any of the wells in the first row) and

properly mixed. Twenty microlitres (20µl) of mixture in each of the wells in the second row were pipetted and placed respectively in the third row of wells and each well properly mixed with twenty microlitres (20µl) of PBS. Twenty microlitres (20µl) of mixture in each of the wells in the third row were pipetted and placed respectively in the fourth row of wells and each well properly mixed with twenty microlitres (20µl) of PBS. This procedure was followed for all the subsequent set of wells. The PBS was required for solubilizing the protein and served as a standard diluent. Twenty microlitres (20µl) prepared dye reagent produced from Bio-Rad Protein Assay Dye Reagent Concentrate were added to each well in the microtitre plate (starting from the first row) and properly mixed. The dye reagent was prepared as shown in Appendix 7. After adding the dye reagent, the microtitre plate was placed in an incubator for 10 minutes at 30ºC to develop colour. A differential colour change of the dye occurred in response to the various concentrations of protein. Absorbances were measured at 595nm. Absorbances measured from the eight standard BSA concentrations were used to produce a standard BSA curve in Appendix 12. This was used to determine protein concentrations in the purified culture filtrates (Reisner *et al*., 1975; Fazakes *et al*., 1963; Sedmack and Grossberg, 1977).

3.6 Determination of Molecular Weights of Proteins in the Purified Culture Filtrates

3.6.1 Running and staining of Electrophoresis Gels

Calculated volumes of purified supernatants of crude extracts from culture filtrates of *Aspergillus niger* and *Rhizopus* species as described in section 3.4 (each carrying 20µg of proteins) were mixed with loading buffer in 1:1 ratio. The loading buffer was prepared as described in Appendix 8. The mixtures were thoroughly mixed by

vortexing and the homogenized mixtures heated at 95°C for 5 minutes in a Dry Bath Incubator. After heating, the mixtures were vortexed again. The mixtures were loaded into wells in cast gel placed in a running buffer (prepared as shown in Appendix 9) using micro-syringes. The cast gel was prepared as shown in Appendix 10. A standard marker produced by Sigma-Aldrich Company, USA, containing proteins of known molecular weights was loaded alongside the samples. The gel was run at 80 V and 100 mA. As soon as the tracking dye entered the resolving gel, the voltage and the current were changed to 100 V and 120 mA to facilitate better running of the proteins through the gel. The electrophoresis was run for three hours (Bolt and Mahoney, 1997). After electrophoresis, the gel was stained in Coomassie Brilliant Blue solution (comprising 40% distilled water, 10% acetic acid, 50% methanol and 0.25% by weight of Coomassie Brilliant Blue R-250; the Coomassie Brilliant Blue R-250 was dissolved in the methanol before adding acetic acid and water) for 2 hours on a shaker. Thereafter, the gel was destained for 2 hours in a solution comprising 67.5% distilled water, 7.5% acetic acid and 25% methanol on a shaker. The solution was replaced from time to time to enhance result.

3.6.2 Determination of Molecular Weights of Proteins

Relative mobilities of proteins were calculated by dividing the distances migrated by the proteins, by the distance migrated by the tracking dye. A linear plot of the relative mobilities versus logarithms of the molecular weights of proteins (in the standard molecular weight marker) was used to estimate the molecular weights of the unknown proteins in the purified culture filtrates obtained as described in section 3.5.1. The standard marker contained: Myosin (200,000), β-galactosidase (116, 250), Serum

albumin (66,200), Ovalbumin (45,000), Carbonic anhydrase (31,000), Trpsin inhibitor (21,500), Lysozyme (14,400) and Aprotinin (6,500).

3.7 Determination of Kmax and Vmax of Glucoamylases

Various concentrations of soluble starch solutions were prepared. The minimum concentration of soluble starch was 0.001% (w/v). The starch solution was prepared by weighing 0.001g of powdered starch (Harris Chemical, England) and dissolving in total volume of 100 ml of boiling distilled water. One millilitre (1ml) of starch solution at a concentration of 0.001% (w/v) was mixed with one millilitre (1ml) crude glucoamylase extract in a small aluminium container at 40°C at a constant stirring speed of 15 rpm. Glucose concentrations were measured every 10 seconds for one minute using TRUEtest glucose metre (manufactured by Home Diagnostics, U.S.A.). The procedure was repeated for starch concentrations of 0.002, 0.003, 0.004 and 0.005 % (w/v). A Graph of glucose concentration versus time was plotted (for each starch concentration) and initial velocity of glucoamylase activity,V, determined from the equation of the curve obtained. Graphs used to determine initial velocities for glucoamylase activities obtained from *Aspergillus niger* and *Rhizopus* species are shown in Appendices 15 and 16 respectively. Hanes –Woolf's plot (shown in Appendices13 and 14) was used to determine maximum forward velocity (Vmax) and Michaelis-Menten constant (Kmax) for glucoamylases produced by *Aspergillus niger* and *Rhizopus* species respectively.

Chapter 4

4.1 Results

4.1.1 Effect of Substrate on Production of Glucoamylase

Results obtained when solid free supernatants produced from culture filtrates of *Aspergillus niger*, *Aspergillus flavus*, *Fusarium oxysporum* and *Rhizopus* species on wheat bran, rice bran, groundnut pod, maize bran and cocoa pod as described in section 3.4 were tested for glucoamylase activity as described in section 3.4.1 are shown in Tables 4.1, 4.2, 4.3, 4.4 and 4.5. Analysis of variance conducted on the results using statistix 9 statistical software shown in appendix 17 showed that, in Figure 4.1, glucoamylase activity of culture filtrate of *Aspergillus niger* was the highest at 48 hours of incubation period followed by that of *Rhizopus* species. The highest glucoamylase activity recorded with *Aspergillus niger* was 2.00 U/ml and that of *Rhizopus* species was 1.99 U/ml (Table 4.1). However, these activities, 2.00 U/ml and 1.99 U/ml were not significantly different. Similarly, at 48 hours of incubation period, glucoamylse activities of culture filtrates of *Aspergillus flavus* and *Fusarium oxysporum* were 0.27 U/ml and 0.28 U/ml respectively which were not significantly different from one another. *Aspergillus niger* and *Rhizopus* species have shorter peak periods than *Fusarium oxysporum* and *Aspergillus flavus*. Hence energies used by *Aspergillus niger* and *Rhizopus* species to produce their respective highest enzyme activities were far less than that used by *Fusarium oxysporum* and *Aspergillus flavus*. Empirically, ratios of glucoamylase activities on wheat bran at 48 hours of incubation period by *Aspergillus niger*, *Rhizopus* species, *Fusarium oxysporum* and *Aspergillus flavus* are 7.41, 7.37, 1.04 and 1.00 respectively. Hence *Aspergillus niger* and *Rhizopus* species have glucoamylase activities almost seven (7) times as high as those

of *Fusarium oxysporum* and *Aspergillus flavus* at 48 hours of incubation period on wheat bran. Comparing *Aspergillus niger* and *Rhizopus* species performance in terms of glucoamylase production on wheat bran, *Aspergillus niger* may be preferred to *Rhizopus* species because of its high enzyme activity associated with it. Economically *Aspergillus niger* and *Rhizopus* species are better producers of glucoamylase than *Fusarium oxysporum* and *Aspergillus flavus* on wheat bran (appendix 17).

Table 4.1 Glucoamylase Activity on Wheat Bran at 30°C

Incubation Period (hours)	*Aspergillus niger* (U/ml)	*Aspergillus flavus* (U/ml)	*Rhizopus* species (U/ml)	*Fusarium oxysporum* (U/ml)
0	0.00	0.00	0.00	0.00
24	1.50	0.13	1.47	0.16
48	2.00	0.27	1.99	0.28
72	1.56	0.37	1.50	0.38
96	1.66	0.22	1.55	0.17
120	1.79	0.16	1.11	0.22

Glucoamylase activities of crude culture filtrates of the four organisms on wheat bran are compared in Figure 4.1 below.

Production of Glucoamylase by Fungal Isolates on Wheat Bran at 30ºC

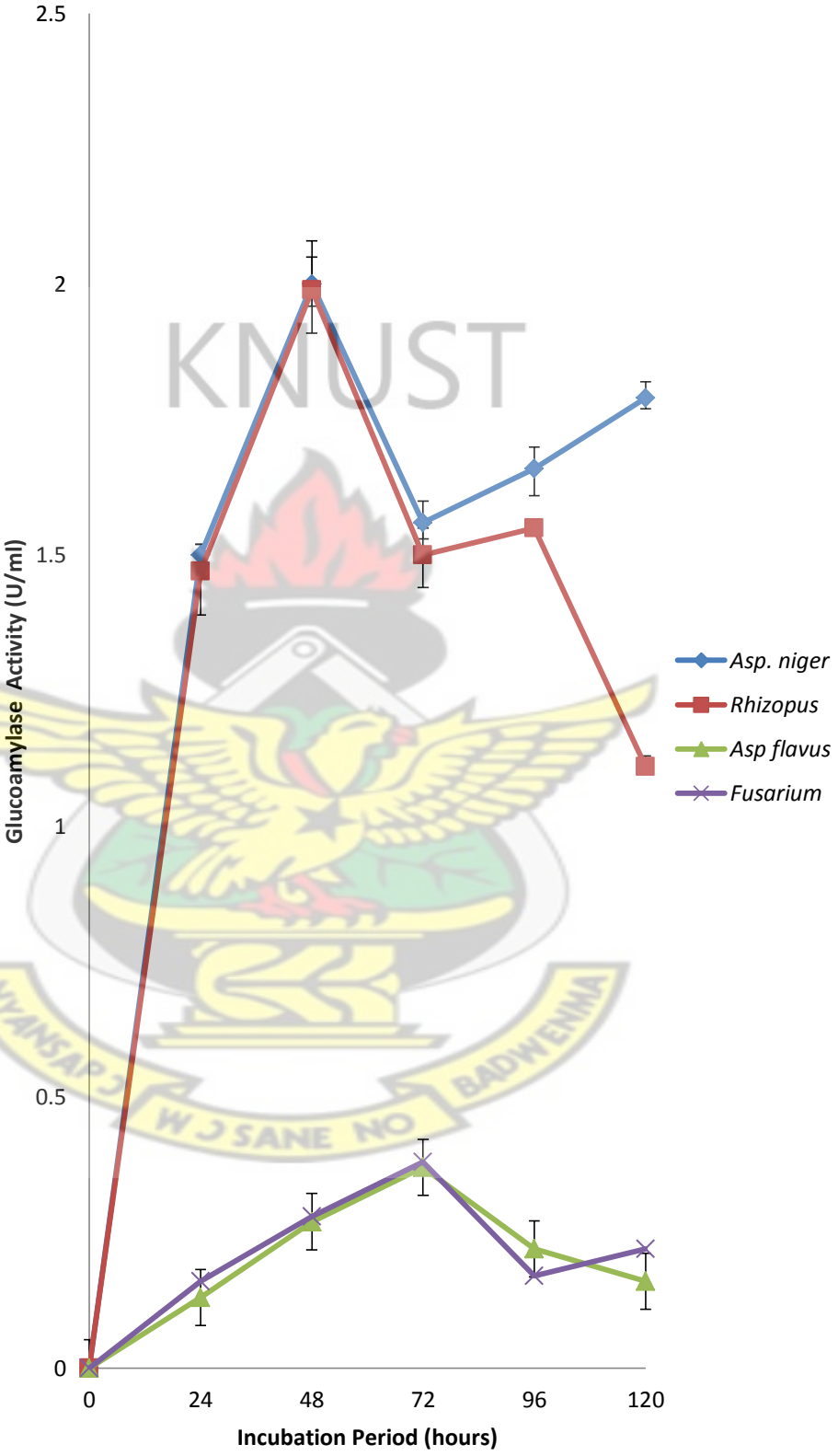


Figure 4.1

Results of glucoamylase production on rice bran by the four fungal isolates are presented in Table 4.2. *Aspergillus niger* gave the highest glucoamylase activity of 1.58 U/ml in 96 hours on rice bran. In *Rhizopus* species the highest glucoamylase activity was 1.55 U/ml recorded in 72 hours. The lowest highest enzyme activities were recorded in culture filtrates of *Aspergillus flavus* and *Fusarium oxysporum* (Figure 4.2). *Rhizopus* species had a shorter peak period than *Aspergillus niger*. Therefore *Rhizopus* species required a lower energy to produce highest enzyme activity of 1.55 U/ml whereas *Aspergillus niger required* a much higher energy to produce highest enzyme activity of 1.58 U/ml. These activities were not significantly different (appendix 17). Hence on rice bran, *Rhizopus* species was the preferred choice for glucoamylase production. *Aspergillus niger* and *Rhizopus* species achieved highest enzyme activities of 2.00 U/ml and 1.99 U/ml respectively on wheat bran with lower energy. On rice bran, *Aspergillus niger* and *Rhizopus* species achieved highest enzyme activities of 1.58 U/ml and 1.55 U/ml respectively with much higher energy. Hence wheat bran was a better supporter of glucoamylase than rice bran since respective highest enzyme activities achieved were significantly different for *Aspergillus niger* and *Rhizopus* species (appendix 17).

Table 4.2 Glucoamylase Activity on Rice Bran at 30°C

Incubation Period (hours)	*Aspergillus niger* (U/ml)	*Aspergillus flavus* (U/ml)	*Rhizopus* species (U/ml)	*Fusarium oxysporum* (U/ml)
0	0.00	0.00	0.00	0.00
24	1.01	0.11	0.89	0.14
48	1.21	0.24	1.11	0.20
72	1.52	0.19	1.55	0.32
96	1.58	0.32	1.50	0.12
120	0.92	0.12	0.94	0.19

Production of Glucoamylase by Fungal Isolates on Rice Bran at 30ºC

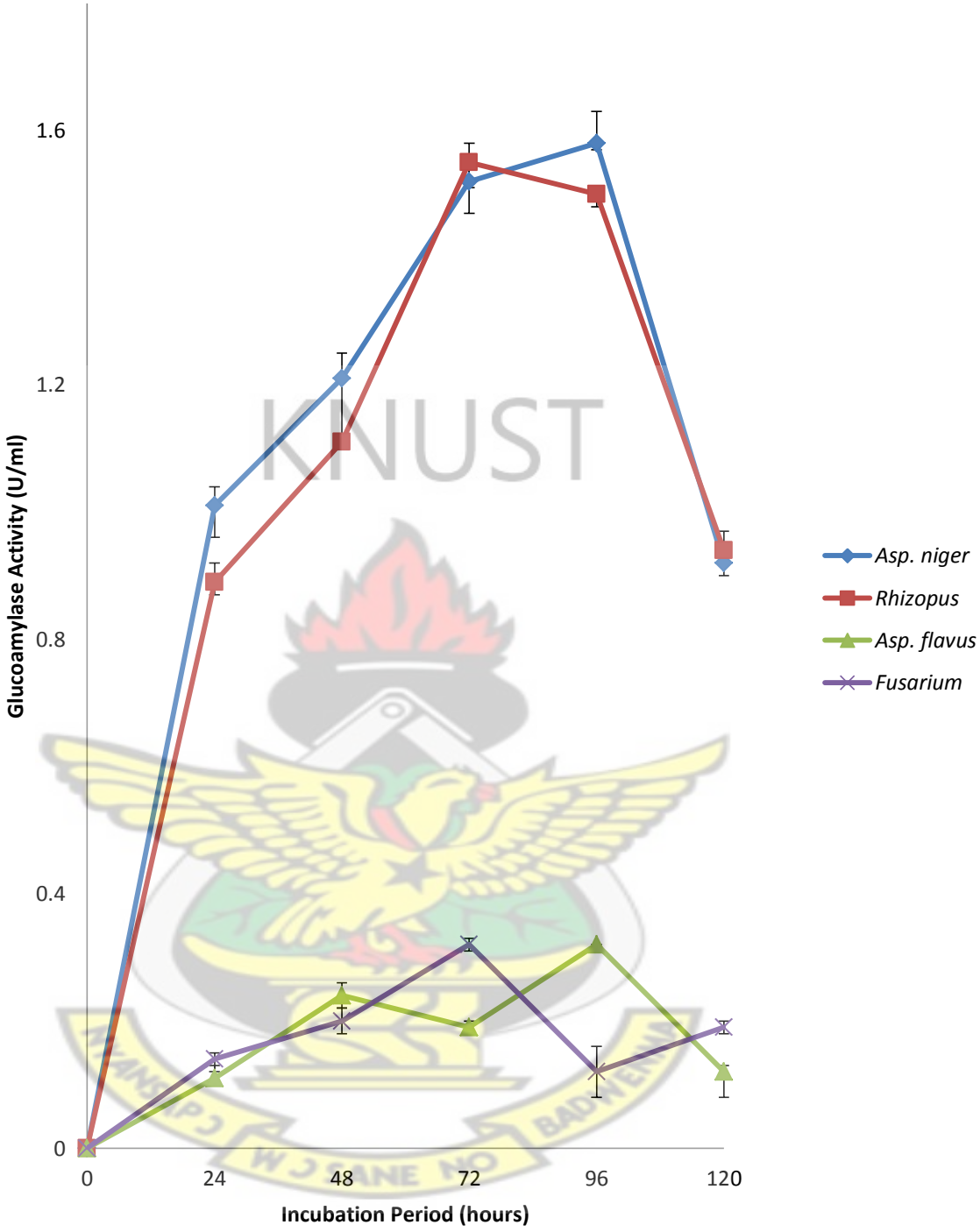

1.6
1.2
0.8
0.4
0
Glucoamylase Activity (U/ml)
0
24
48
72
96
120
Incubation Period (hours)
Asp. niger
Rhizopus
Asp. flavus
Fusarium

Figure 4.2

Results of glucoamylase production on groundnut pod by the four fungal isolates are presented in Table 4.3. *Aspergillus niger* gave the highest glucoamylase activity of 1.39 U/ml in 96 hours. *Rhizopus* species followed with glucoamylase activity of 1.33 U/ml in 72 hours. *Fusarium oxysporum* and *Aspergillus flavus* came third with glucoamylase activities of 0.32 U/ml in 48 and 72 hours respectively (Figure 4.3). *Rhizopus* species had a shorter peak period than *Aspergillus niger*. Hence, *Rhizopus* species used a lower energy to produce its highest enzyme activity, 1.33 U/ml, whereas *Aspergillus niger* used a much higher energy to produce its highest enzyme activity, 1.39 U/ml. These enzyme activities, 1.33 U/ml and1.39 U/ml, were not significantly different (appendix 17). Economically, *Rhizopus* species may be preferred to *Aspergillus niger* for enzyme production because of its lower energy associated with it. *Aspergillus niger* and *Rhizopus* species achieved highest enzyme activities of 1.58 U/ml and 1.55 U/ml respectively on rice bran. On groundnut pod, *Aspergillus niger* and *Rhizopus* species achieved highest enzyme activities of 1.39 U/ml and 1.33 U/ml respectively. Hence, rice bran was a better supporter of glucoamylase than groundnut pod (appendix 17)

Table 4.3 Glucoamylase Activity on Groundnut Pod at 30°C

Incubation Period (hours)	*Aspergillus niger* (U/ml)	*Aspergillus flavus* (U/ml)	*Rhizopus* species (U/ml)	*Fusarium oxysporum* (U/ml)
0	0.00	0.00	0.00	0.00
24	0.44	0.11	0.42	0.14
48	0.83	0.24	0.80	0.32
72	1.11	0.32	1.33	0.24
96	1.39	0.19	1.11	0.19
120	1.17	0.19	0.83	0.19

Production of Glucoamylase by Fungal Isolates on Groundnut Pod at 30ºC

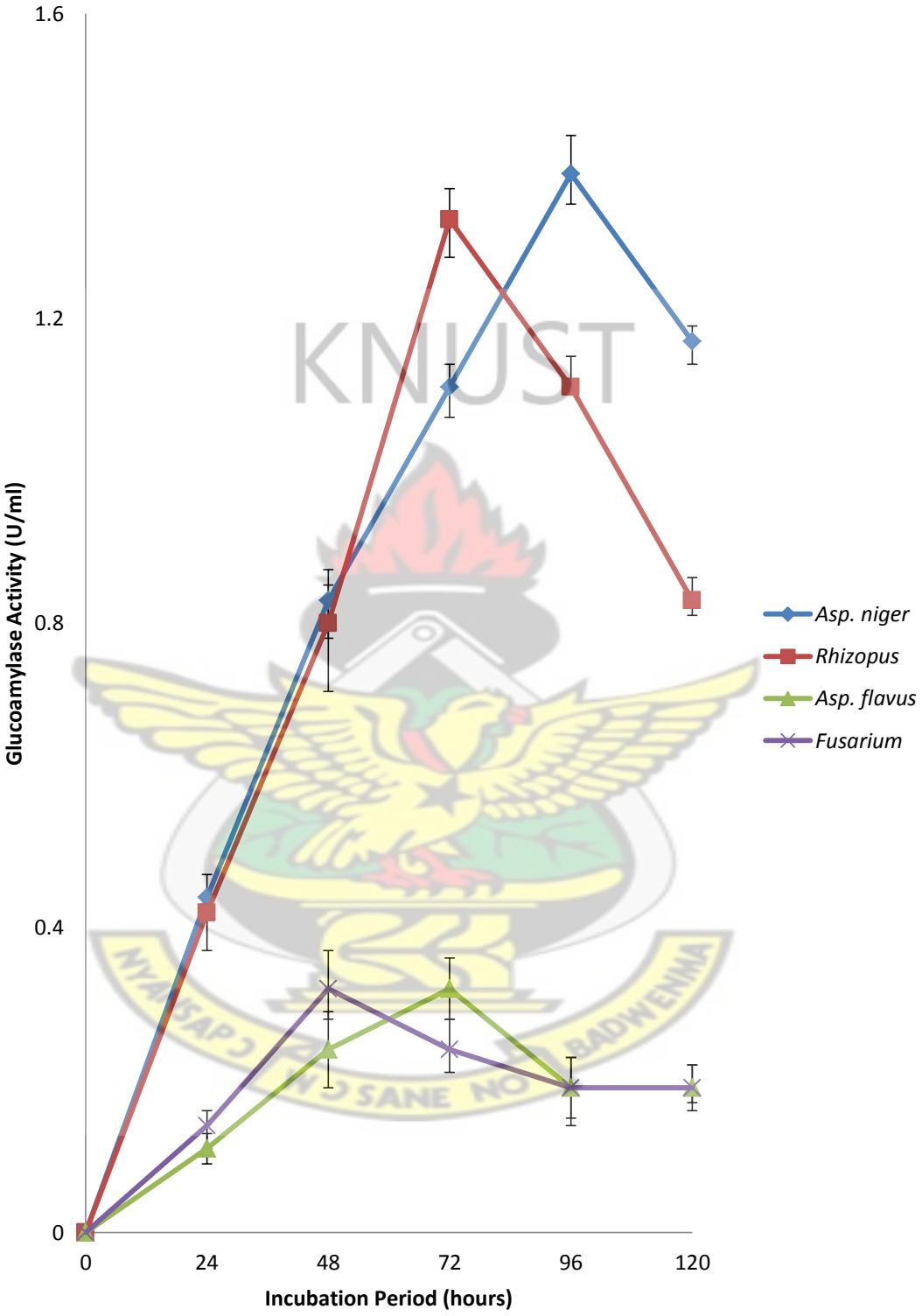


Figure 4.3

Results of glucoamylase production on maize bran by the four fungal isolates are presented in Table 4.4. *Aspergillus niger* registered the highest glucoamylase activity of 0.38 U/ml in 72 hours when culture filtrates were tested for glucoamylase activity. *Rhizopus* species gave the second highest glucoamylase activity of 0.21 U/ml in 96 hours. The third highest glucoamylase activity came from *Aspergillus flavus* with glucoamylase activity of 0.13 U/ml in 72 hours. The lowest highest glucoamylase activity was recorded from the culture filtrate of *Fusarium oxysporum* (Figure 4.4). *Aspergillus niger* had a shorter peak period than *Rhizopus* species. Therefore *Aspergillus niger* required a lower energy to produce its highest enzyme activity of 0.38 U/ml. *Aspergillus niger* was a better producer of glucoamylase than *Rhizopus* species on maize bran since their highest enzyme activities were significantly different (appendix 17). On groundnut pod, *Aspergillus niger* and *Rhizopus* species achieved highest enzyme activities of 1.39 U/ml and 1.33 U/ml respectively. On maize bran, respective highest enzyme activities achieved were 0.38 U/ml and 0.21 U/ml. Therefore groundnut pod was a better supporter of glucoamylase than maize bran (appendix 17).

Table 4.4 Glucoamylase Activity on0 Maize Bran at 30°C

Incubation Period (hours)	*Aspergillus niger* (U/ml)	*Aspergillus flavus* (U/ml)	*Rhizopus* species (U/ml)	*Fusarium oxysporum* (U/ml)
0	0.00	0.00	0.00	0.00
24	0.14	0.08	0.13	0.07
48	0.28	0.09	0.17	0.09
72	0.38	0.13	0.15	0.10
96	0.22	0.13	0.21	0.08
120	0.17	0.08	0.11	0.08

Production of Glucoamylase by Fungal Isolates on Maize Bran at 30ºC

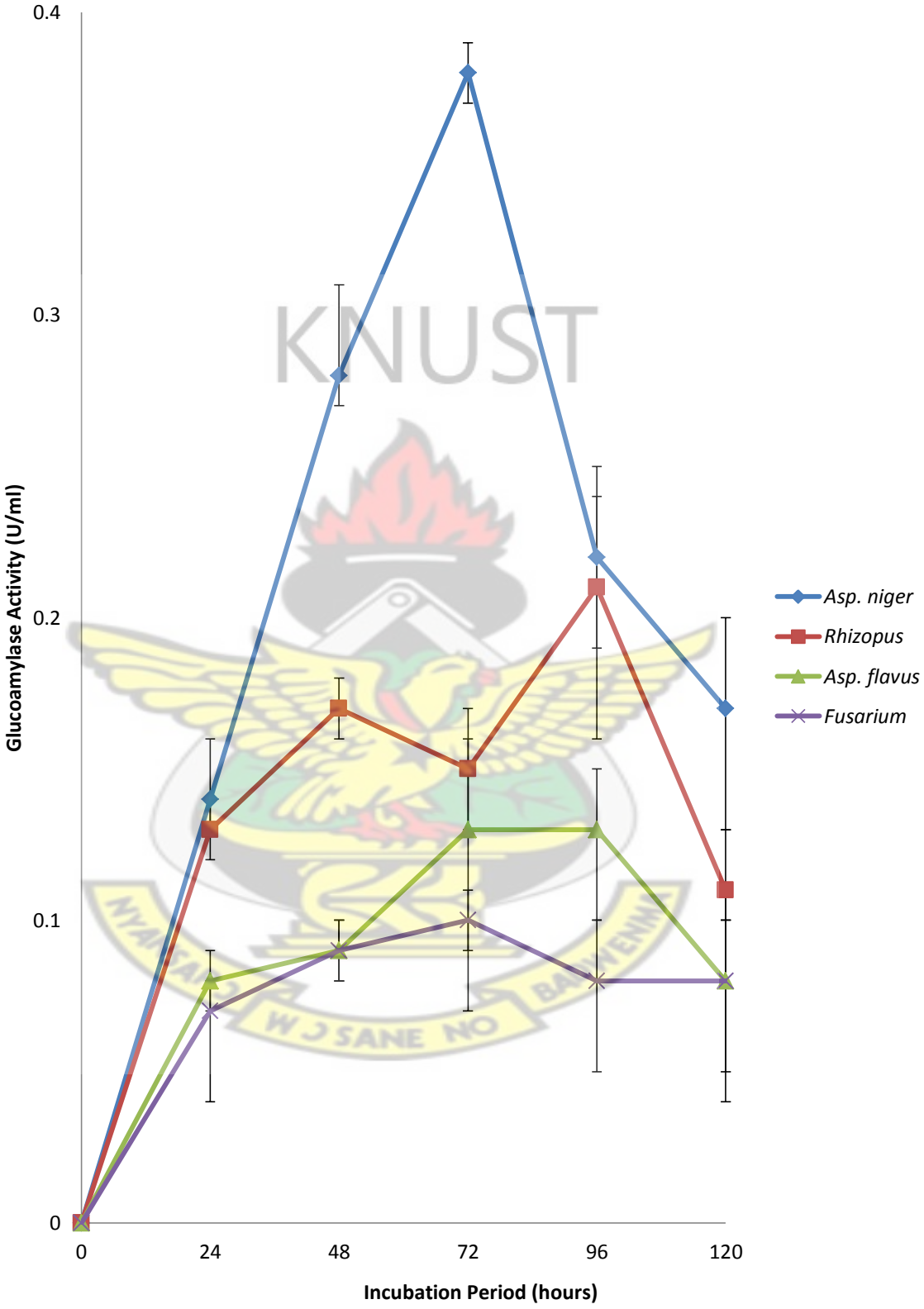


Figure 4.4

Results of glucoamylase production on cocoa pod by the four fungal isolates are presented in Table 4.5. Both *Aspergillus niger* and *Rhizopus* species registered highest glucoamylase activities of 0.13 U/ml in 48 hours. *Aspergillus flavus* followed with highest glucoamylase activity of 0.08 U/ml in 72 hours. *Fusarium oxysporum* gave the lowest highest glucoamylase activity of 0.07 U/ml in 48 hours (Figure 4.5). On cocoa pod, either *Aspergillus niger* or *Rhizopus* species could be used for glucoamylase production since energy needed in either case to produce highest enzyme activity was the same and highest enzyme activities were not significantly different. Highest enzyme activity achieved on cocoa pod with *Aspergillus niger* was 0.13 U/ml. On maize bran, highest enzyme activity achieved with *Aspergillus niger* was 0.38 U/ml. Hence maize bran was a better supporter of glucoamylase than cocoa pod (appendix 17).

Table 4.5 Glucoamylase Activity on Cocoa Pod at 30°C

Incubation Period (hours)	*Aspergillus niger* (U/ml)	*Aspergillus flavus* (U/ml)	*Rhizopus* species (U/ml)	*Fusarium oxysporum* (U/ml)
0	0.00	0.00	0.00	0.00
24	0.11	0.05	0.12	0.03
48	0.13	0.06	0.13	0.07
72	0.12	0.08	0.11	0.04
96	0.12	0.07	0.12	0.05
120	0.11	0.06	0.12	0.04

Production of Glucoamylase by Fungal Isolates on Cocoa Pod at 30°C

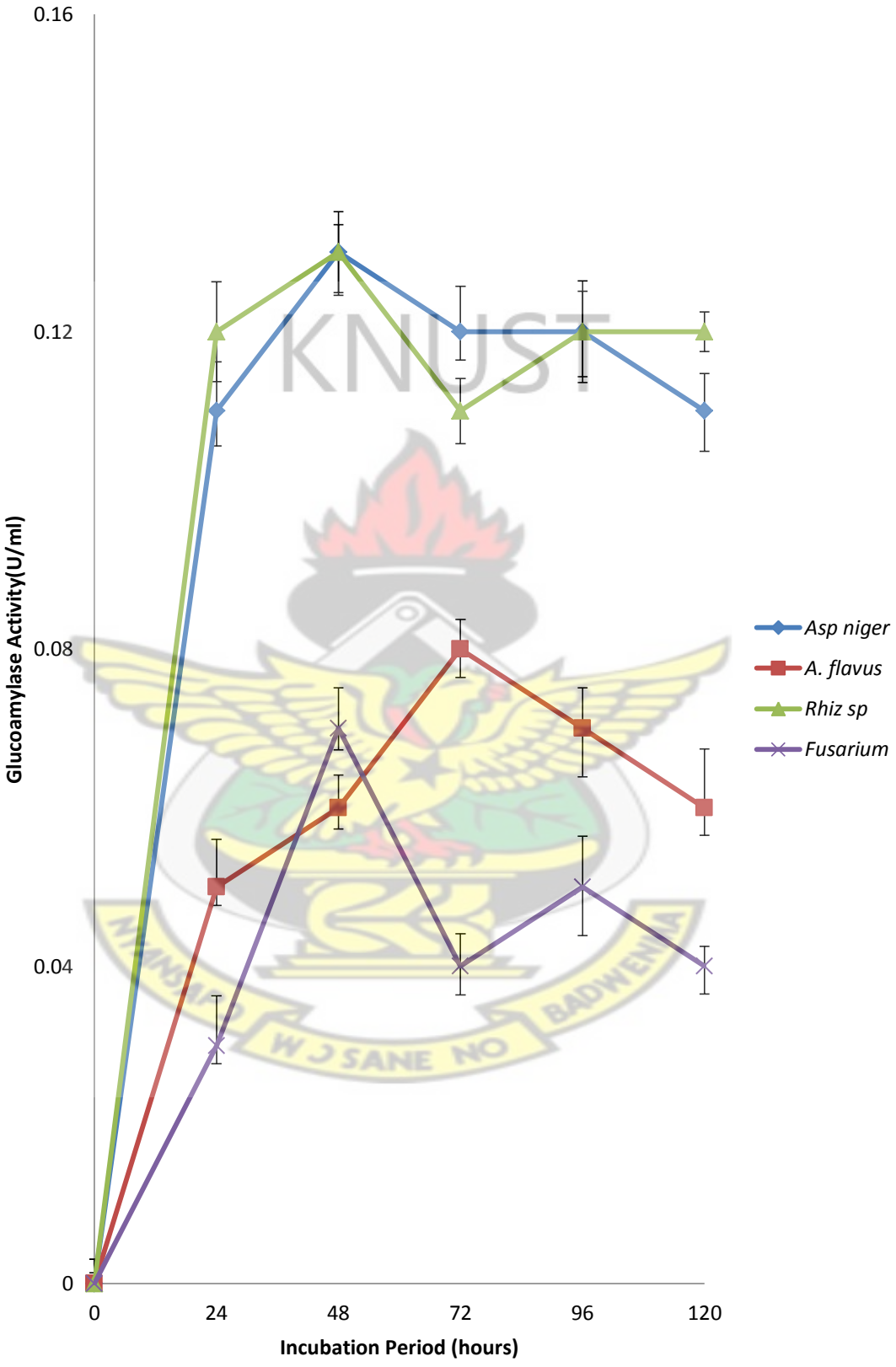


Figure 4.5

4.1.2 Effect of Temperature on Production of Glucoamylase on Wheat Bran

Results of glucoamylase activities obtained from crude culture filtrates of *Aspergillus niger* and *Rhizopus* species obtained as outlined in section 3.4.2 on wheat bran are presented in appendix 18. Crude culture filtrate of *Aspergillus niger* produced at 40ºC had the highest glucoamylase activity of 3.50 U/ml in 48 hours. Crude culture filtrate of *Rhizopus* species produced at 40ºC had glucoamylase activity of 3.00 U/ml in 48 hours. In this work, the optimum temperature for glucocoamylase production by both organisms was 40ºC (Figure 4.6).

KNUST

Effect of Temperature on Production of Glucoamylase on Wheat Bran by *Aspergillus niger* and *Rhizopus* species

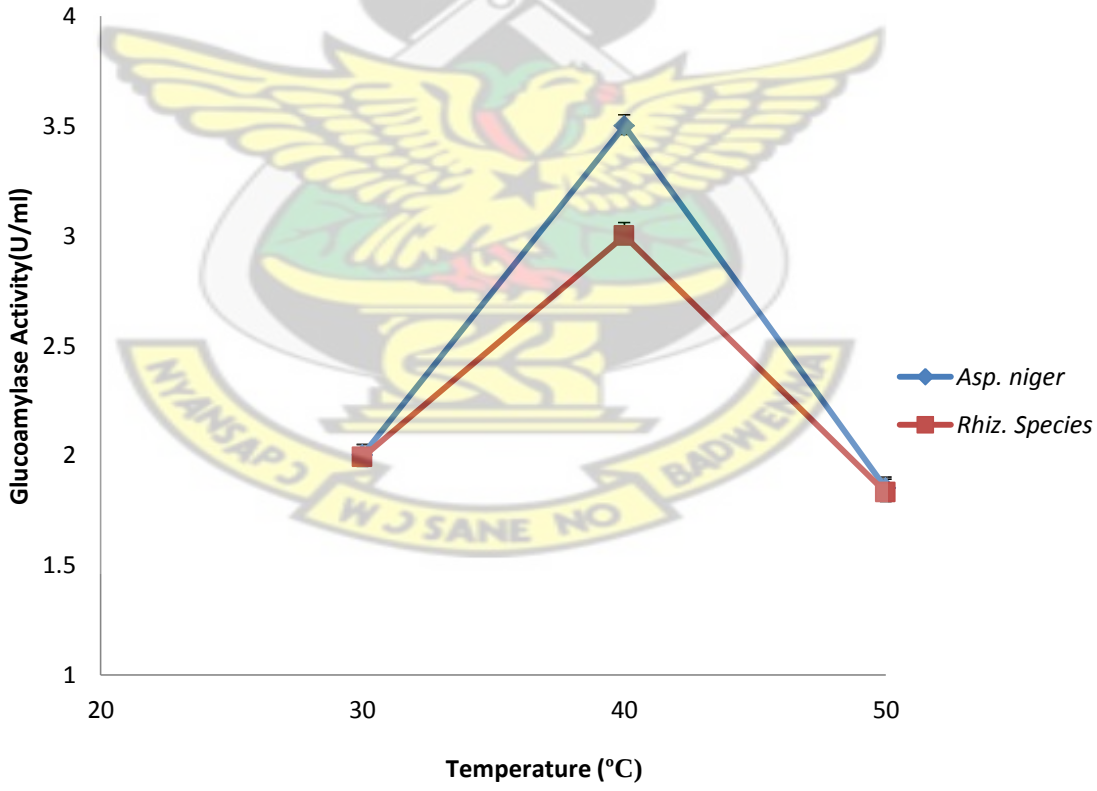


Figure 4.6

4.1.3 Effect of pH on Production of Glucoamylase on Wheat Bran

In experiments to test the effects of pH, crude enzyme from *Aspergillus niger* gave the highest glucoamylase activity of 3.34 U/ml at pH 5.0 shown in appendix 19. When pH was increased beyond 5.0, activity declined to 2.60 U/ml at pH 6.0 (Figure 4.7) and further down to 2.24 U/ml at pH 7.0. Activity however, increased at pH 8.0 to 3.22 U/ml. Crude enzyme from *Rhizopus* species gave highest glucoamylase activity of 3.11 U/ml at pH 4.5. When pH was increased beyond 4.5, activity declined to 1.64 U/ml at pH 6.0.

KNUST

Effect of pH on Production of Glucoamylase on Wheat Bran by *Aspergillus niger* and *Rhizopus* species

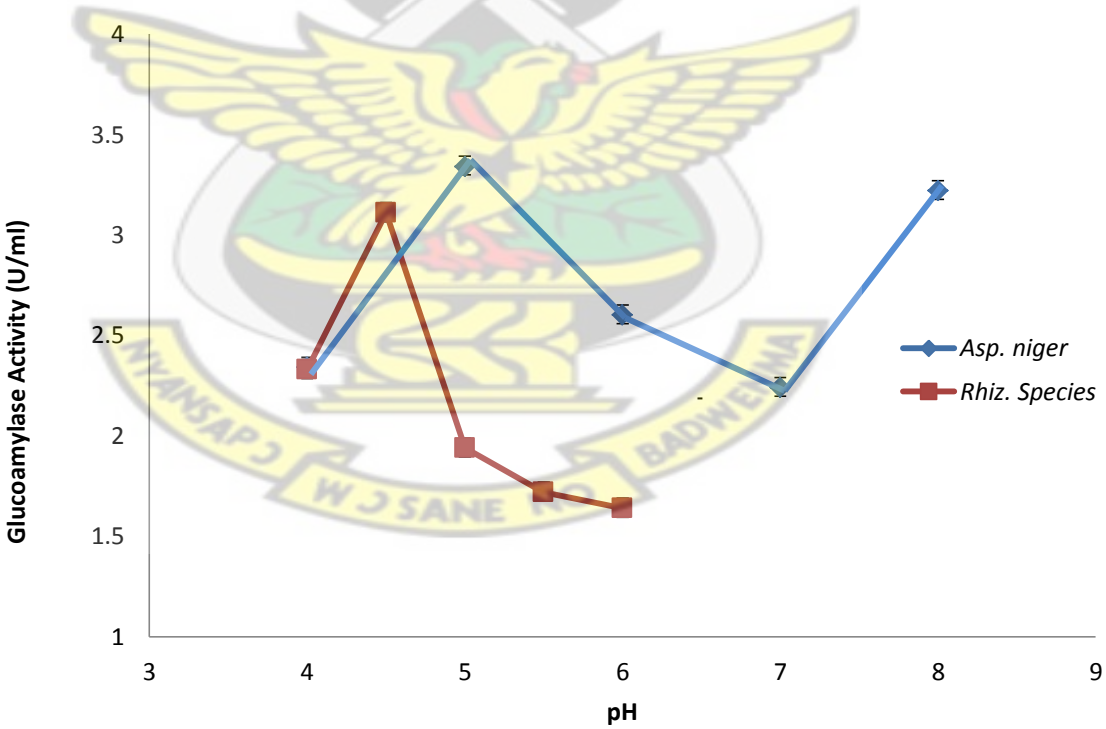


Figure 4.7

4.1.4 Effect of Nitrogen on Production of Glucoamylase on Wheat Bran by *Aspergillus niger*

In experiments to determine the effects of nitrogen on glucoamylase activity, enzyme activity increased when nitrogen concentration was increased from 0.1g/l at activity of 3.59 U/ml to 0.2 g/l at activity of 5.00 U/ml shown in appendix 20. Further increases beyond 0.2 g/l resulted in a decline in enzyme activity to 2.68 U/ml at nitrogen concentration of 0.4 g/l (Figure 4.8).

KNUST

Effect of Nitrogen on Production of Glucoamylase on Wheat Bran by *Aspergillus niger*

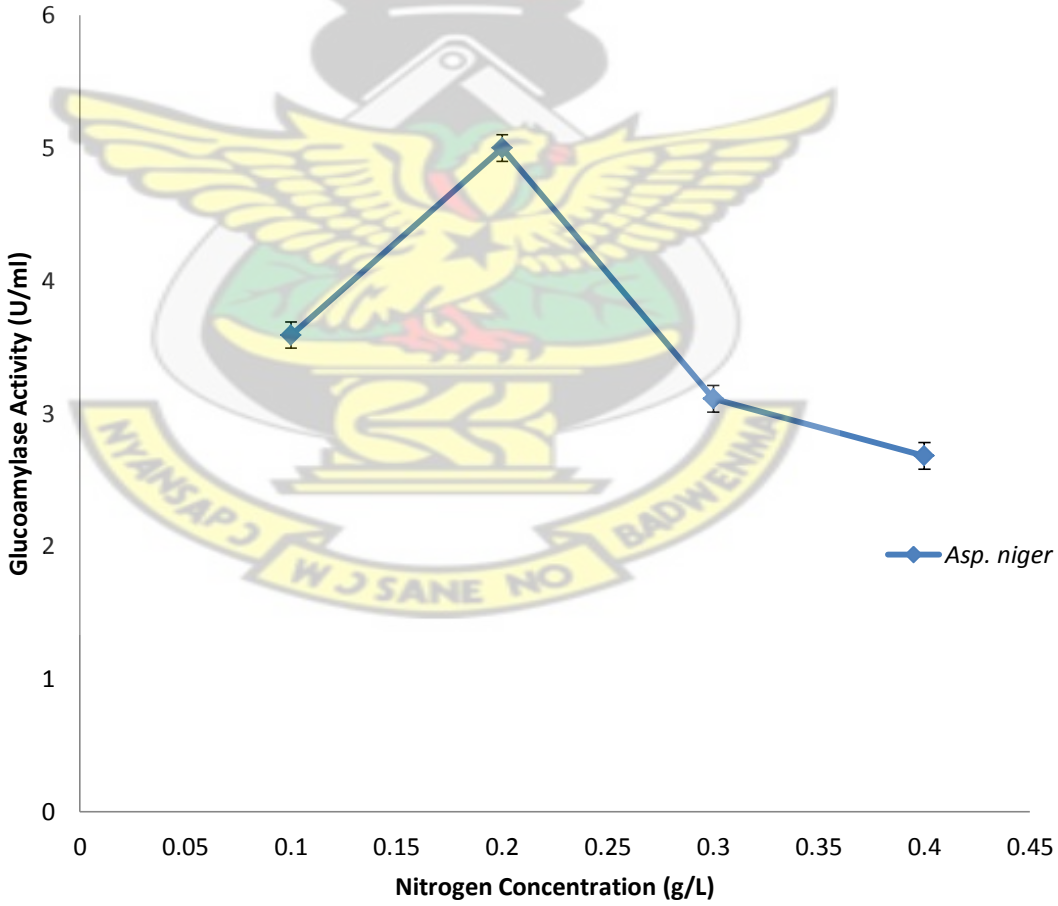


Figure 4.8

4.1.5 Effect of Incubation Period on Glucoamylase Production on Wheat Bran at the Optimum Regimes

Results obtained when crude culture filtrates of *Aspergillus niger* and *Rhizopus* species obtained as described in section 3.4.5 were tested for glucoamylase activity are presented in appendix 21. Crude culture filtrate of *Aspergillus niger* gave the highest glucoamylase activity of 6.66 U/ml in 18 hours of incubation period at optimum conditions of: (a) incubation temperature of 40°C, (b) pH 5.0 and (c) Nitrogen concentration of 0.2 g/l. Glucoamylase activity declined to 3.65 U/ml in 30 hours of incubation period and continuously decreased to 3.12 U/ml in 54 hours of incubation period (Figure 4.9). Crude culture filtrate of *Rhizopus* species also gave highest glucoamylase activity of 4.44 U/ml in 18 hours of incubation period at optimum conditions of: (a) incubation temperature of 40°C and (b) pH 4.5. Glucoamylase activity declined to 3.22 U/ml in 48 hours of incubation period and further declined to 1.67 U/ml in 54 hours of incubation period.

KNUST

Effect of Incubation Period on Glucoamylase Production on Wheat Bran at the Optimum regimes

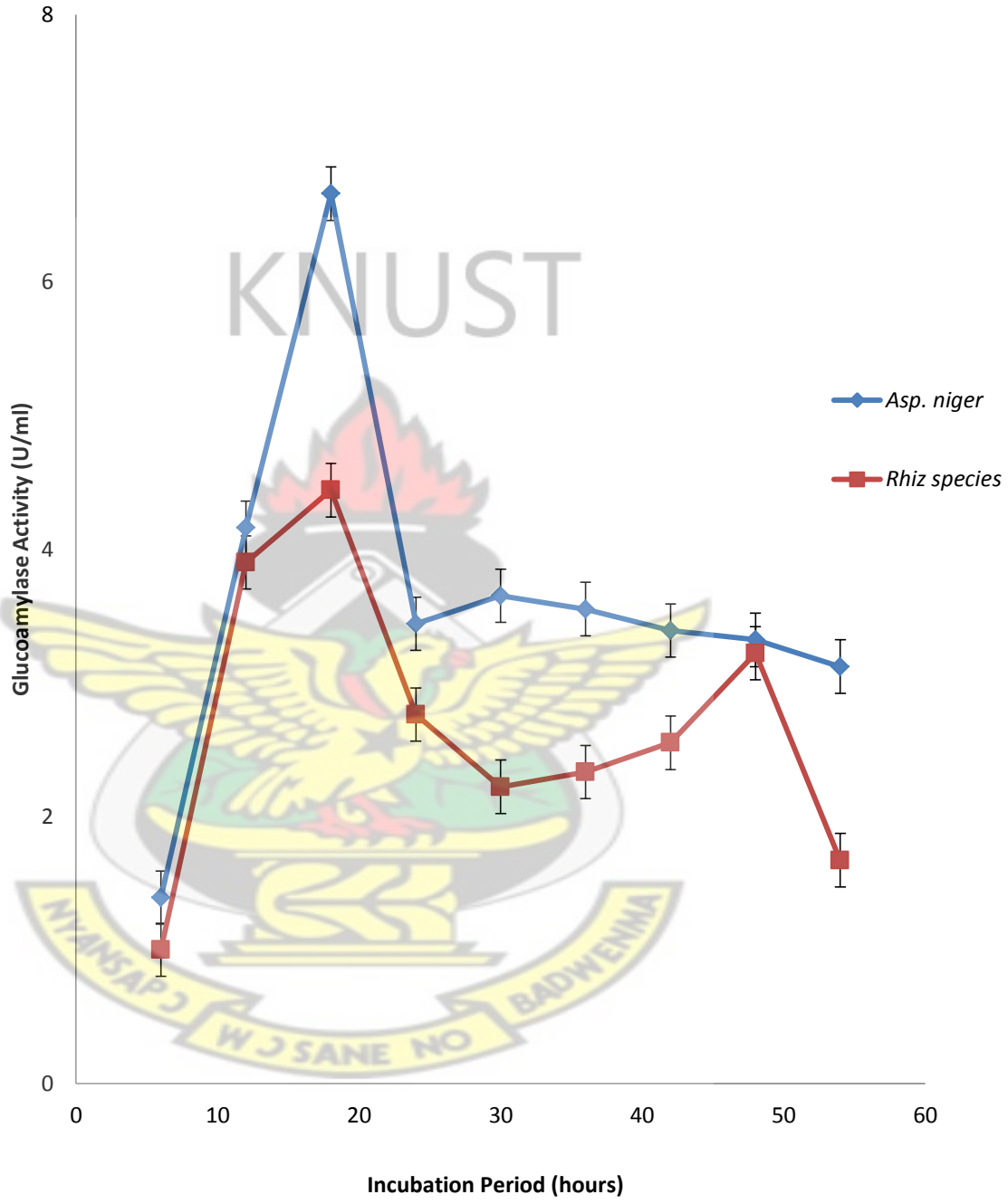


Figure 4.9

KNUST

4.2 Determination of Molecular Weights of Glucoamylase Proteins Produced by *Aspergillus niger* and *Rhizopus* species

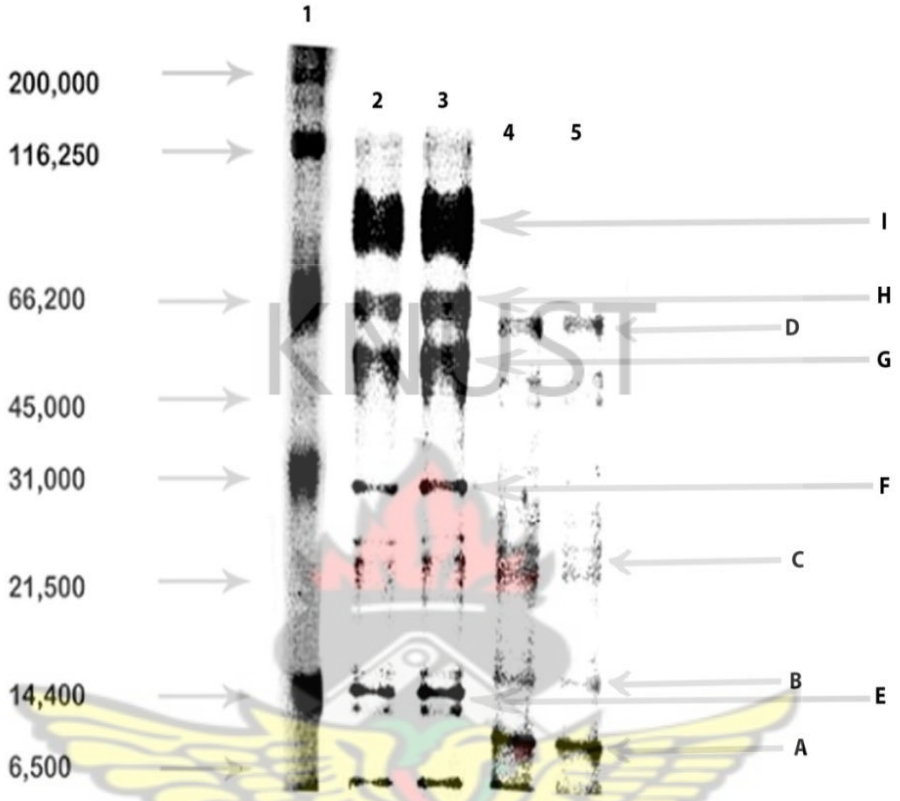


Figure 4.10 SDS-Polyacrylamide Gel with Protein Bands

Figure 4.10 is the picture of the electrophoresis gel produced after running purified culture filtrates of *Aspergillus niger* and *Rhizopus* species produced at their respective optimum conditions. Proteins in the standard marker used are shown in the 1st lane. Lanes 2 and 3 and 4 and 5 were produced from purified culture filtrates of *Rhizopus* species and *Aspergillus niger* respectively. Relative distances and molecular weights of proteins in the purified culture filtrates of *Aspergillus niger* and *Rhizopus* species determined from the developed gel are presented in Tables 4.6 and 4.7 respectively.

Table 4.6 Molecular Weights of Proteins in *Aspergillus niger* Culture Filtrate

Aspergillus niger		
Relative Distance	Log (MW)	Estimated Molecular Weight (Daltons)
0.36774	4.78872	61478.04
0.58710	4.47240	29675.63
0.69032	4.32356	21064.93
0.85161	4.09098	12330.48

KNUST

In Figure 4.10, the bands named A, B, C and D in the *Aspergillus niger* lane have molecular weights: 12330.48, 21064.93, 29675.63 and 61478.04 Daltons respectively.

Table 4.7 Molecular Weights of Proteins in *Rhizopus* species Culture Filtrate

Rhizopus species		
Relative Distance	Log (MW)	Estimated Molecular Weight (Daltons)
0.23226	4.984081	96400.90
0.34839	4.816622	65557.38
0.41935	4.714297	51796.13
0.59355	4.463101	29046.97
0.70968	4.295641	19753.38

In Figure 4.10, the protein bands identified as E, F, G, H and I in the *Rhizopus* species lane, have molecular weights: 19753.38, 29046.97, 51796.13, 65557.38 and 96400.90 Daltons respectively.

4.2.1 Determination of Kmax and Vmax of Glucoamylases Produced from *Aspergillus niger* and *Rhizopus* species

Results obtained when graphs of glucose concentration versus time (shown in Appendices 15 and 16) were plotted to determine initial velocity of glucoamylase activity (for each starch concentration) as described in section 3.7 are presented in Tables 4.8 and 4.9 for *Aspergillus niger* and *Rhizopus* species respectively. Concentration of starch prepared as described in section 3.7 is represented as [S]. Initial velocity of glucoamylase enzyme activity obtained from the graph is represented as V. [S]/V represents the ratio of starch concentration to initial velocity of glucoamylase enzyme activity. Kmax represents Michaelis-Menten constant of the glucoamylase enzyme and Vmax, the maximum velocity achieved by glucoamylase enzyme-starch reaction.

Table 4.8 Determination of Kmax and Vmax for Glucoamylase from *Aspergillus niger*

[S] (g/l)	V (g/l.min)	[S]/V (min)
0.005	2.107	0.002373
0.010	2.158	0.004634
0.015	2.255	0.006652
0.020	2.272	0.008803
0.025	2.320	0.010780

Table 4.9 Determination of Kmax and Vmax for Glucoamylase from *Rhizopus* species

[S] (g/l)	V (g/l.min)	[S]/V (min)
0.005	2.254	0.002218279
0.010	2.327	0.004297379
0.015	2.333	0.00642949
0.020	2.376	0.008417508
0.025	2.438	0.010254307

KNUST

A graph of $\frac{[S]}{V}$ versus [s] (Hanes –Woolf's plot i.e. $\frac{[S]}{V} = \frac{K_m}{V_m} + \frac{1}{V_m}[s]$) gives a slope of 1/Vmax represented as $\frac{1}{V_m}$ and intercept of Kmax/Vmax represented as $\frac{K_m}{V_m}$.

Glucoamylase enzyme produced by *Aspergillus niger* has Kmax = 0.0009548 g/l and Vmax = 2.387 g/l.min (using values in Table 4.8). Glucoamylase enzyme produced from *Rhizopus* species has Kmax = 0.0007443 g/l and Vmax = 2.481 g/l.min (using values in Table 4.9). Glucoamylase enzyme produced from *Aspergillus niger* has Kmax = 0.0009548 g/l which is greater than Kmax = 0.0007443 g/l obtained from glucoamylase enzyme produced by *Rhizopus* species. Hence, glucoamylase enzyme produced from *Aspergillus niger* has a higher affinity for starch or required a higher amount of starch for it to become saturated and that Vmax = 2.387 g/l.min was reached at relatively higher starch concentration.

Chapter 5

5.0 Discussions

The four fungal species *Aspergillus niger*, *Rhizopus* species, *Fusarium oxysporum* and *Aspergillus flavus* investigated in this study were all able to produce glucoamylase on the substrates wheat bran, maize bran, rice bran, groundnut pod and cocoa pod. The amounts of glucoamylase produced by the fungal species varied on the different substrates. *Aspergillus niger* produced higher amounts of glucoamylase on each of the substrates when compared to the other fungal species tested in this study. *Aspergillus niger* has been shown to be an active producer of glucoamylase and is being exploited or used on large scale for industrial production of the enzyme. In an investigation similar to what is being reported on, Varalakshmi *et al*., (2009) and Suganthi *et al*., (2011) reported that *Aspergillus niger* produced glucoamylase more actively than several fungal species they investigated on different substrates. Imai *et al*., (1994) also reported that *Aspergillus niger* is a very useful fungus in the industrial production of glucoamylase. Imai and colleagues in the same study also found *Rhizopus* species to be very useful for industrial production of glucoamylase. *Rhizopus* species investigated in this study was next to *Aspergillus niger* when its glucoamylase production capacity was compared with the other fungal species (Appendix 22). With respect to the substrates used, wheat bran was the best followed by rice bran (appendix 23).

The *Aspergillus niger* and *Rhizopus* species investigated in this study could be depended on for the production of glucoamylase for industrial purposes.

In this study, the best activities achieved with *Aspergillus niger* and *Rhizopus* species on wheat bran were respectively 6.66 U/ml and 4.44 U/ml. In similar studies, Ellaiah

et al., (2002) and Sun *et al*., (2009) respectively reported glucoamylase activities of about 37.08 and 16.22 times higher on wheat bran than what were achieved in this study.

The isolates used by these workers possibly could be mutants with multiple copies of the gene for the production of glucoamylase. It is important that the local isolates of *Aspergillus niger* and *Rhizopus* species identified in this study are improved on to make them more efficient producers of glucoamylase.

Temperature, pH, nitrogen concentration and incubation period of culture media influenced glucoamylase production in this study. In this study, increasing temperature from 30ºC to 40ºC led to an increase in glucoamylase activity. There was a decline in glucoamylase activity when the temperature of the culture mixture was increased to 50ºC for all fungal species studied. Deshmukh *et al*., (2011) also demonstrated that temperature influenced the production of glucoamylase. In their study, it was found that increasing the temperature of the culture mixtures from 30ºC to 40ºC increased glucoamylase activities significantly. Further increase in temperature to 50ºC decreased glucoamylase activities.

In this study, highest glucoamylase activities were recorded at pH 5 for *Aspergillus niger* and pH 4.5 for *Rhizopus* species (appendix 19). Ellaiah *et al*., (2002) demonstrated that pH influenced glucoamylase activity. In their study, it was reported that the highest glucoamylase activity was achieved at pH 5 for *Aspergillus niger*. Work carried out by Suntornsuk and Hang, (1997) on effect of pH on glucoamylase activity showed that highest glucoamylase activities were achieved at pH between 4 and 5 for *Rhizopus* species.

Optimum nitrogen concentration recorded in this study was 0.2 g/l (appendix 20). Barton *et al*., (1972) produced highest glucoamylase activities at nitrogen concentration of 5g/l.

In this study, highest glucoamylase activities were achieved with *Aspergillus niger* and *Rhizopus* species on wheat bran at 18 hours of incubation period (appendix 21). Deshmukh *et al*., (2011) demonstrated that incubation period influenced glucoamylase activity. In their work, highest glucoamylase activity was achieved at 72 hours of incubation period.

By comparing the best glucoamylase activities achieved in this study with what have been reported by other workers, activities in this study are low. For industrial usage and profitability, the local fungal isolates identified in this study (i.e. *Aspergillus niger* and *Rhizopus* species) would have to be improved genetically or otherwise to make them better producers of glucoamylase. Improving other parameters such as substrates, temperature, pH, nitrogen, incubation period and composition of substrates to achieve higher glucoamylase activity may be the best option to consider if the local fungal isolates are to be used for enzyme production.

It is recommended that further investigation to define optimum carbon requirements, temperature, pH, incubation period and nitrogen concentrations among others are carried out aimed at increasing the levels of glucoamylase activities produced by the local isolates of fungi used in this study.

In the studies to determine the molecular weights of glucoamylases produced by *Aspergillus niger* and *Rhizopus* species, proteins with molecular weights 61.48, 29.68, 21.06 and 12.33 KDa were identified from culture filtrates of *Aspergillus niger*. Sixty (60) and 61 KDa proteins in culture filtrates of *Aspergillus niger* have been identified

as glucoamylase proteins by a number of workers (Boel *et al*., 1984; Wingard Jr *et al*., 1979). The 61 KDa protein identified in the culture filtrates of *Aspergillus niger* used in this study may be the possible protein responsible for the glucoamylase activity in the culture filtrates. Proteins identified in culture filtrates of the *Rhizopus* species used in this study had molecular weights 96.40, 65.56, 51.80, 29.05 and 19.75 KDa. Norouzian *et al*., (2006) identified glucoamylases of molecular weights 96.50, 66.00 and 52.00 KDa from the culture filtrates produced by the *Rhizopus* species they worked with. It is therefore possible that the proteins with molecular weights of 96.40 and 65.56 KDa identified in the culture filtrates of the *Rhizopus* species used in this study were responsible for the detected glucoamylase activity.

Measured Kmax and Vmax values for glucoamylase produced from *Aspergillus niger* in this study are 0.0009548 g/l and 2.387 g/l. min respectively. For *Rhizopus* species, the measured Kmax and Vmax values are 0.0007443 g/l and 2.481 g/l. min respectively. The higher value of Kmax for glucoamylase of *Aspergillus niger* means glucoamylase produced by *Aspergillus niger* has a lower binding affinity for starch than that produced by *Rhizopus* species. Therefore, at maximum velocity of the glucoamylase-starch reaction, glucoamylase of *Aspergillus niger* would contain more starch molecules than that of *Rhizopus* species. Hence Kcat or turnover number or maximum number of starch molecules converted to glucose per active site of glucoamylase enzyme per unit time when the glucoamylase enzyme of *Aspergillus niger* is saturated is expected to be higher than that of *Rhizopus* species. Therefore, glucoamylase activity of *Aspergillus niger* is expected to be higher. Some reported Kmax values for *Aspergillus niger* and *Rhizopus* species are 3.5 g/l (Selvakumar *et al*., 1996) and 12.2 g/l (Suntornsuk and Hang, 1997) respectively.

Chapter 6

6.0 Conclusions

In conclusion, the studies showed that glucoamylase can be produced by the four native fungal isolates, *Aspergillus niger*, *Aspergillus flavus*, *Rhizopus* species and *Fusarium oxysporum*. The work showed that all the five solid substrates - wheat bran, rice bran, groundnut pod, maize bran and cocoa pod could potentially be used for the production of glucoamylase by Solid-State Fermentation process with wheat bran being the best among them. The studies pointed out that the nature of substrate, incubation period, incubation temperature, pH of the culture medium and the kind of microorganism used all affected production of glucoamylase in Solid-State Fermentation. The results, however, provided valuable information for production of glucoamylase using *Aspergillus niger* and *Rhizopus* species on wheat bran. Glucoamylase production using *Aspergillus niger* was found to be affected by nitrogen. Under optimum growth conditions, *Aspergillus niger* yielded maximum enzyme activity of 6.66 U/ml in 18 hours of incubation period at incubation temperature of 40ºC, nitrogen concentration of 0.2 g/l and at pH 5.0. Whereas glucoamylase produced from *Rhizopus* species gave maximum enzyme activity of 4.44 U/ml in 18 hours of incubation period at incubation temperature of 40ºC and at pH 4.5. Molecular weights of proteins determined by SDS-PAGE of glucoamylase of *Rhizopus* species were 96.40, 65.56, 51.80, 29.05 and 19.75 KDa whilst those of *Aspergillus niger* were 61.48, 29.68, 21.06 and 12.33 KDa. Kinetic studies also revealed Kmax = 0.0009548 g/l and Vmax = 2.387 g/l. min for crude culture filtrate of *Aspergillus niger* and Kmax = 0.0007443 g/l and Vmax = 2.481 g/l. min for crude culture filtrate of *Rhizopus* species.

6.1 Recommendations

(a) *Aspergillus niger* and *Rhizopus* species used in this work have shown that they have the capacity to produce reasonable quantities of glucoamylase on small substrate sizes. It is recommended that a scaling up of the process to increase the production of glucoamylase by these organisms receives important attention. After scaling up studies, the two organisms can be used to produce economic quantities of glucoamylase for industrial fermentation.

(b) Wheat bran was the best substrate identified in this study. However, it is recommended that any future work should pay more attention to rice bran because it is abundant, locally available at cheaper cost.

KNUST

References

Aiyer, P. V., Arts, S. P. T., and College, S.; (2005). Amylases and their Applications. African Journal of Biotechnology, 4: 1525–1529.

Anto, H., Trivedi, U.P., Patel, K.C.; (2006).Glucoamylase Production by Solid-State Fermentation using Rice Flake Manufacturing Waste Products as Substrate. Bioresource Technology, 97: 1161-1166.

Bahl, B.S., Tuli, G.D., and Arun, Bahl; (1997). Essentials of Physical Chemistry. Bahl, B.S., Tuli, G.D., and Arun, Bahl (24thEd.). Volume 2, pp. 747-750.

Bairoch, A.; (2000). The Enzyme Database in 2000. Nucleic Acids Research, 28(1):304-5.

Banks, G.T., Binns, F., and Cutcliffe, R.L.; (1976). Recent Development in the Production and Industrial Applications of Amylolytic Enzymes Derived from Filamentous Fungi. Progress Industrial Microbiology, 6: 95-139.

Barton, L. L., Georgi C. E., and Lineback, D. R.; (1972). Effect of Maltose on Glucoamylase Formation by *Aspergillus niger*. Journal of Bacteriology, 111(3): 771–7.

Baysal, Z., Uyar, F., and Aytekin, C.; (2003). Solid-State Fermentation for Production of α-amylase by a Thermotolerant *Bacillus Subtilis* from Hot-Spring Water. Process Biochemistry, 38: 1665–1668.

Bennett, T. P., and Frieden, E.; (1969). Modern Topics in Biochemistry, pg. 43-45. Macmillan, London.

Bertolin, T.E., Schmidell, W., Maiorano, A.E., Casara, J., and Costa, J. A.; (2003). Influence of Carbon, Nitrogen and Phosphorus Sources on Glucoamylase Production by *Aspergillus awamori* in Solid-State Fermentation. Z. Naturforsch. C, 58: 708 – 712.

Bhargav, S., Panda, B. P., Ali, M., and Javed, S.; (2008). Solid-State Fermentation: An Overview. Chemical Biochemical Engineering, 22 (1):49–70.

Bhatti, H. N., Rashid, M. H., and Nawaz, R.; (2007). Optimization of Media for Enhanced Glucoamylase Production in Solid-State Fermentation by *Fusarium solani*. Food Technology and Biotechnology, 45(1): 51–56.

Biesebeke, Te, Record,R. E., Biezen,van N., Heerikhuisen,M., Franken, A., Punt, P.J., and Hondel,C.A.; (2005). Branching Mutants of *Aspergillus oryzae* with Improved Amylase and Protease Production on Solid Substrates. Applied Microbiology Biotechnology, 69: 44-50.

Boel, E., Hansen, M.T., Hjort, I., Hoegh, I., and Fill, N.P.; (1984). Two Different Types of Iintervening Sequences in the Glucoamylase Gene from *Aspergillus niger*. Embo Journal, 3: 1581-1585.

Boel, E., Hjort, I., Svensson, B., Norris, F. K.E., and Fiil, N.P.; (1984). Glucoamylase G1and G2 from *Aspergillus niger* are Synthesized from Two Different but Closely Related mRAs. Embo Journal, 3: 1097-1102.

Bolster, De, M.W.G.; (1997). Glossary of Terms Used in Bioinorganic Chemistry. International Union of Pure and Applied Chemistry. European Journal of Biochemistry, 69: 1251-1303.

Bolt, M.W., and Mahoney, P.A.; (1997). High-Efficiency Blotting of Proteins of Diverse Sizes Following Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis. Analytical Biochemistry, 247(2):185–92.

Boyer, and Rodney; (2002). "6". Concepts in Biochemistry (2nd ed.). New York, Chichester, Weinheim, Brisbane, Singapore, Toronto.; John Wiley and Sons, Inc.. pp. 137–8.

Byrne, G. S., and Ward, O. P.; (1989). Effect of Nutrition by Pellet Formation by *Rhizopus arrhizus*. Biotechnology and Bioengineering, 33 (7): 912–914.

Campos, L., and Félix, R.C.;(1995). Purification and Characterization of Glucoamylase from *Humicol agrisea*. Applied Environmental Microbiology, 61(6): 2436-2438.

Cereia, M., Guimarães, L. H. S., Peixoto-Nogueira, S. C., Jorge, J. A., Terenzi, H. F., Greene, L. J., and Polizeli, M. D. L. T. M.; (2006). Glucoamylase Isoform (GAII) Purified from a Thermophilic Fungus *Scytalidium thermophilum* 15.8 with Biotechnological Potential. African Journal of Biotechnology, 5: 1239–1245.

Chang, Raymond; (2005). Physical Chemistry for the Biosciences. Sausalito (CA): University Science Books. p. 311-347.

Chen, L.H., Kenyon, G.L., Curtin, F., Harayama, S., Bembenek, M.E., Hajipour, G., and Whitman, C.P.; (1992). 4-Oxalocrotonate Tautomerase, an Enzyme Composed of 62 Amino Acid Residues Per Monomer. Journal Biological Chemistry, 267 (25): 17716–21.

Coutinho, P.M., and Reilly, P.J.; (1997). Glucoamylase, Structural, Functional and Evolutionary Relationships. Biochemistry and Molecular Biology, 29: 334-347.

Couto, S., Rodrıguez, M., and Sanroman, A.; (2006). Application of Solid-State Fermentation to Food Industry. Journal of Food Engineering, 76: 291–302.

KNUST

Deshmukh, Kshipra, B., Sandeepsingh, A., Taur, Makrand, N., Cherekar, Mangesh, N., Kothari, and Anupama, Pathak, P.; (2011).Process Optimization, Purification and Characterization of Glucoamylase from Different *Sorghum* Varieties. Journal of Chemistry and Pharmacy Research, 3(2):732-737.

Didier, Azard, and Jacquesf, Baldensper; (1982). Enzymes from *Aspergillus izenrzebergi* (*A. niger* group): Purification and Characterization of Amylases from Solid and Liquid Cultures. Carbohydrate Research, 107: 231-241.

Ellaiah, P., Adinarayana, K., Bhavani, Y., Padmaja, P., and Srinivasulu, B.; (2002). Optimization of Process Parameters for Glucoamylase Production Under Solid State Fermentation by a Newly Isolated *Aspergillus* species. Process Biochemistry, 38(4): 615–620.

Fagerstrom, Richard, Arja, Vainio, Kari, Suoranta, Tina, Pakula, Nisse, Kalkkinen, and Helena, Torkkeli; (1990). Comparison of Two Glucoamylases from Hormoconis resinae. Journal of General Microbiology, 136: 913-920.

Fazakes de St Groth, S.; (1963). A Procedure for Rapid and Sensitive Staining of Protein. Biochemistry and Biophysics, 71:377-391.

Fersht, Alan; (1985). Enzyme Structure and Mechanism (2nd Ed.). San Francisco: W.H. Freeman. pp. 50–2.

Fisher, Z., Hernandez, Prada, J.A., Tu, C., Duda, D., Yoshioka C. H., Govindasamy, L., Silverman, D.N., and McKenna, R.;(2005). Structural and Kinetic Characterization of Active-Site Histidine as a Proton Shuttle in Catalysis by Human Carbonic Anhydrase II. Journal of Biochemistry, 44 (4): 109–115.

KNUST

Fogarty, W.M. and Kelly, C.T.; (1980). Amylases, Amyloglucosidases and Related Glucanases. In: Microbial Enzymes and Bioconversions (Ed. Rose, A. H.,). London, Academic Press, pp. 115-170.

Fujio, Y., and Morita, H.; (1996). Improved Glucoamylase Production by *Rhizopus* species A-l 1 Using Metal-Ion Supplemented Liquid Medium. Journal of Fermentation Bioengineering, 82(6): 554–557.

Garrett, R.H., and Grisham, C.M.; (1999). Enzyme Kinetics (2nd Ed.). Biochemistry.Saunders College Publishing, Orlando, Florida. pp. 426–427.

Ge´linas, P., and Barrette, J.; (2006). Short Communication Protein Enrichment of Potato Processing Waste Through Yeast Fermentation. Bioresource Technology, 98(5):1138-43

Grajek,W ., and Grevais,P.; (1987). Influence of Water Activity on the Enzyme Biosynthesis and Enzyme Activities Produced by *Trichoderma viride* TS in solid-state fermentation. Enzyme and Microbiol Technology, 9: 658-662.

Grisham, Charles, Reginald, M., Garrett, H.; (1999). Biochemistry: Handbook of Amylases and Related Enzymes (2nd Ed.). Saunders College Publications. Pergamon Press, Oxford, pp. 117–120.

Hata, Y., Ishida, H., Kojima, Y., Ichikawa, E. K. A., Suginami, K., and Imayasu, S.; (1997). Comparison of Two Glucoamylases Produced by *Aspergillus oryzae* in Solid-State Culture and in Submerged Culture. Journal of Fermentation and Bioengineering, 84: 532-537.

Hesseltine, C. W., Doelle,; (1977). Solid State Fermentation-Part 1.Process Biochemistry, 12: 24-27.

Horváthová, V., Janeček, Š., and Šturdík, E.; (2001). Amylolytic Enzymes : Molecular Aspects of Their Properties. General Physiology and Biophysics, 20: 7–32.

Huang, M. Y., Bungay, H. R.;(2004). Microprobe Measurements of Oxygen Concentrations in Mycelial Pellets. Biotechnology and Bioengineering, 24:1193– 1197.

Ibba, M., and Soll, D.; (2000). Aminoacyl-tRNA Synthesis. Annual Review of Biochemistry, 69: 617–50.

Imai, Y., Suzuki, M., Masamoto, M., and Nagayasu, K.; (1994).Glucoamylase Production of *Aspergillus oryzae* in Fed-Batch Culture Using a Statistical Regression Model. Journal of Fermentation and Bioengineering, 78(4): 310–314.

Irfan, Muhammad, Muhammad, Nadeem, and Quratualain, Syed; (2012). Media Optimization for Amylase Production in Solid State Fermentation of Wheat Bran by Fungal Strains. Journal of Cell and Molecular Biology, 10(1): 55-64.

Jaeger, K.E., and Eggert, T.; (2004). Enantioselective Biocatalysis Optimized by Directed Evolution. Current Opinion in Biotechnology, 15 (4): 305–13.

Jafari-Aghdam, J., Khajek, K., Ranjbar, B., and Nemat-Gorgani, M.; (2005). Deglycosylation of Glucoamylase from *Aspergillus niger*: Effects on Structure, Activity and Stability. Biochemistry and Biophysics, 1750: 61-68.

James, J.A., and Lee, B.H.; (1997). Glucoamylases: Microbial Sources. Industrial and Molecular Biology. Journal of Food Biochemistry, 21:1-52.

KNUST

Joshi, V.K., Pandey, A., and Sandhu, D.K.; (1999). Waste Treatments in Fermentation Technology. Pakistan Journal of Botany, 2:1291-1338.

Kareem, S. O., Akpan, I., Popoola,T. O. S., and Sanni,L. O.; (2011). Activated Charcoal - A Potential Material in Glucoamylase Recovery. Enzyme Research Vol. 2011.

Kaur, P., Grewal, H.S., and Kocher, G.S.; (2003). Production of α-amylase by *Aspergillus niger* using Wheat Bran in Submerged and Solid State Fermentations. Indian Journal of Microbiology, 43: 143–145.

Konig, B., Schugerl, K., and Seewald, C.; (1982). Strategies for Penicillin Fermentation in Tower-Loop Reactors. Biotechnology and Bioengineering, 24 (2): 259–280.

Koshland, D.E.;(1958). Application of a Theory of Enzyme Specificity to Protein Synthesis. Process National Academic Science, 44(2): 98-104.

Koukab, Rana, Ikram-ul-Haq, and Hamad, Ashraf; (2005). Production of Amyloglucosidase by UV Irradiated Strain of *Aspergillus niger*. Using Solid State Fermentation. Journal of Food Technology, 3(1): 68-71.

Kubicek, C.P., and Rohr, M.; (1986). Citric Acid Fermentation. Critical Reviews in Biotechnology, 3(4):331-373.

Liao, Wei, Yan, Liu, Craig, Frear, and Shulin, Chen; (2007). A New Approach of Pellet Formation of a Filamentous Fungus – *Rhizopus oryzae*. BioresourceTechnology, 98:3415–3423.

Madihah, M.S., Ariff, A.B., Khalil,M.S., Suraini,A.A., and Karim,M.I.A.; (2000). Partial Purification and Some Properties of α-amylase and Glucoamylase obtained as By-Product from Direct Fermentation of Sago Starch to Solvent by Clostridium acetobutylicum. Pakistan Journal of Biological Science, 3:744-749.

Mattey, M.; (1992).The Production of Organic Acids. Critical Reviews in Biotechnology, 12 (1-2):87-132.

Meijer, Susan; (2008). Creation of an Organic Acid Production Platform in *Aspergillus niger* Using a System-Level Approach. Applied Microbiology and Biotechnology.

Haiyan, S., Xiangyang, G., Lu, W., Pingjuan, Z., and Ming P.; (2009). Microbial Production of Raw Starch Digesting Enzymes. African Journal of Biotechnology, 8 (9): 1734-1739.

Miller, Gail, Lorenz; (1959). Use of Dinitrosalicylic Acid Reagent for Determination of Reducing Sugar.Analytical Chemistry, 31 (3): 426–428.

Mishra, B.K., Anju, and Arora, Lata; (2004). Optimization of a Biological Process for Treating Potato Chips Industry Wastewater Using a Mixed Culture of *Aspergillus foetidus* and *Aspergillus niger*. Bioresource Technology. 94: 9–12.

Mitra, P., and Chandra, A. L. (1994). Solid State Fermentation (ed. Pandey, A.), Wiley Eastern Publishers, New Delhi, pp. 134–136.

Monique, A., Mennink-Kersten, S. H., Peter, Donnelly, J., and Paul, Verweij, E.; (2004). Detection of Circulating Galactomannan for the Diagnosis and Management of Invasive Aspergillosis. Lancet Infect Dis., 4(6): 349-57.

Moo-Young, M., Moreira, A. R., Tengerdy, R. P.; (1983). Principles of Solid-Substrate Fermentation, In: JE Smith, DR Berry, B. Kristiansen (ed), The filamentous fungi, vol. 4 (fungal Technology), Edward Anold, London, pp 117-144.

Mudgett, R. E.; (1986). Solid-State Fermentations. In: A.L. Demain and N.A. Solomon (eds). Manual of Industrial Microbiology and Biotechnology. American Society for Microbiology, Washington, DC, pp. 66-83.

Narahara,H., J.; (1997). Solid-State Fermentation: An Overview. Fermentation Technology, 55:254-256.

Norouzian, D., Akbarzadeh, A., Scharer, J. M., and Moo-Young,M.; (2006).Fungal Glucoamylases. Biotechnology Advances, 24(1): 80 – 85.

Oberoi, Harinder, Singh, Krishan, L., Kalra, Dewinder, Singh, Uppal, and Sanjeev, Kumar, Tyagi; (2007). Effects of Different Drying Methods of Cauliflower Waste on Drying time, Colour Retention and Glucoamylase Production by *Aspergillus niger* NCIM 1054. International Journal of Food Science and Technology, 42: 228–234.

Ocloo, F. C. K., and Ayernor, G. S.; (2010). Production of Alcohol from Cassava Flour Hydrolysate. Journal of Brewing and Distilling, 1(2): 15–21.

Oriol, E., Raimbault, M., Roussons, S., Gonzales, G. V.; (1988). Applied Microbiology. Journal of Biotechnology, 27: 498-503.

Oznur, Koc, and Kubilay, Metin ; (2010). Purification and Characterization of a Thermostable Glucoamylase Produced by *Aspergillus flavus* HBF34. African Journal of Biotechnology, 9(23): 3414-3424.

KNUST

Pandey, A., Selvakumar, P., Ashakumary, L.; (1994).Glucoamylase Production by *Aspergillus niger* on Rice Bran is Improved by Adding Nitrogen Sources. World Journal of Microbiology and Biotechnology, 10 (3): 348-349.

Pandey, A., Nigam, P., Soccol, C., Soccol, V., Singh, D., Mohan, R.; (2000) Advances in Microbial Amylases. Biotechnology and Applied Biochemistry, 31: 135–152.

Pandey, A., Radhakrishna, S.; (1993). The Production of Glucoamylase by *Aspergillus niger* NCIM 1245. Process Biochemistry, 28(3):305-9.

Pandey, A., Selvakumar, P., and Ashakumary, L.; (1996). Performance of a Column Bioreactor for Glucoamylase Synthesis by *Aspergillus niger* in Solid State Fermentation. Process Biochemistry, 3(1): 43–46.

Pandey, A., Selvakumar, P., Soccol, C.R., and Nigam, P.; (1999). Solid State Fermentation for the Production of Industrial Enzymes.Current Science, 77(1):149 - 62.

Pandey, Ashok, and Radhakrishnan, S.; (1992). Packed-bed Column Bioreactor for Production of Enzyme. Enzyme Microbiology Technology, 14: 486 - 488.

Pandey, Ashok, Selvakumar, P., Carlos, Soccol, R., and Poonam, Nigam; (1990). Solid State Fermentation for the Production of Industrial Enzymes. Current Science, 34:11-19.

Pandey, Ashok, Selvakumar,P., Carlos, Soccol, R., and Poonam, Nigam; (1991).Solid StateFermentation for the Production of Industrial Enzymes. Bioresearch Technology, 37: 169–172.

Pandey, Ashok; (1991). Aspects of Design of Fermenter in Solid State Fermentation. Process Biochemistry, 26: 355-361.

KNUST

Papagianni, M..; (2004). Fungal Morphology and Metabolite Production in Submerged Mycelia Processes. Biotechnology Advances, 22 (3):189-259.

Papagianni, M., Mattey, M., and Kristiansen, B.; (1994). Morphology and Citric Acid Production of *Aspergillus niger* PM 1. Biotechnology Letters, 16 (9): 929-934.

Paulchamy, C.; (2008). Solid-State Cultivation of *Aspergillus niger* NCIM 548 for Glucoamylase Production on Groundnut Shell. Journal of Microbiology, 5: 1-6.

Pavezzi, F. C., Gomes, E., and Silva, R.; (2008).Production and Characterization of Glucoamylase from Fungus *Aspergillus awamori* Expressed in Yeast Saccharomyces Cerevisiae using Different Carbon Sources. Brazilian Journal of Microbiology, 39:108-114.

Pazur, J.H., Liu, B.L., and Miskiel, F.J.; (2010). Comparison of the Properties of Glucoamylases from *Rhizopus niveus* and *Aspergillus niger*. Biotechnology and Applied Biochemistry, 12: 63–78.

Pedersen, H., and Nielsen, J.; (1999). The Influence of Nitrogen Sources on the α-amylase Productivity of *Aspergillus oryzae* in Continuous Cultures. Applied Microbiology and Biotechnology, 53(3): 278-281.

Peter, van de Vondervoort,J.I.;(2007). Mutagenesis and Mitotic Recombination in *Aspergillus niger*, An Expedition from Gene to Genome. Production on Agro residues.International Journal of Life Sciences, 4: 16–25.

Prosser, J. I.; (1994). Kinetics of Filamentous Growth and Branching, In: Grow NA and Gadd GM (eds.). The Growing Fungus, Champman and Hall, London, UK, pp. 301-318.

KNUST

Raghavarao, K.S.M.S., Ranganathan, T.V., and Karanth, N.G.; (2003). Some Engineering Aspects of Solid State Fermentation. Journal of Biochemistry Engineering, 13:127–35.

Ramesh, M. V., and Lonsane, B. K.; (1990). Critical Importance of Moisture Content in Amylase Production by *Bacillus licheniformis* M 27 in Solid State Fermentation. Applied Microbiology and Biotechnology, 33: 501–505.

Reisner, A. H., Nemes, P., and Bucholtz, C.; (1975). A Rapid and Sensitive Method for the Quantitation of Microgram Quantities of Protein Utilizing the Principle of Protein-Dye Binding. Analytical Biochemistry, 64: 509-516.

Robert, Sabin,D., and Bruce, Wasserman,P.; (1987). A Continuous Spectrophotometric Screening Assay for Glucoamylase. Journal of Agricultural Food Chemistry, 35: 649-651.

Rodnina, M.V., and Wintermeyer, W.; (2001). Fidelity of Aminoacyl-tRNA Selection on the Ribosome: Kinetic and Structural Mechanisms. Annual Review of Biochemistry, 70: 415–35.

Rose, H.A., (1980). Microbial Enzymes and Bioconversions. Economic Microbiology, 5: 116–117.

Roskoski, Robert; (2007). Michaelis-Menten Kinetics. Biochemical Biophysical Research Communications, 1-10.

Ruijter, G. J. G., Van de Vondervoort, P.J . I., and Visser, J.; (1999). Oxalic Acid Production by *Aspergillus niger*: An Oxalate-Non-Producing Mutant Produces Citric Acid at pH 5 and in the Presence of Manganese. Microbiology, 145:2569-2576.

Saha, B.C., and Zeikus, J.G.; (1989). Microbial Glucoamylases. Brazilian Journal of Chemical Engineering, 41(2): 57–64.

KNUST

Savir, Y., and Tlusty, T., Scalas, Enrico: (2007). Conformational Proof reading: The Impact of Conformational Changes on the Specificity of Molecular Recognition. 2(5).

Sedmack, J. J., and Grossberg, S. E.; (1977). A rapid, Sensitive and Versatile Assay for Protein using Coomassie. Analytical Biochemistry, 79: 544 -552.

Selvakumar, Ashakumary, L., P., and Pandey, Ashok; (1994). Solid State Fermentation. (Ed. Pandey, A.), Wiley Eastern Publishers, New Delhi, pp. 33–37.

Selvakumar, P., Ashakumary, L., and Pandey, Ashok; (1996). Solid State Fermentation for the Production of Industrial Enzymes. Journal of Scientific and Industrial Research, 55: 443-449.

Selvakumar, P., Ashakumary, L., and Pandey, Ashok; (1998). Biosynthesis of Glucoamylase from *Aspergillus niger* by Solid-State Fermentation using Tea Waste as the Basis of a Solid Substrate. Bioresearch Technology, 65: 83–85.

Shevelev, IV, Hubscher, U.; (2002).The 3' 5' Exonucleases.National Review Molecular Cell Biology. 3 (5): 364–76.

Shivaramakrishnam, S., Ganghadharan, D., Nampoothiri, K.M., Soccol C.R., and Pandey, A. ; (2007). Alpha Amylase Production by *Aspergillus oryzae* Employing Solid-State Fermentation. Journal of Scientific and Industrial Research, 66: 621-626.

Shuler, Michael, L. and Kargi, Fikret; (2002).Bioprocess Engineering (2nd Ed.). Basic Concepts. pp. 57 – 96.

KNUST

Singh, H., and Soni, S.K.; (2001). Production of Starch-Gel Digesting Amyloglucosidase by *Aspergillus oryzae* HS-3 in Solid State Fermentation. Process Biochemistry, 37: 453–459.

Smith, A.L.; (1997). Dictionary of Biochemistry and Molecular Biology (4th Ed.). Oxford [Oxfordshire]: Oxford University Press. p.508.

Smith, S.; (1994). The Animal Fatty Acid Synthase: One Gene, One Polypeptide, Seven Enzymes. Journals of Life Science, 8 (15): 1248–59.

Suganthi, R., Benazir, J.F., Santhi, R., Ramesh, Kumar, V., Anjana Hari Nitya, Meenakshi, Nidhiya, K. A., Kavitha, G., and Lakshmi, R.; (2011). Amylase Production by *Aspergillus niger* Under Solid State Fermentation using Agro Industrial Wastes. International Journal of Engineering Science and Technology, 3(2).

Sun, Haiyan, Xiangyang,Ge, Lu, Wang, Pingjuan, Zhao, and Ming, Peng; (2009).Microbial Production of Raw Starch Digesting Enzymes. African Journal of Biotechnology, 8 (9) : 1734-1739.

Suntornsuk, W., and Hang,Y. D.; (1997).Purification and Characterization of Glucoamylase from *Rhizopus oryzae* Mutant. Journal of Science Society, 23: 199-208.

Svensson, B., Pedersen, T. G., Svendsen, I., Sakai, T., and Ottesen, M.; (1982). Production and Purification of a Granular-Starch-Binding Domain of Glucoamylase 1 from *Aspergillus niger*. Carlsberg Research Communications, 47, 55-69.

KNUST

Swift, R. J., Karandikar, A., Griffen, M., Punt, P. J., Van den Hondel, C. A., Robson, G. D., Trinci, P.; (2000). The Effect of Organic Nitrogen Sources on Recombinant Glucoamylase Production by *Aspergillus niger* in Chemostat Culture. Fungal Genetics and Biology, 31(2): 125–33.

Takahashi, T., Kato, K., Ikegami, Y., and Irie, M.; (1985).Production and Purification of a Granular-Starch-Binding Domain of Glucoamylase 1 from *Aspergillus niger*. Journal of Biochemistry, 98: 663-671.

Takahashi, T., Tsuchida, Y., and Irie, M.; (1982). Glucoamylases G1 and G2 from *Aspergillus niger* are Synthesized from Two Different but Closely Related mRNAs. Journal of Biochemistry, 92: 1623-1633.

Tengerdy, R. P.; (1996). Bioreactors for Solid State Fermentation of Lignocellulosics. Journal of Scientific and Industrial Research, 55: 313-316.

Tymoczko, John, L., Stryer, Berg, Tymoczko, Stryer, Lubert, Berg, Jeremy, Mark; (2002).Biochemistry. San Francisco: W.H. Freeman. ISBN0-7167-4955-6.

Varalakshmi, K.N., Kumudini, B.S., Nandini, B.N., Solomon J., Suhas, R., Mahesh, B., and Kavitha, A.P.; (2009). Production and Characterization of α-amylase from *Aspergillus niger* JGI 24 Isolated in Bangalore.Polish Journal of Microbiology, 58: 29-36.

Vasella, A., Davies, G.J., and Bohm, M.; (2002). Glycosidase Mechanisms. Current Opinion in Chemical Biology, 5: 619–29.

Wadhwa, M., Kaushal, S.S., and Bakshi, M.P.S.; (2005).Nutritive Evaluation of Vegetable Wastes as Complete Feed for Goat Buck. Small Ruminant Research, 64 (3): 197-310.

KNUST

Wang, X.J., Bai,G., and Liang,Y. X.; (2006).Optimization of Multienzyme Production by Two Mixed Strains in Solid-State Fermentation.Applied Microbiology and Biotechnology, 73: 533 – 540.

Ward, O. P.; (1988). Fermentation Biotechnology: Principles, Processes and Products (ed. A). Wiseman, Chapman and Hall, New York,1988, p. 5.

Wessel, D., and Flugge, U.I.; (1984). A Method for the Quantitative Recovery of Protein in Dilute Solution in the Presence of Detergents and Lipids. Analytical Biochemistry, 138: 141 – 143.

Wingard, Jr, L.B., Katchalski-Katzir, Golstein, L., Aunstrup, K.; (1979). Production, Isolation and Economics of Extracellular Enzymes.Applied Biochemistry. Bioengineering.Volume 2. Academic Press, New York.

Zambare, V.; (2010). Solid State Fermentation of *Aspergillus oryzae* for Glucoamylase Production on Agro residues. International Journal of Life Science, 4:16-25.

Zaldivar-Aguero J.M., Badino Jr. AC, Vilaça PR, Facciotti MCR and Schmidell W (1997) Influence of phosphate concentrations on glucoamylase production by *Aspergillus awamori* in submerged culture. Brazilian Journal of Chemical Engineering, 14:1-4

KNUST

Appendices

Appendix 1

Aspergillus Complete Medium

Composition of one litre ACM in (g/L) is:

$(NH_4)_2SO_4$: 5.0; $Na_2HPO_4 \cdot 12H_2O$: 3.8; KH_2PO_4 3.5; $MgSO_4$ 0.5and yeast extract 0.1 at pH 5.0.

KNUST

Appendix 2

Mineral Salts Solution for Growing *Rhizopus* species

Composition of one litre (1L) of mineral salts solution for growing *Rhizopus* species in (g/L) is:

$(NH_4)_2SO_4$ 20.0; K_2HPO_4 3.0; NaCl 5.0; $MgSO_4$ 5.0; Na_2HPO_4 2.0 and $CaCl_2$ 1.0 at pH 4.5.

Appendix 3

Mineral Salts Solution for Growing *Fusarium oxysporum*

Composition of one litre (1L) of mineral salts solution for growing *Fusarium oxysporum* in (g/L) is: Trisodium Citrate 2.5; $(NH_4)_2SO_4$ 4.0; NH_4NO_3 2.0; KH_2PO_4 5.0 and $MgSO_4 \cdot 7H_2O$ 0.2 at pH 5.0.

Appendix 4

Citric Acid Buffer

Composition of one litre (1L) of Citric Acid buffer solution in (g/L) is:

Citric Acid 4.01 and Sodium Citrate 8.56 at pH 4.6.

Appendix 5

KNUST

3, 5-Dinitrosalicylic acid (DNS) Reagent

DNS reagent was prepared by adding 300g salts of sodium potassium tartrate dissolved in 500 ml of distilled water to 10 g salts of 3, 5-Dinitrosalicylic acid (DNS) dissolved in 200 ml of 2M NaOH solution and the total volume made to 1000 ml with distilled water.

Appendix 6

Concentrations of BSA prepared by Dilution

mg/ml	2.0	1.0	0.5	0.25	0.125	0.0625	0.03125	0.015625	0.0078125

Appendix 7

Preparation of Bio-Rad Protein Assay Dye Reagent Concentrate (Dye Reagent)

Dye reagent was prepared by diluting one (1) part Bio-Rad Protein Assay Dye Reagent Concentrate with four (4) parts distilled deionized water. The reagent obtained was filtered through Whatman filter to remove particulates and kept at room temperature.

Appendix 8

Loading Buffer

Laemmli loading buffer (2X) was prepared by adding the following together: 9.5 ml of distilled water, 2.5 ml of 0.5 M Tris-HCl (pH 6.8), 2.0 ml of Glycerol, 4.0 ml of 10% (w/v) SDS (Sodium dodecyl Sulphate), 1.0 ml of 2-Mercaptoethanol (added under hood) and 1.0 ml of 1.5% (w/v) Bromophenol Blue. The final mixture obtained was stirred and the pH adjusted to 6.8 using 2M HCl and 2M NaOH.

KNUST

Appendix 9

Running Buffer

Tris-glycine buffer (1X) was prepared by putting the following together: 2.120 g of Tris Base (25 mM Tris base; MW 121.14g), 9.984 g of Glycine (190 mM glycine; MW 75.07g) and 0.7g of SDS (0.1% W/V; MW 288.38g). Sterilized distilled water was added and stirred to form a homogeneous solution. The total volume was made up to 1000 ml and the pH adjusted to 8.3 using 2M HCl and 2M NaOH.

Appendix 10

Cast Gel

The cast gel contained separating and stacking gels. The separating gel was first cast followed by the stacking gel to form the cast gel. Separating and stacking gels were prepared as follows:

Separating Gel:

A 10% separating gel was prepared by adding the following together: 2.5 ml of 40% Acrylamide/Bis (29:1), 2.5 ml of 1.5M Tris-HCl (pH 8.8), 4.8 ml of distilled water and 100 µl of 10% SDS and mixture obtained stirred to achieve homogeneity and degassed. 100 µl of 10% APS and 10 µl of TEMED solutions were added simultaneously to the homogeneous solution and quickly placed in the gel caster (Bolt and Mahoney, 1997).

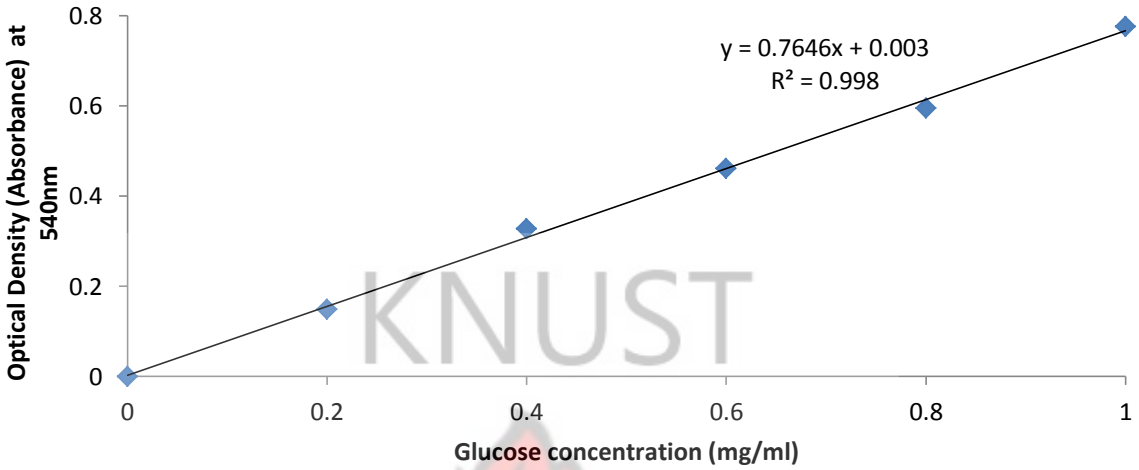
KNUST

Stacking Gel:

A 4% stacking gel was prepared by adding the following together: 1.0 ml of 40% Acrylamide/Bis (29:1), 2.5 ml of 0.5M Tris-HCl (pH 6.8), 6.4 ml of distilled water and 100 µl of 10% SDS. The mixture produced was stirred thoroughly to achieve homogeneity and degassed. 100 µl of 10% APS and 10 µl of TEMED solutions were added simultaneously and the final solution obtained placed on top of the polymerized separating gel in the gel caster and the comb fixed (Bolt and Mahoney, 1997).

Appendix 11

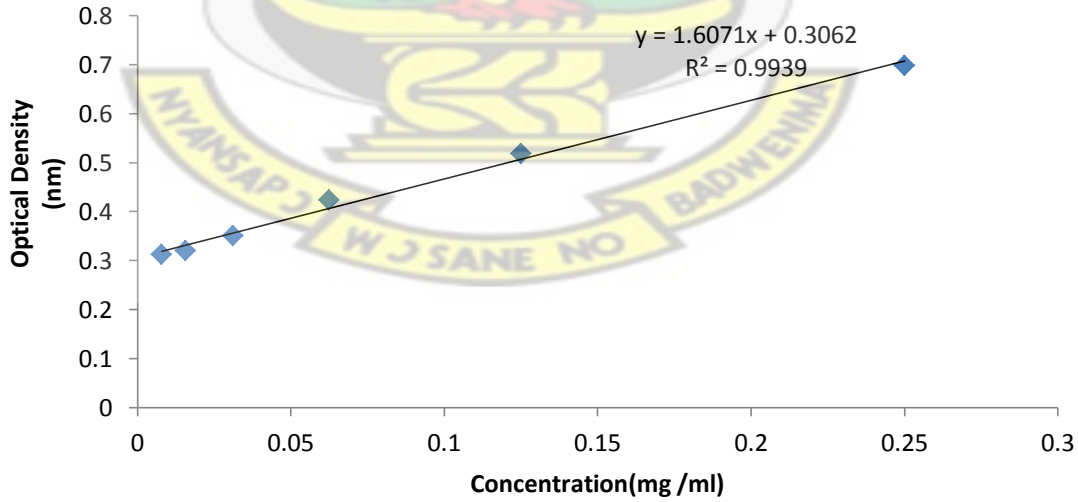
Standard Glucose Curve produced after (Miller, 1959)



KNUST

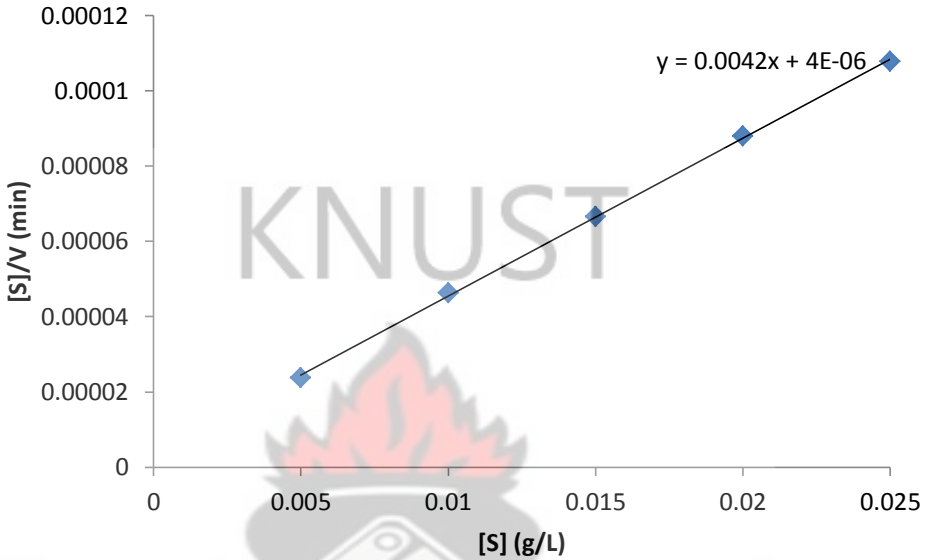
Appendix 12

Standard BSA Curve produced after (Bradford *et al.*, 1976)



Appendix 13

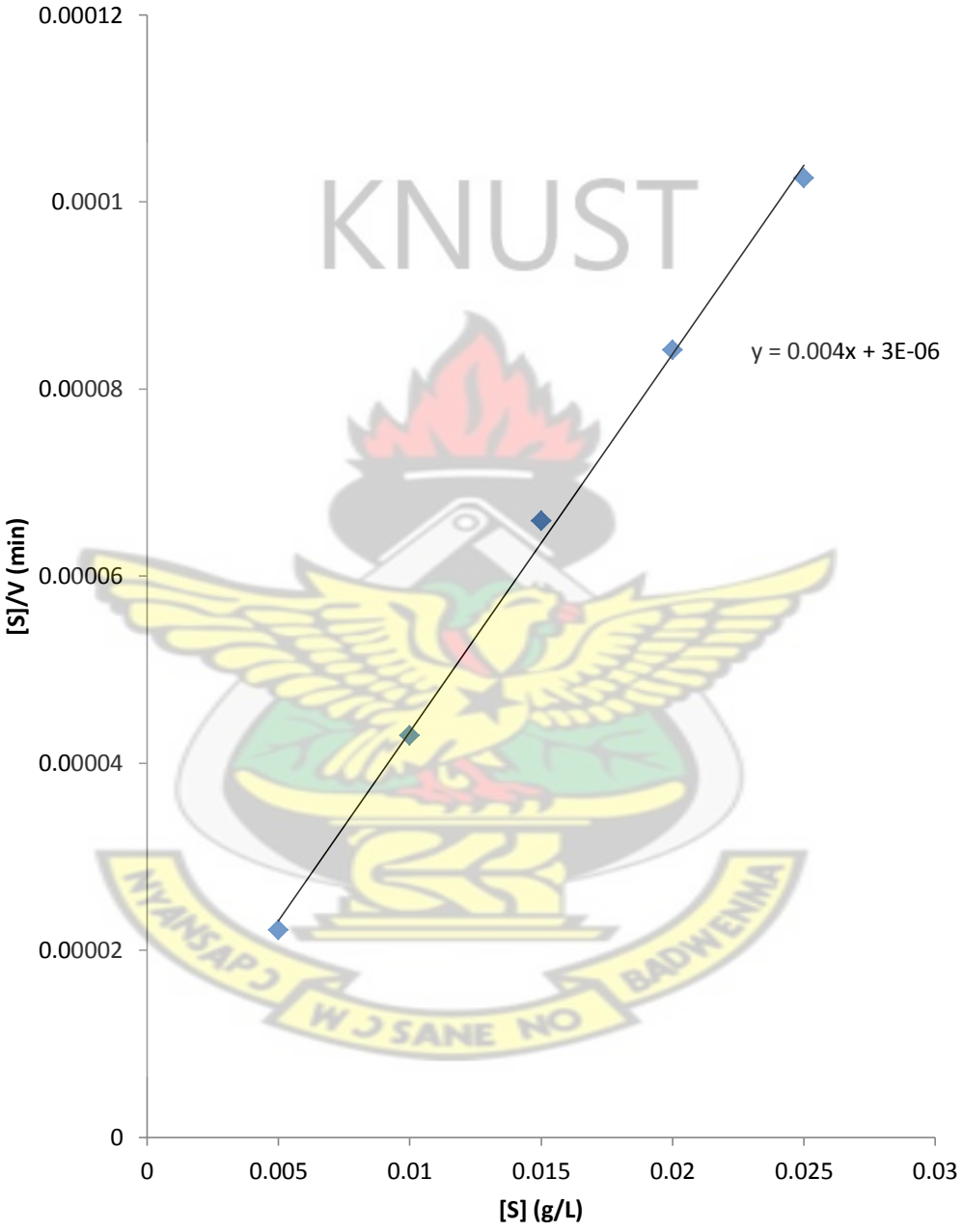
Hanes-Woolf's plot to determine Vmax and Kmax for glucoamylase enzyme produced from *Aspergillus niger* culture filtrate using starch as substrate



KNUST

Appendix 14

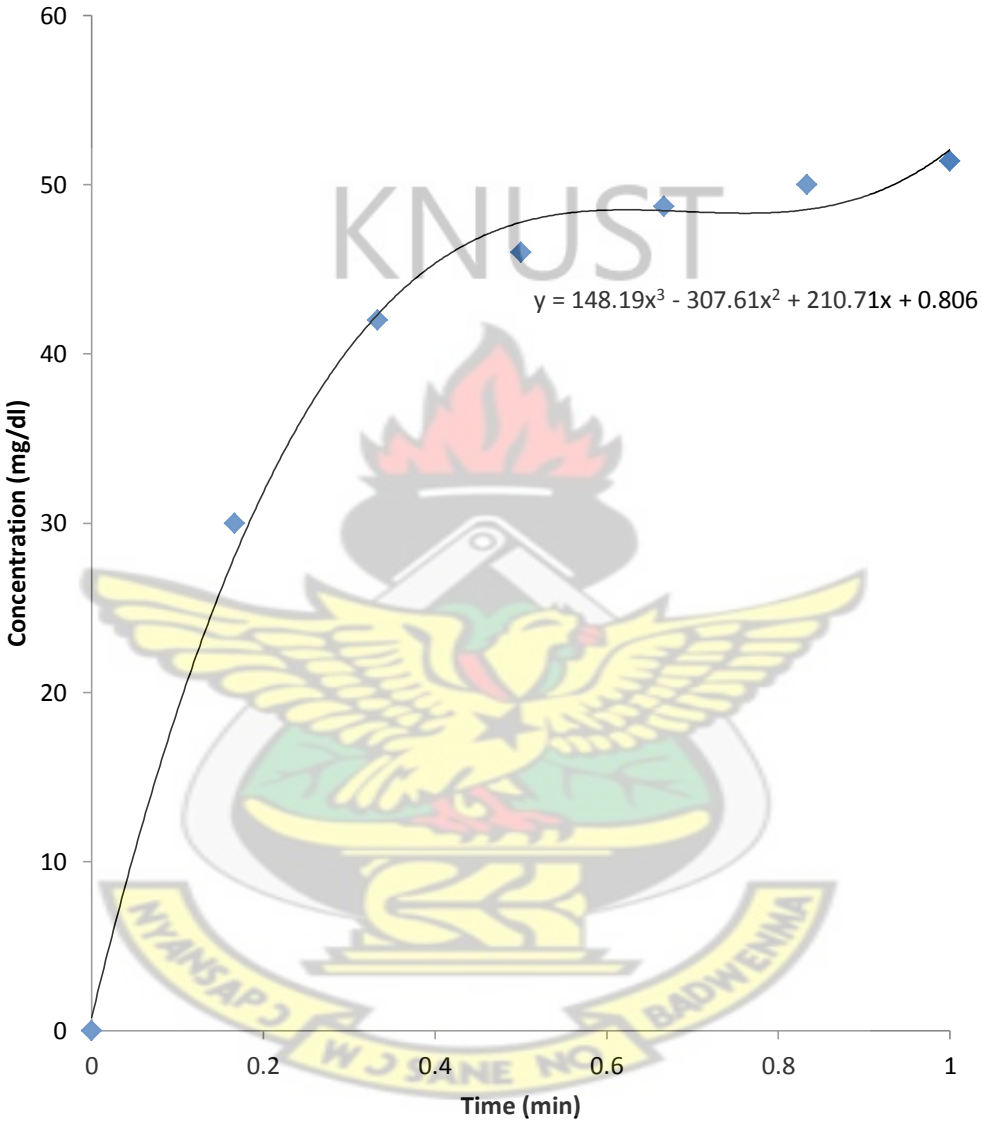
Hanes-Woolf's plot to determine Vmax and Kmax for glucoamylase enzyme produced from *Rhizopus* species culture filtrate using starch as substrate



KNUST

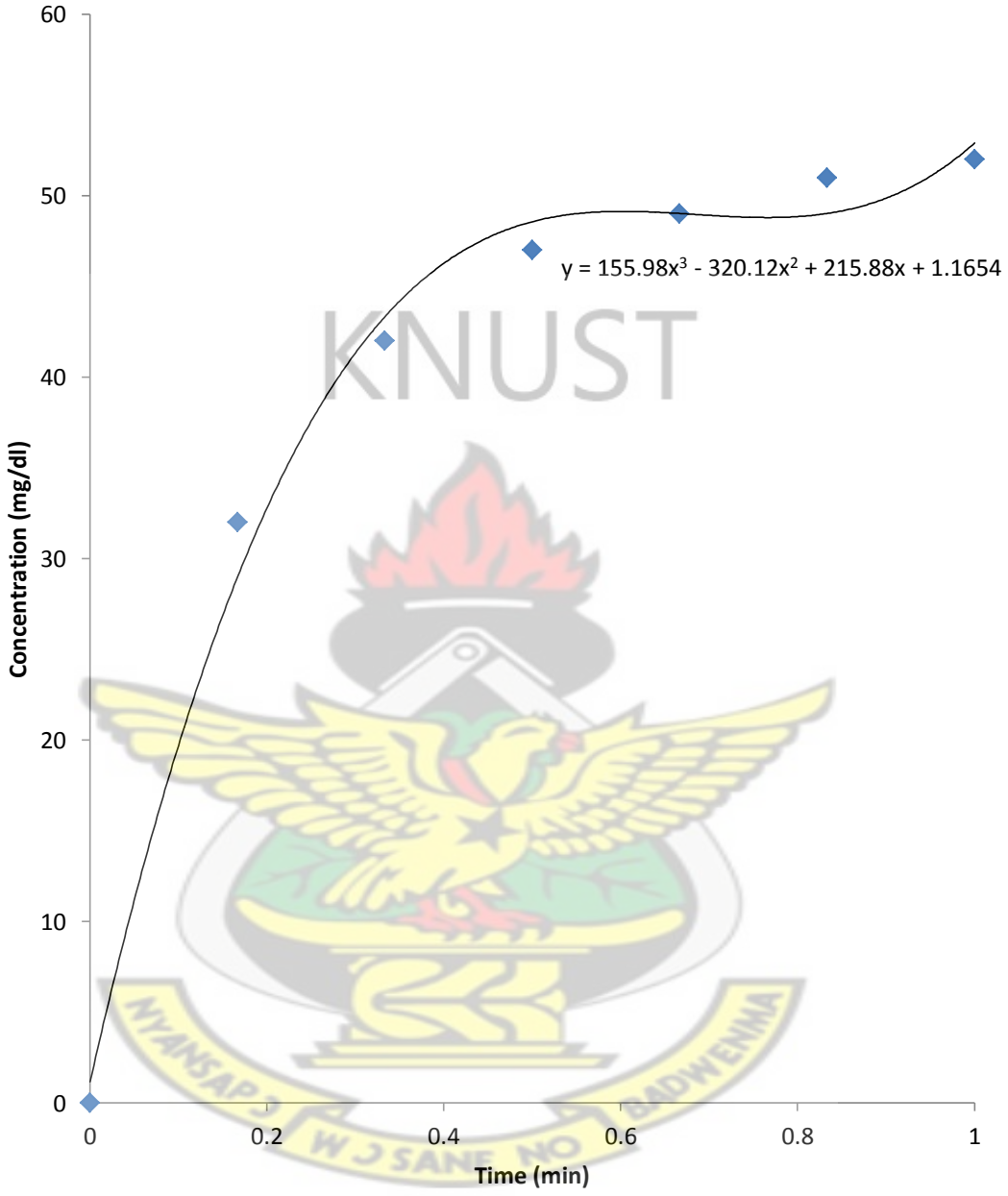
Appendix 15

Determination of initial velocity, V, for glucoamylase enzyme produced from culture filtrate of *Aspergillus niger* at starch concentration of 0.001%



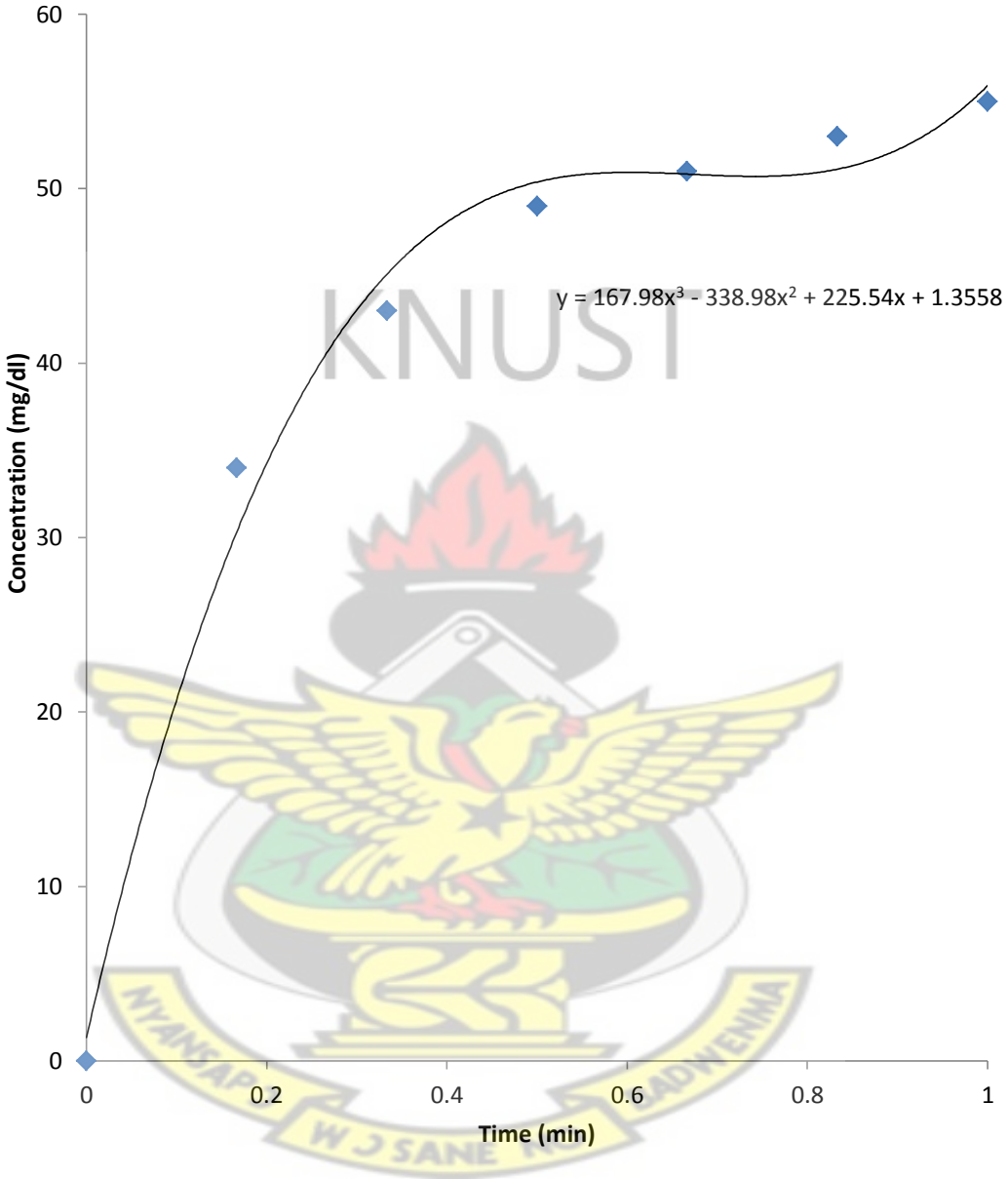
$V = \frac{d[P]}{d[t]} = -\frac{d[S]}{d[t]} = V_1 = 210.7 = 2.107$ g/Lmin at time t = 0.

Determination of initial velocity,V, for glucoamylase enzyme produced fromculture filtrate of *Aspergillus niger* at starch concentration of 0.002%



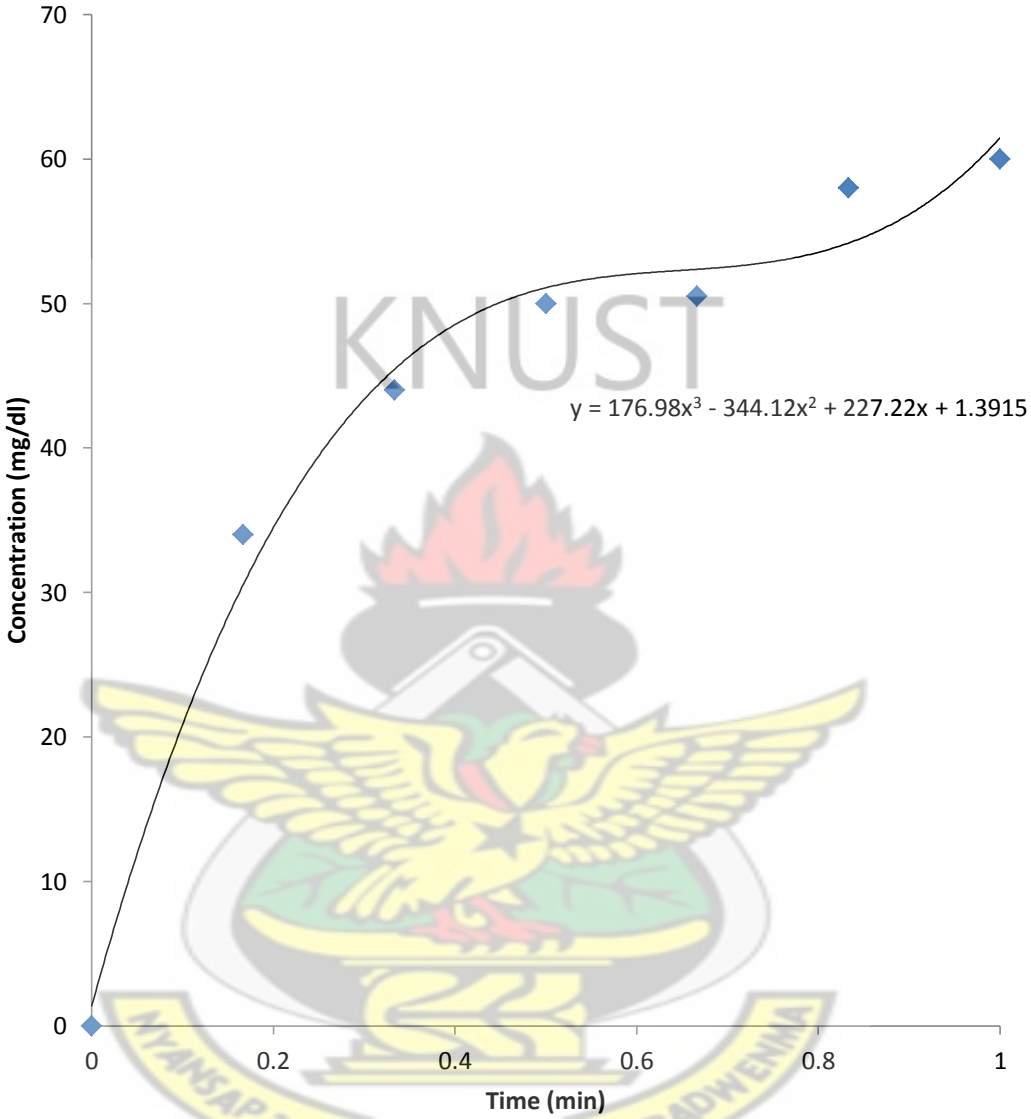
$V = \frac{d[P]}{d[t]} = -\frac{d[S]}{d[t]} = V_2 = 215.8 = 2.158$ g/Lmin at time t = 0.

Determination of initial velocity,V, for glucoamylase enzyme produced fromculture filtrate of *Aspergillus niger* at starch concentration of 0.003%



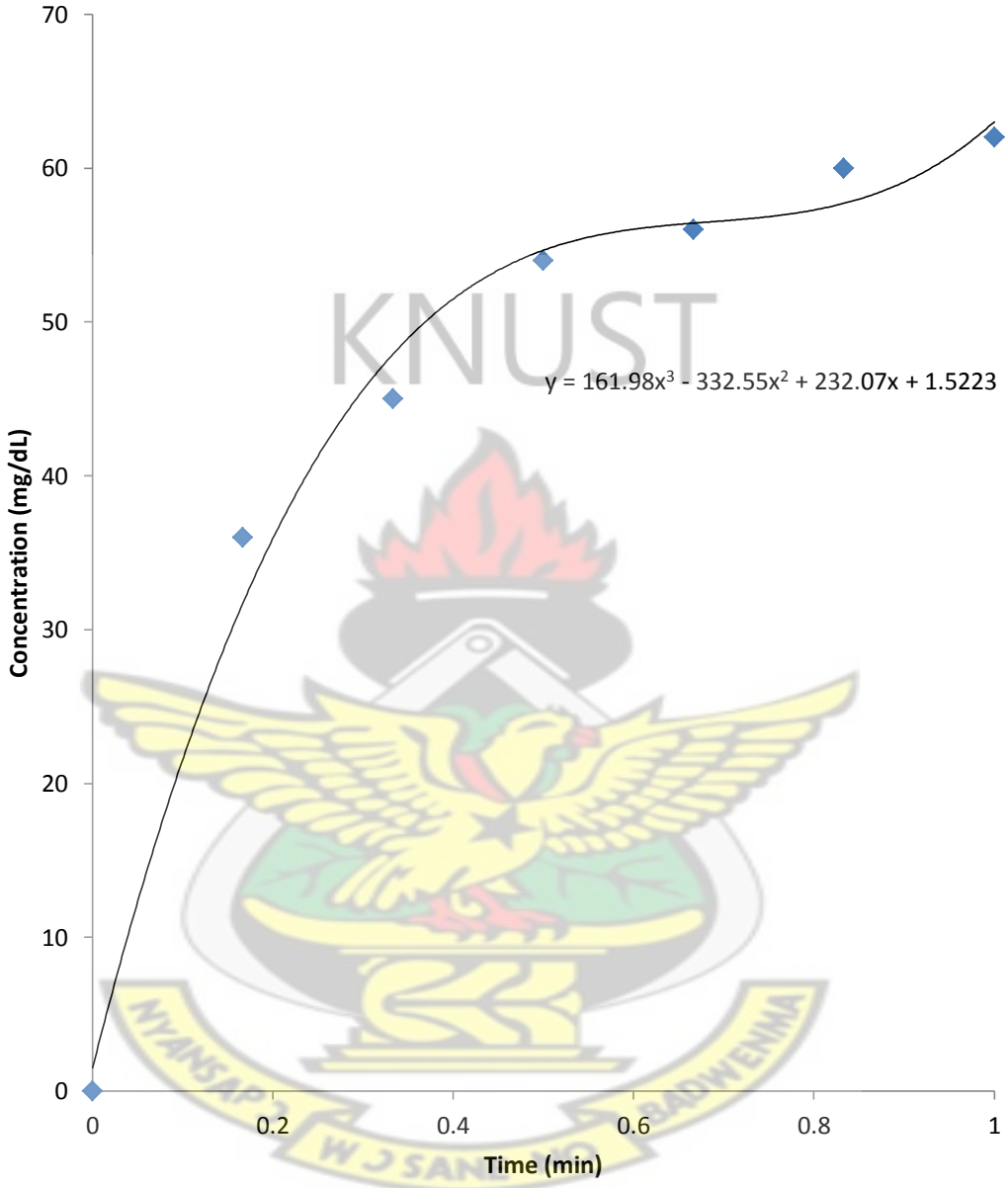
$V = \frac{d[P]}{d[t]} = -\frac{d[S]}{d[t]} = V_3 = 225.5 = 2.255$ g/Lmin at time t = 0.

Determination of initial velocity,V, for glucoamylase enzyme produced fromculture filtrate of *Aspergillus niger* at starch concentration of 0.004%



$V = \frac{d[P]}{d[t]} = -\frac{d[S]}{d[t]} = V_4 = 227.2 = 2.272$ g/Lmin at time t = 0.

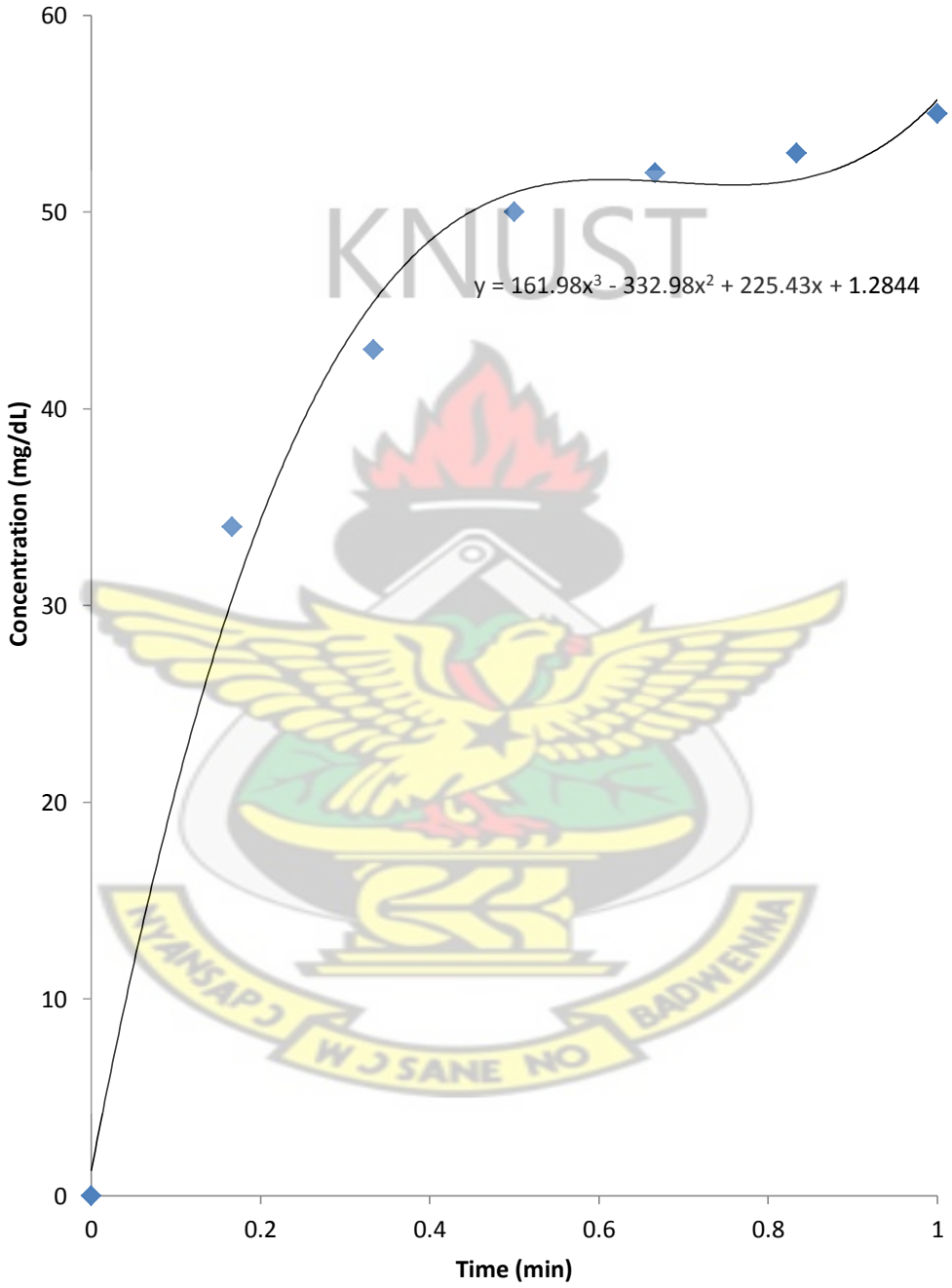
Determination of initial velocity, V, for glucoamylase enzyme produced from culture filtrate of *Aspergillus niger* at starch concentration of 0.005%



$V = \frac{d[P]}{d[t]} = -\frac{d[S]}{d[t]} = V_5 = 232.0 = 2.320$ g/Lmin at time t = 0.

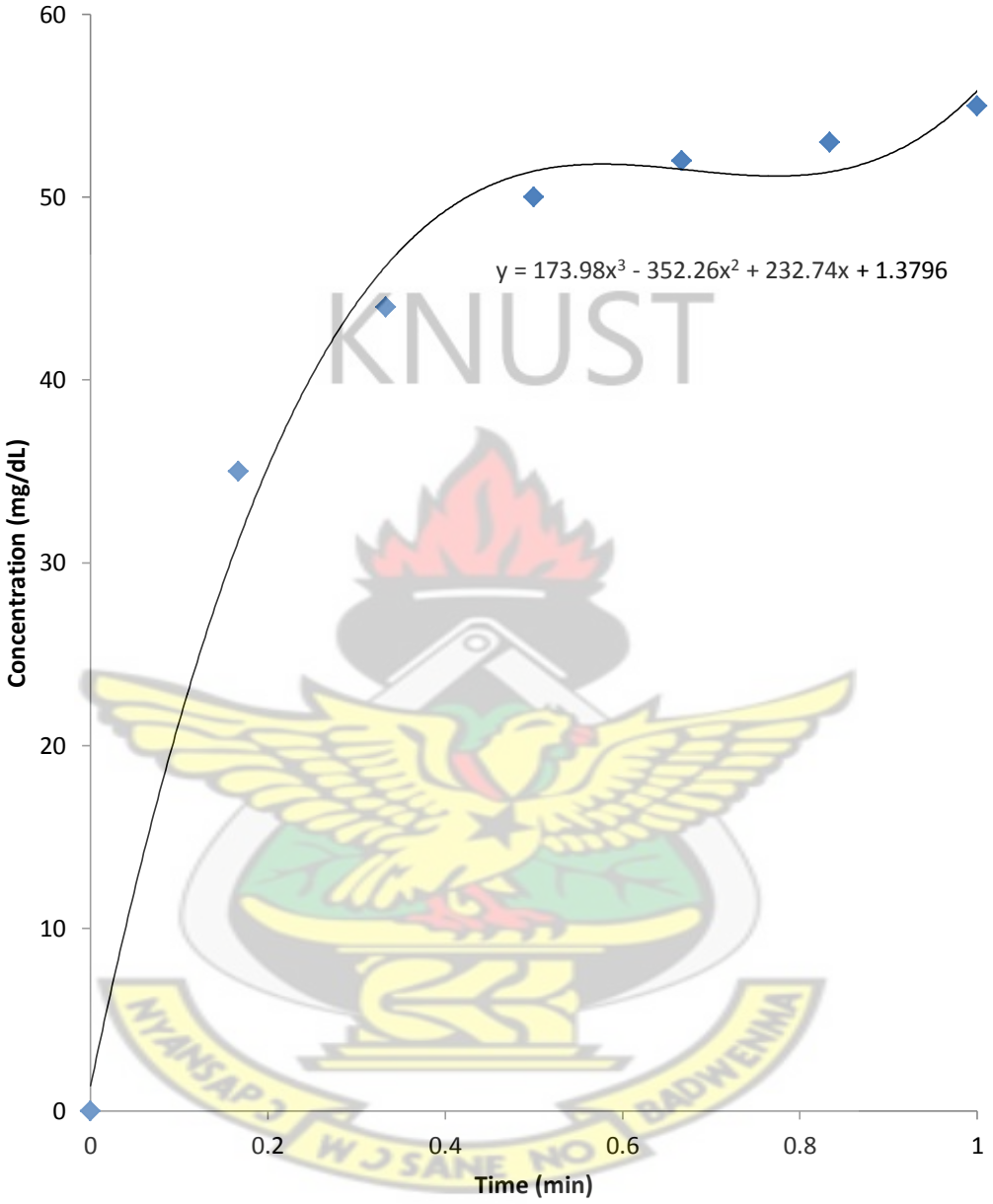
Appendix 16

Determination of initial velocity, V, for glucoamylase enzyme produced from culture filtrate of *Rhizopus* species at starch concentration of 0.001%



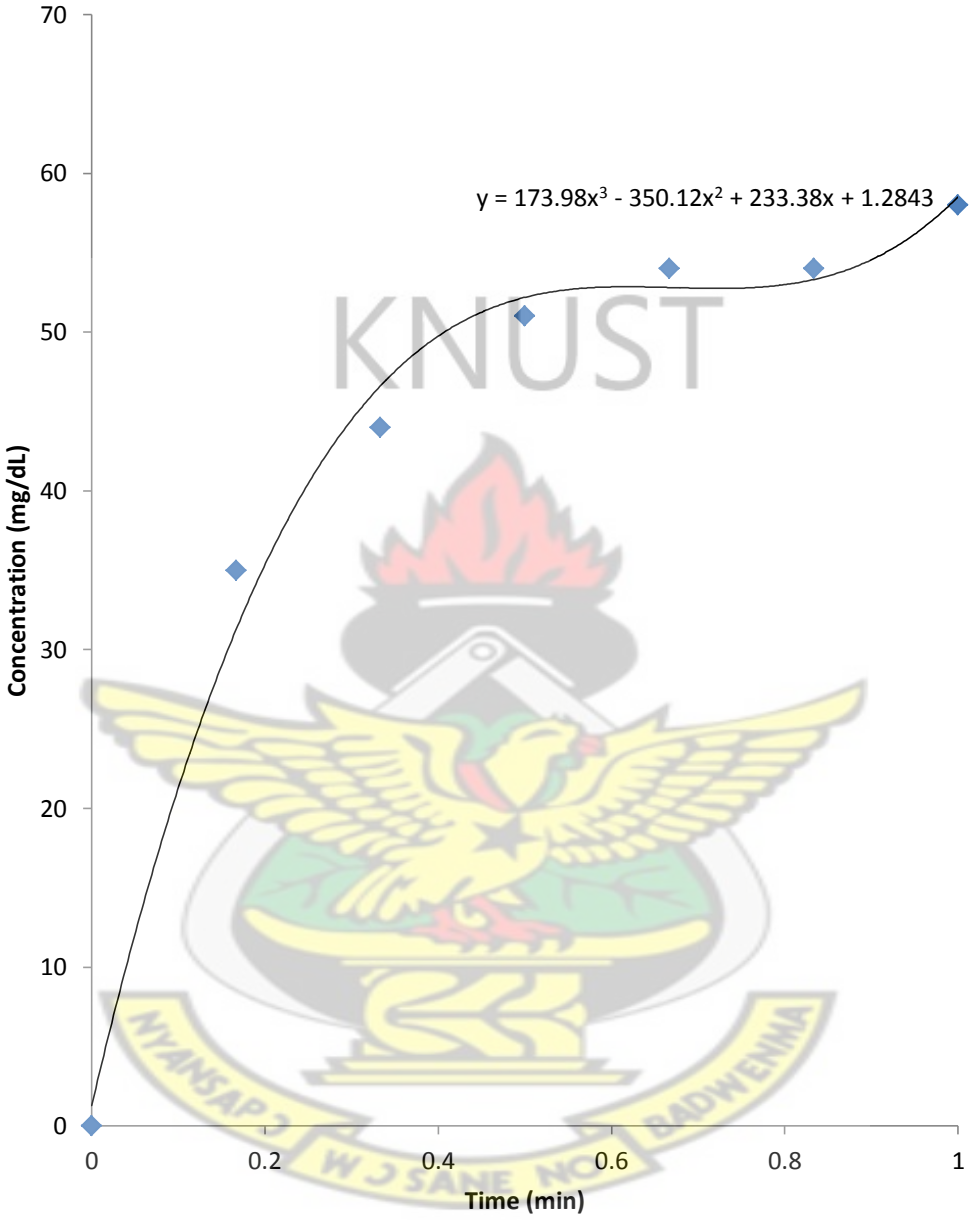
$V = \frac{d[P]}{d[t]} = -\frac{d[S]}{d[t]} = V_1 = 225.4 = 2.254$ g/Lmin at time t = 0.

Determination of initial velocity, V, for glucoamylase enzyme produced from culture filtrate of *Rhizopus* species at starch concentration of 0.002%



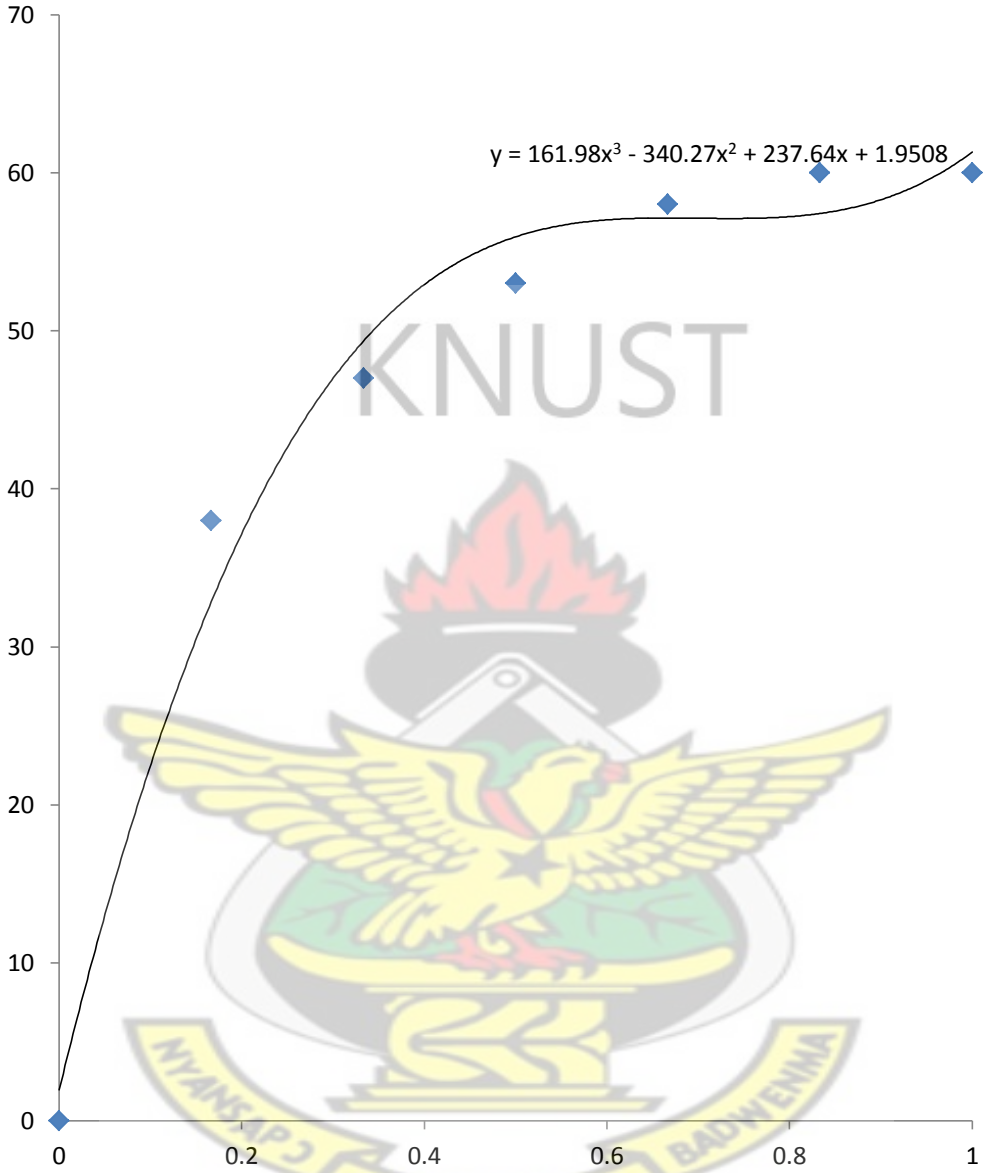
$V = \frac{d[P]}{d[t]} = -\frac{d[S]}{d[t]} = V_2 = 232.7 = 2.327$ g/Lmin at time t = 0.

Determination of initial velocity, V, for glucoamylase enzyme produced from culture filtrate of *Rhizopus* species at starch concentration of 0.003%



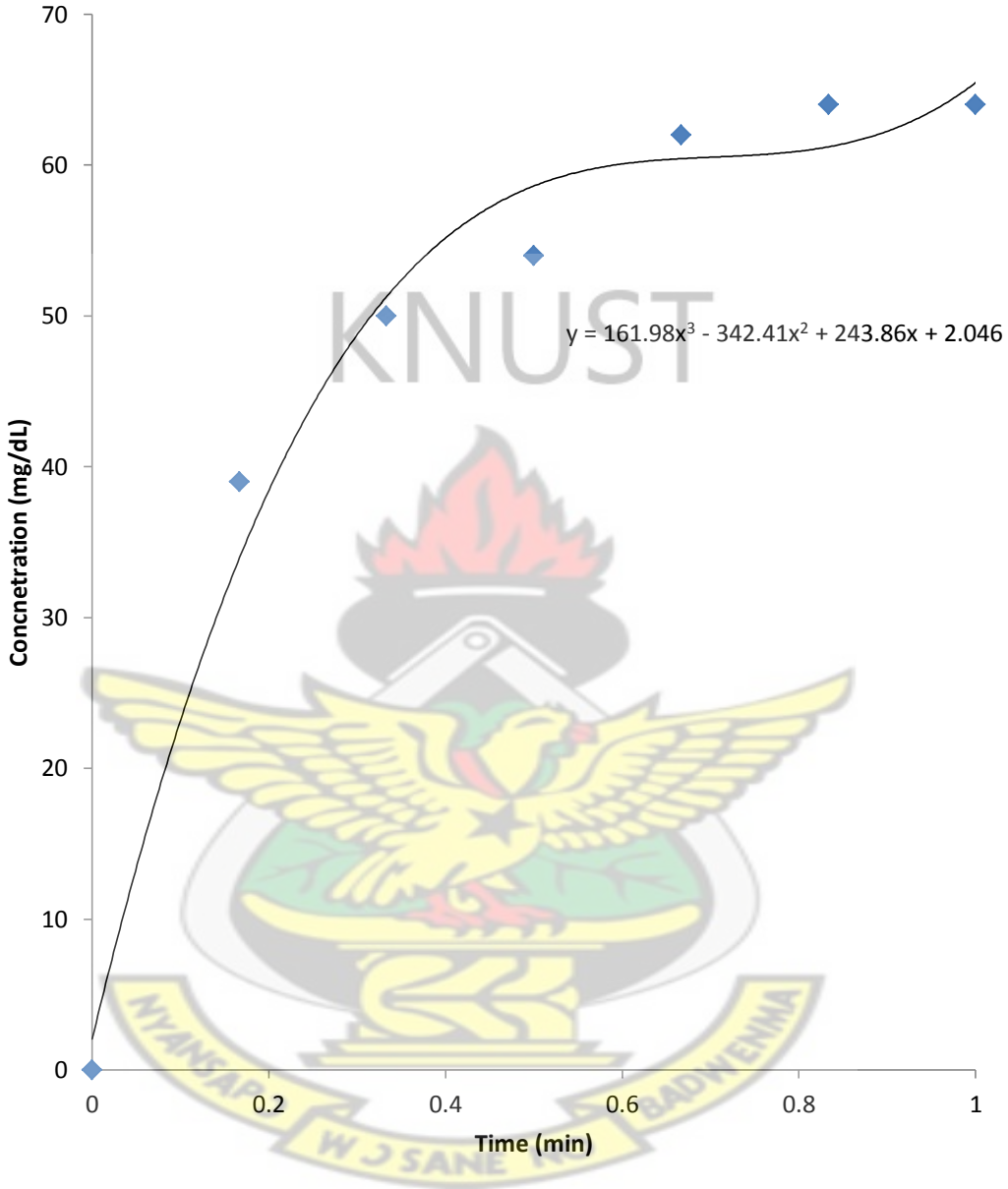
$V = \frac{d[P]}{d[t]} = -\frac{d[S]}{d[t]} = V_3 = 233.3 = 2.333$ g/Lmin at time t = 0.

Determination of initial velocity, V, for glucoamylase enzyme produced from culture filtrate of *Rhizopus* species at starch concentration of 0.004%



$V = \frac{d[P]}{d[t]} = -\frac{d[S]}{d[t]} = V_4 = 237.6 = 2.376$ g/Lmin at time t = 0.

Determination of initial velocity, V, for glucoamylase enzyme produced from culture filtrate of *Rhizopus* species at starch concentration of 0.005%



$V = \frac{d[P]}{d[t]} = -\frac{d[S]}{d[t]} = V_5 = 243.8 = 2.438$ g/Lmin at time t = 0.

Appendix 17

Table 4.11 Combined Effects of Organism, Substrate and Incubation Period on Glucoamylase Production

Organism	Substrate	Incubation period (hours)				
		24 hrs	48 hrs	72 hrs	96 hrs	120 hrs
Rhizopus sp.	Wheat bran	1.47	1.99	1.50	1.55	1.11
	Rice bran	0.89	1.11	1.55	1.50	0.94
	Groundnut Pod	0.42	0.80	1.33	1.11	0.83
	Maize bran	0.13	0.17	0.15	0.21	0.11
	Cocoa Pod	0.12	0.13	0.11	0.12	0.12
Asp. niger	Wheat bran	1.50	2.00	1.56	1.66	1.79
	Rice bran	1.01	1.21	1.52	1.58	0.92
	Groundnut Pod	0.44	0.83	1.11	1.39	1.17
	Maize bran	0.14	0.28	0.38	0.22	0.17
	Cocoa Pod	0.11	0.13	0.12	0.12	0.11
Fusarium oxy	Wheat bran	0.16	0.28	0.38	0.17	0.22
	Rice bran	0.14	0.20	0.32	0.12	0.19
	Groundnut Pod	0.14	0.32	0.24	0.19	0.19
	Maize bran	0.07	0.09	0.10	0.08	0.08
	Cocoa Pod	0.03	0.07	0.04	0.05	0.04
Asp. flavus	Wheat bran	0.13	0.27	0.37	0.22	0.16
	Rice bran	0.11	0.24	0.19	0.32	0.12
	Groundnut Pod	0.11	0.24	0.32	0.19	0.19
	Maize bran	0.08	0.09	0.13	0.13	0.08
	Cocoa Pod	0.05	0.06	0.08	0.07	0.06
	F.Pr	**< 0.001**	**< 0.001**	**< 0.001**	**< 0.001**	**< 0.001**
	$LSD_{(0.01)}$	**0.140**	**0.140**	**0.140**	**0.140**	**0.140**
	%CV	**10.2**				

KNUST

Appendix 18

Table 4.12 Combined Effects of Incubation Period and Temperature on Glucoamylase Production

Incubation period (hours)	Temperature (°C)	*Aspergillus niger*	*Rhizopus* sp.
		Glucoamylase activity (U/ml)	Glucoamylase activity (U/ml)
24	30	1.50	1.47
48	30	2.00	1.99
72	30	1.56	1.50
96	30	1.66	1.55
120	30	1.79	1.11
	F. Pr	**< 0.001**	**< 0.003**
	LSD (0.01)	**0.218**	**0.485**
	%CV	**5.0**	**12.3**
24	40	1.94	1.78
48	40	3.50	3.00
72	40	3.00	2.50
96	40	2.22	2.61
120	40	2.16	1.67
	F. Pr	**< 0.001**	**< 0.001**
	LSD (0.01)	**0.265**	**0.229**
	%CV	**4.0**	**3.8**
24	50	1.13	1.17
48	50	1.39	1.33
72	50	1.85	1.83
96	50	1.50	1.44
120	50	1.28	1.42
	F. Pr	**< 0.001**	**< 0.001**
	LSD (0.01)	**0.053**	**0.052**
	%CV	**1.4**	**1.4**

KNUST

Appendix 19

Table 4.13 Combined Effects of Incubation Period and pH on Glucoamylase Production

Inc. period (hours)	*Aspergillus niger*		Inc. period (hours)	*Rhizopus sp.*	
	pH	Glucoamylase activity (U/ml)		pH	Glucoamylase activity (U/ml)
24	4.0	1.89	24	4.0	1.20
48	4.0	2.34	48	4.0	2.22
72	4.0	1.40	72	4.0	2.33
96	4.0	0.63	96	4.0	1.34
120	4.0	1.23	120	4.0	1.30
	F. Pr	**< 0.001**		**F. Pr**	**< 0.001**
	LSD (0.01)	**0.117**		**LSD (0.01)**	**0.098**
	%CV	**3.0**		**%CV**	**2.2**
24	5.0	1.93	24	4.5	2.78
48	5.0	3.34	48	4.5	3.11
72	5.0	2.50	72	4.5	2.35
96	5.0	2.61	96	4.5	1.78
120	5.0	1.61	120	4.5	1.85
	F. Pr	**< 0.001**		**F. Pr**	**< 0.001**
	LSD (0.01)	**0.120**		**LSD (0.01)**	**0.031**
	%CV	**1.9**		**%CV**	**0.5**
24	6.0	2.60	24	5.0	1.94
48	6.0	2.21	48	5.0	1.61
72	6.0	1.94	72	5.0	0.92
96	6.0	1.67	96	5.0	0.62
120	6.0	1.58	120	5.0	0.96
	F. Pr	**< 0.001**		**F. Pr**	**< 0.001**
	LSD (0.01)	**0.046**		**LSD (0.01)**	**0.035**
	%CV	**0.9**		**%CV**	**1.1**
24	7.0	2.24	24	5.5	1.72
48	7.0	1.62	48	5.5	1.48
72	7.0	0.68	72	5.5	0.94
96	7.0	0.68	96	5.5	0.58
120	7.0	0.93	120	5.5	0.85
	F. Pr	**< 0.001**		**F. Pr**	**< 0.001**
	LSD (0.01)	**0.046**		**LSD (0.01)**	**0.042**
	%CV	**1.5**		**%CV**	**1.4**
24	8.0	3.22	24	6.0	1.64
48	8.0	1.26	48	6.0	1.59
72	8.0	0.89	72	6.0	1.37
96	8.0	0.69	96	6.0	1.39
120	8.0	0.99	120	6.0	1.28
	F. Pr	**<.001**		**F. Pr**	**<.001**
	LSD (0.01)	**0.138**		**LSD (0.01)**	**0.045**
	%CV	**3.8**		**%CV**	**1.2**

Appendix 20

Table 4.14 Combined Effects of Incubation Period and Nitrogen Concentration on Glucoamylase Production

Incubation period (hours)	*Aspergillus niger*	
	Nitrogen concentration (g/L)	Glucoamylase activity (U/ml)
24	0.1	1.84
48	0.1	3.59
72	0.1	2.58
96	0.1	2.78
120	0.1	2.34
	F. Pr	**< 0.001**
	LSD $_{(0.01)}$	**0.228**
	%CV	**3.4**
24	0.2	3.31
48	0.2	5.00
72	0.2	3.67
96	0.2	3.64
120	0.2	3.38
	F. Pr	**< 0.001**
	LSD $_{(0.01)}$	**0.116**
	%CV	**1.2**
24	0.3	1.67
48	0.3	3.11
72	0.3	2.33
96	0.3	2.66
120	0.3	2.66
	F. Pr	**< 0.001**
	LSD $_{(0.01)}$	**0.048**
	%CV	**0.7**
24	0.4	1.62
48	0.4	2.56
72	0.4	2.64
96	0.4	2.68
120	0.4	2.35
	F. Pr	**< 0.001**
	LSD $_{(0.01)}$	**0.038**
	%CV	**0.6**

Appendix 21

Table 4.15 Combined Effects of Incubation Period and Optimum Regimes on Glucoamylase Production on Wheat Bran

Incubation period (hours)	Glucoamylase activity (U/ml)	
	Aspergillus niger	*Rhizopus sp.*
6	1.39	1.00
12	4.16	3.90
18	6.66	4.44
24	3.44	2.76
30	3.65	2.22
36	3.55	2.33
42	3.39	2.55
48	3.32	3.22
54	3.12	1.67
F. Pr	**< 0.001**	**< 0.001**
$LSD_{(0.01)}$	**0.045**	**0.086**
%CV	**0.5**	**1.4**

KNUST

Appendix 22

Table 4.16 Main Effects of Microorganism on Glucoamylase Production

Microorganism	Glucoamylase activity (U/ml)
Rhizopus species	0.80
Aspergillus niger	0.86
Fusarium oxysporum	0.16
Aspergilus flavus	0.17
F.Pr	**< 0.001**
$LSD_{(0.01)}$	**0.028**
%CV	**13.3**

Appendix 23

Table 4.17 Main Effects of Substrate on Glucoamylase Production

Substrate	Glucoamylase activity (U/ml)
Wheat bran	1.31
Rice bran	0.97
Groundnut pod	0.77
Maize bran	0.18
Cocoa pod	0.10
F.Pr	**< 0.001**
$LSD_{(0.01)}$	**0.031**
%CV	**13.3**

KNUST

Appendix 24

Calculation of Glucoamylase Activity

One unit of glucoamylase activity (U) is defined as that amount of enzyme that will produce one micromole of glucose per minute. Let c_{sp} represent glucose concentration (concentration of glucose produced by the enzymatic reaction). Let the volume of the mixture comprising citric acid, starch, enzyme and DNS be v_{sp} in millilitres. Then milligrams of glucose produced in the 10 minutes by the enzymatic reaction = $(c_{sp} \times v_{sp})$. One micromole (1µmole) of glucose is produced from 0.18016 mg salt of glucose. Therefore $(c_{sp} \times v_{sp})$ mg of glucose produced $\frac{c_{sp} \times v_{sp}}{0.18016}$ micromoles of glucose. These micromoles of glucose were produced in 10 minutes by one millilitre (1ml) of enzyme. So micromoles of glucose produced per minute (U) per millilitre of enzyme = $\frac{c_{sp} \times v_{sp}}{0.18016 \times 10}$ U/ml at a given temperature and pH. Hence, one unit (U/ml) of glucoamylase activity is defined as the amount of enzyme that releases one micromole (1µmole) of reducing sugar as glucose per minute under assay conditions.

KNUST

Appendix 25

Deriving the Michaelis-Menten Equation

The substrate S reversibly associates with the enzyme E in a first step, and some of the resulting complex ES is allowed to break down and yield the product P and the free enzyme back.

$$E + S \underset{K_r}{\overset{K_f}{\rightleftarrows}} ES \xrightarrow{K_{cat}} E + P \quad \ldots\ldots\ldots (a)$$

Where K_f , K_r and K_{cat} are constants for substrate binding, unbinding and conversion to product respectively. Indeed, K_{cat} also known as the turnover number is the maximum number of substrate molecules converted to product per enzyme molecule per second.

Some implicit assumptions:

(1) As long as the initial velocity is considered, molecules of substrate converted to product can be neglected. Hence, concentration of substrate at time t following time zero [S] is far greater than the concentration of substrate converted to product [P]. Hence [S] ≫ [P]. Total molecules of the substrate at the start $[S]_0$ must be equal to free molecules of substrate $[S]_{free}$ plus molecules of substrate in the complex ES plus molecules of substrate in the product [P].

$[S]_0 = [S]_{free} + [ES] + [P]$, which approximates to $[S]_0 = [S]$.

(2) The concentration of substrate is in large excess over that of the enzyme

i.e.[S] ≫ [E]. $[E]_{Total} = [E]_{free} + [ES]$ ……. (b)

Note that possible formation of a complex EP is neglected.

At steady state approximation, the rate of production of ES in equation (a) must equal its rate of breakdown:

Rate of formation of [ES] = Rate of consumption of [ES].

$$\text{i.e.} K_f[E][S] = K_r[ES] + K_{cat}[ES] \quad \ldots\ldots\ldots \text{(c)}$$

Rearranging equation (c) gives the concentration of ES as:

$$[ES] = \frac{K_f[E][S]}{[K_r]+[K_{cat}]} \ldots\ldots \text{(d)}$$

The fraction, F of the enzyme in the form ES is given by:

$$F = \frac{[ES]}{[E]+[ES]} \ldots\ldots\ldots \text{(e)}$$

Combining equations (d) and (e) gives:

$$F = \frac{[S]}{\{(K_r + K_{cat})/K_f\} + [S]} \ldots\ldots\ldots\ldots \text{(f)}$$

The maximum velocity Vmax occurs when the enzyme is saturated. That is, when all enzyme molecules are tied up with S, or when all the enzyme molecules are in the form ES. Therefore, in a situation where some free enzyme molecules are present, rate of product formation V would be:

$$V = V_{max} F \quad \ldots\ldots\ldots \text{(g)}$$

Combining equations (f) and (g) gives:

$$V = V_{max}\left(\frac{[S]}{\{(K_r + K_{cat})/K_f\} + [S]}\right) = V_{max}\left(\frac{[S]}{K_m + [S]}\right) \quad \ldots\ldots\ldots \text{(h)}$$

Where $K_m = (K_r + K_{cat})/K_f$ and Vmax may be defined as $V_{max} = K_{cat}[E]_{Total}$. Since Vmax occurs when [ES] = $[E]_{Total}$. K_{cat} is a first order rate constant and $[E]_{Total}$ is the initial concentration of the enzyme before any binding occurred. Equation (h) is known as Michaelis-Menten equation.

KNUST